RADIOBIOLOGISCH INSTITUUT T.N.O.

PROCEEDINGS

of the

INTERNATIONAL SYMPOSIUM ON BONE MARROW THERAPY AND CHEMICAL PROTECTION IN IRRADIATED PRIMATES

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PREFACE

This symposium was sponsored by the Organization for Health Research T. N. O. which enabled its Radiobiological Institute to act as host for the meetings.

Financial grants for a number of participants (from the United States) were generously provided by the Netherlands Government.

These two gestures were of invaluable help to the organizers in achieving their object which was to assemble as many as possible of the small group of investigators who have personal experience with bone marrow transplantation and chemical protection in primates for a detailed exchange of experience

An opening speech was given by Prof. Dr. A. Querido, professor of Internal Medicine at Leyden University and member of the Board of the Radiobiological Institute, on behalf of its chairman Prof. Dr. J. W. Tesch.

The recorded discussions were condensed by an editorial committee consisting of the following members of the symposium: H. Balner, D. W. van Bekkum, B. G. Crouch, R. R. Overman, L. M. van Putten, O. Vos. In order to minimize the time required for publication, the condensed version of the discussions was prepared without consulting the discussants.

Because of the nature of the offset printing procedure employed the authors alone are responsible for the contents of their papers.

The bulk of the secretarial work required to produce this volume has been performed by Mrs. M. J. Kooistra and Mrs. T. Moelker of the Radiobiological Institute who have deserved the deepest gratitude of the participants and the organizers.

THE ORGANIZING COMMITTEE

D. W. van Bekkum
B. G. Crouch
R. R. Overman

OPENING SPEECH BY PROF. DR. A. QUERIDO

It is a great privilege for me to welcome you all at this International Symposium on Bone Marrow Therapy and Chemical Protection in Irradiated Primates. I would not have this privilege if there was not the unfortunate situation that Prof. J. W. Tesch, the president of the Organization for Health Research T. N. O. (which organization as you know sponsors this meeting) was unable to be present today, because he is abroad. He regretted very much indeed that he was prevented from attending this meeting and has sent his best wishes. I appreciate very much that he has chosen me to say a few words at the opening of this symposium, because some of the problems you study have also my interest. I am referring to those that are more related to bone marrow transplantation, than to irradiation protection. They cover an extremely interesting field of research, which field (needless to say to you) shows difficulties which sometimes seem to be insurmountable. They are different from most field of medical or biological research, because they are not related to adaptation of the organism to its external environment, but mainly to the confrontation of the organism in its internal environment to tissues with a different genetic structure.

The problems which have been encountered in these experiments are known to you, who are all distinguished workers in this area of research. One could play with the idea that these difficulties could be overcome if it were possible to transform the genetic structure of the graft tissue, so that the next generation of cells was identical to those of the receiving organism. There are recently reports that human immature erythrocytes or human bone marrow can be induced to produce an abnormal hemoglobin. This achievement, however, only concerns the induction of another protein, not the production of new cells with different genetical information. It seems to be hardly appropriate therefore to consider it as a serious

future possibility.

As an outsider I have the impression that effort in the past years has been directed mainly to changing the host and fairly little to altering the graft, for making their coexistance possible. Very agressive methods have been used to make the host willing to accept the graft, as e. g. lethal irradiation. It seems of what I see in the literature that more refined techniques are being developed now directed towards specific destruction of lymphoid cells, which may open new possibilities for study. It appears that also more attention is given to the production of experimental runt disease in non-irradiated adult animals, which will give an opportunity to study more deeply the conditions under which runt disease may or may not develop.

Although it looks that the practical use of homologous bone marrow transplantation hardly can be achieved, the basic research of its problems appears to me as an outsider to be still wide open.

The Organization for Health Research T. N. O. therefore is most happy to sponsor this very timely symposium and is very grateful indeed to you all for accepting the invitation. We are glad that our Ministry of Social Affairs and Public Health provided the means for it, not in the least because many of the Dutch workers have enjoyed hospitality and support from you. As Dutch we take pride in the fact that the Medical Biological Laboratory T. N. O. as early as 1954 started the work with rodents in Holland and contributed considerably to problems related to bone marrow transplantation; and that in cooperation with the new Radiobiological Institute T. N. O., supported very liberally by the Organization for Health Research T. N. O. and by Euratom, work is continuing both with rodents and primates. As a representative of Leyden University, situated not more than 20 miles from these Dutch laboratory workers, I am happy to say that we already enjoy a close cooperation in common fields of interest which early 1963 (after

construction of semipermanent facility on our premises) will even be closer in the field of bone marrow transplantation. It is this combination of basic research and the study of clinical application to which we are looking forward very much indeed.

Ladies and Gentlemen, with these opening remarks I hope to have indicated how warmly we welcome this symposium and your personal presence in Rijswijk. Also how grateful we are to all those who made it possible. On behalf of the Organization for Health Research T. N. O., I wish you great success.

INTRODUCTION TO THE SYMPOSIUM

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The past 15 years have seen an almost incredible activity from laboratories all over the world in the search for agents and methods to prevent or combat radiation injury. The large majority of the workers has employed radiation sickness in various rodent species in their experimental set ups and usually 30-day mortality has been the end point of these tests. Since 1949 two main lines of research have developed:

1. The so-called chemical protection starting with the discovery of the protective effect of cyanide in mice by Herve and Bacq (1949) and that of cysteine by Patt and collaborators (1949) and
2. the bone marrow treatment emerging from the classical studies of Jacobson and colleagues (1949) on the effect of spleen shielding, also in irradiated mice.

To date we count over 600 papers on various aspects of chemical protection which is a conservative estimate, and probably no less on subjects concerned with or related to the transplantation of hemopoietic cells in irradiated animals.

Among the numerous chemical substances that are now known to possess protective activity are at least a dozen, which - if administered in suitable doses before the irradiation - may easily change 100% mortality into complete survival in mice as well as in some other rodent species. However dramatic this effect appears to be, it is now also known

that the protective activity of these most effective compounds does not exceed a dose reduction of about 50 per cent, the dose reduction being defined as

$$(D_1 - D_2) / D_1 \times 100 \text{ per cent}$$

in which D_1 = radiation dose administered to protected animals and

D_2 = radiation dose causing quantitatively the same mortality in non-protected animals.

In spite of continued efforts to develop new series of protective chemicals this limit of achievable protection has not been surpassed.

The radiation doses which are usually administered in those types of experiments cause death from bone marrow aplasia or from denudation of the intestinal mucosa. In both cases a proliferating cellular system is damaged by direct destruction of cells or by the blocking of cell multiplication. In other words, death is the result of a failure of the surviving cells of these systems to multiply at a sufficiently high rate; however, the initial protection of a relatively small number of cells may provide the organism with a sufficient number of descendant cells to enable it to survive the critical period which is several days or weeks later.

Although much knowledge has been accumulated on the mechanisms of chemical protection and notwithstanding the demonstrated effectiveness of certain protectors in mice, the applications to date in the field of clinical medicine and preventive medicine have been very limited indeed.

Obviously this is because the compounds that have been shown to be most effective in mice do not seem to be suitable for application in man because of the expected toxic effects.

Several classes of protective compounds - e. g. the biological amines - have been shown to act by producing anoxia (v. d. Meer et al., 1962) and this

may have provided serious objections against their application in humans. It should be stressed however that it is not the anoxia of the brain or the heart tissues which determines survival following irradiation but the anoxia which existed in hemopoietic cells and the epithelial cells of the gut during the irradiation. The drugs which induce anoxia specifically in these cells in mice may not necessarily exert exactly the same influences in man.

Furthermore it cannot be predicted that protective activity and toxicity of a substance will show the same quantitative relationship in man as in mice in view of the profound differences between the two species with regard to their reactions to a number of pharmacological agents.

These are only a few of the most obvious arguments which lead me to the conclusion not only that studies in infrahuman primates are required before the results obtained with irradiated rodents are brought to a clinical trial but also that maybe a much larger part of the search for useful protective agents should be performed directly in monkeys. There is even some question as to the feasibility of large scale screening programs in mice when the object is to discover protective agents that can be used in man.

Unfortunately, the number of studies on the action of chemical protective agents with the monkey as the experimental animal has been extremely limited. Only a few groups, notably in the United States, have been actively interested in this problem.

The reasons for this are not difficult to recognize. Only very few laboratories in the world combine the facilities for both the handling of a considerable number of monkeys and the irradiation of monkeys with interest in this aspect of radiobiology plus the adequate financial resources to support such an extremely expensive research project.

We are fortunate to have the experts from these few laboratories present during this meeting. Their papers will provide us with material for a complete session on this subject.

I hope that the data to be presented will provide us with the material for a fruitfull discussion of at least the following points.

1. What evidence has been accumulated so far to support the conclusion that the pharmacological effects of known radioprotective substances in monkeys resemble the effects observed in humans.
2. Does anoxia of a relatively small proportion of the hemopoietic system (or the intestinal epithelium) induced by either physical or chemical means in the monkey result in a similar degree of protection as it is supposed to afford in mice?
3. To what extent is pre-irradiation sanitation of monkeys required for the performance of dependable investigations on chemical protection?

In the field of bone marrow transplantation to date, the situation is somewhat different. Again the bulk of available information has been collected in mice and the mechanism of the therapeutic effectiveness of hemopoietic tissue cell injection has been elucidated in this species.

A satisfactory if not abundant amount of information is available on a variety of aspects of bone marrow transplantation in mice, such as the initial distribution and localization of the injected cells, the dynamics of the repopulation of the hemopoietic and immunological systems by donor type cells, the factors which influence the establishment and the persistence of the chimeric state and many other aspects.

The complications occurring after a successful take of the transplant known as secondary disease have been studied in detail and it is now generally acknowledged that these are caused primarily by an immunological reaction of the graft versus the host. Already various effective methods have been reported to prevent and to treat secondary disease in mice and the interest of the bone marrow transplantation workers is shifting to a large extent towards the problem of immunological tolerance. These studies have stimula-

ted an immense revival of activities in the fields of transplantation biology, immunology and hematology and it may well be that the most valuable consequences of this activity are still to come.

From the point of view of treatment of radiation disease the main conclusions from rodent studies can be summarized as follows:

1. Bone marrow treatment constitutes an effective method of treatment of lethally irradiated animals up to radiation doses which cause death from intestinal damage.

2. Even with homologous bone marrow a reasonable degree of protection can be obtained, provided suitable host donor combinations are selected.

3. Mortality from secondary disease can be largely prevented by using proper donor material either by selection of donor strains or by the employment of fetal liver cells. Secondary disease, once established, can be treated quite effectively by continuous administration of antibiotics up to the end of the 3rd month. When the drugs are withdrawn after the end of that period, fatalities will usually not reappear although in some degree of secondary disease may persist (van Bekkum and Vos, 1961). In other words, if the animals survive the critical period of about 100 days, the chances are good for a much prolonged survival and there is evidence accumulating that this favourable state of affairs is due to the development of partial or complete specific immunological tolerance on the part of the graft towards host tissue antigens (van Bekkum et al., in press). Investigations are under way in a number of research centres with the aim of finding methods to promote the development of this tolerant condition.

4. Finally the problem of preservation of hemopoietic cells seems to be largely solved as far as the irradiated mouse system is concerned.

This abundance of information, as well as the relatively optimistic prospects provided by the mouse experiments, has induced several clinicians to apply bone marrow transplantation and whole body irradiation to human patients. Unfortunately the results untill now have been far less promising, although it is not fair to call them disappointing in all respects.

Let us briefly examine the clinical achievements in the same order as has just been done with the mouse data and compare them with the results of experiments obtained with irradiated monkeys.

1. Bone marrow treatment is probably similarly effective in man in the treatment of the lethal irradiation syndrome as far as autologous and isologous bone marrow transplantations are concerned. Although definite proof of the effectiveness of these types of bone marrow treatment cannot be provided in man, partially because a suitable untreated control group of patients is not available, there is very good circumstantial evidence from several clinics concerning the efficacy of this type of treatment (Kurnick, 1961; Thomas, 1961). Any remaining uncertainty seems to be largely removed by the results obtained with autologous bone marrow transplantation in lethally irradiated monkeys. When fresh bone marrow cells were used protection has been observed following doses up to 925 r of X-radiation. The minimal effective number of cells injected was slightly below 10^8/kg, which would indicate a requirement for about 2×10^9 cells as a reliable number for the treatment of patients under similar conditions. As in mice, no particular hazard can be attributed to the use of autologous marrow and this treatment is becoming rapidly introduced as a method permitting the administration of otherwise dangerous amounts of chemotherapeutics and irradiation in cancer therapy (Clifford et al., 1961; Miller and Diamond, 1961; Woodruff and Nolan, 1961; Kurnick, 1961; Jones et al., 1960; Conrad and Crosby,

1960).

2. Homologous bone marrow administration is supposed to have favourably influenced the course of the radiation sickness in some of the Yugoslave patients treated in Paris in 1958, but in these cases a temporary take occurred and the period of donor cell proliferation was rather limited (Mathé et al., 1959). This may well be exactly the optimal situation in the treatment of whole body irradiation in man, since all cases in which a more prolonged proliferation of the donor cells was demonstrated have succumbed to what is apparently a severe form of secondary disease.

   In lethally irradiated monkeys conditions in terms of X-ray dose and cell numbers injected, have been established to obtain takes of homologous bone marrow cells in a reproducable way, many of the resulting chimeras have developed fatal secondary disease with a maximum survival time of 65 days in our laboratory (Crouch et al., 1961). The clinical symptoms as well as the pathological changes observed in these monkeys show great resemblance to the findings in man and the disease in primates as a group presents quite a different picture from the secondary disease following bone marrow transplantation as it occurs in mice. Obviously, this necessitates a great deal of further research with monkeys instead of rodents as the experimental animals before the clinical application of homologous bone marrow transplantation can be safely resumed. With regard to the risks involved in the treatment of patients exposed to midlethal doses of radiation with homologous bone marrow, additional investigation with larger numbers of monkeys is badly needed, particularly in view of the unfavourable results reported by one group of workers (Newsome and Overman, 1960)

3. Essentially no reliable information has been supplied so far on the pre-

vention and the treatment of secondary disease in humans. The selection of proper host-donor combinations has still no suitable basis and it is even impossible to predict how fast the identification of transplantation antigens in man will proceed. As to the use of fetal material the optimal age of the fetuses which are to provide the donor cells is a matter of pure speculation and it remains to be seen whether fetuses of the proper age will contain a sufficient number of hemopoietic cells to effectuate an homologous graft. Investigations of these problems in the monkey have started only recently as will be reported during this symposium (van Putten, 1962).

4. The only dependable criterion of viability of frozen marrow cells is the protection test, as long as no other methods to determine the capacity for unlimited proliferation of the cells are available. Since this criterion cannot be used with human material we have essentially no absolute proof that the techniques that are presently being used for the preservation of human bone marrow provide optimal survival of the frozen cells. There is a possibility that some of the disappointing clinical experiences are due to inadequate preservation of the proliferating capacity of the injected cells. It is not at all certain that the conditions found to be adequate for mouse bone marrow will also be optimal for human bone marrow. Here again rather large scale investigations with monkeys seem to be indicated.

With regard to the use of homologous bone marrow it is my personal view that the outcome of the investigations performed so far has been disappointing - to say the least - and it seems very hard indeed to devise a short way out of the present impasse.

The organizers of this symposium have felt that the time has come to consider in as great a detail as possible the causes for these failures.

This means that we intend to emphasize during the discussions not so much the few positive results available but rather the numerous pitfalls which appear to halt our progress and if possible to discuss means of overcoming these difficulties.

One of the most depressing aspects of experimentation with monkeys involving whole body irradiation is the circumstance that these animals suffer from a variety of diseases which need intensive and prolonged treatment. Some of these diseases can be cured or kept under satisfactory control but others are found to recur following the irradiation, thus severely interfering with the experiment. Many of the animals in fact are found to have been unsuitable for this type of experiment afterwards.

In rodent work it is now becoming generally accepted that the absence of a number of specific pathogens is one of the most important factors which determine the reliability of radiation experiments in particular of long term ones. As a consequence many laboratories are now in the process of leaving the conventional laboratory animal for what it is and are changing to disease free or specific pathogen free stock.

We are fortunate that a number of our colleagues who have gained great experience in the sanitation of large monkey colonies have joined us for this meeting so that we shall be in a position to have a full discussion of all the problems related with improving the fittness of monkeys to be irradiated. One of the most pertinent questions to be decided upon seems to me the following:

Should we continue to concentrate our efforts on changing the freshly captured monkey into a disease free laboratory animal in the limited time available before it becomes too old for experiments or do we have to change this system radically and begin with the raising of disease free stock starting with animals born in the laboratory?

LADIES AND GENTLEMEN,

I have mentioned to you only a few of the problems that we might discuss in the next few days. I leave it to you to raise many more.

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BONE MARROW TRANSPLANTATION IN THE PROTECTION OF PRIMATES AGAINST RADIATION[1]

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Recently Congdon (1962) reviewed the literature on bone marrow transplantation in radiation injury. Several authors have observed that some animals which were protected with homologous bone marrow against lethal doses of radiation will survive without developing secondary disease. In our hands, the percentage of mice which thus escape secondary disease is constant for any given genetic combination and experimental condition. One of the important problems of radiation biology seems to be the elucidation of conditions responsible for the escape from secondary disease. Since important species differences exist in this regard, monkeys appeared to be a logical choice for experimental subjects in the hope that the results will approximate those expected in humans. The purpose of this study was to compare autologous and homologous bone marrow protection in monkeys against lethal doses of radiation and to study problems associated with secondary disease.

[1]Supported by grants from the National Institutes of Health, U.S. Public Health Service and the American Cancer Society.

Table 1 shows the effect of homologous and autologous bone marrow transplantation upon radiation injury induced with 700 r whole body radiation in 2.5 - 3.5 kilo Rhesus monkeys.

TABLE 1

EFFECT OF HOMOLOGOUS BONE MARROW TRANSPLANTATION ON LETHALITY OF 700 r WHOLE BODY RADIATION IN 2.5-3.5 KG MACACA MULATTA

Type and No. of bone marrow cells transplanted	None	Homologous			Autologous
		0.25×10^9	$1.2\text{-}2.1 \times 10^9$	$2.2\text{-}3.3 \times 10^9$	$0.25\text{-}0.50 \times 10^9$
No. Survived/Total No.	0/11	2/8	7/12	3/6	6/8
Mean Time of Death of Non-Survivors	12 days	10 days	15 days	31 days (12, 20, 62)	14 days
Survivors Observed for	-	24 months	24 months	12 months	24 months

A 2 Mev resonant transformer generator (General Electric) was used which produces an essentially uniform field strength in the working area. Homologous marrow was obtained from donor monkeys sacrificed by exsanguination under nembutal anesthesia. Each recipient received bone marrow from only one donor. Autologous bone marrow was obtained from surgically disarticulated hind limbs and occasionally from surgically removed ribs.

The marrow was stored at 4° C in Eagle's medium containing streptomycin, ATP penicillin, streptokinase, streptodornase and heparin. Twenty-four hours after the operation, the animals were irradiated and twenty-four hours later, bone marrow was re-injected either into the marrow cavity of the tibia or intravenously. It appears that with homologous bone marrow, survival rates improve as higher numbers of viable cells are injected. The protective effect of autologous bone marrow seems to be superior to that of homologous bone marrow at equal viable cell doses. The group which received $1.2-2.1 \times 10^9$ bone marrow cells was selected for an experiment to be reported later, in which bone marrow reserves were tested with E. coli lipopolysaccharide challenge. Since lipopolysaccharides may influence the survival time of radiated monkeys, this group may not be strictly comparable to the others. Smith (1957, 1958) described that Salmonella typhosa endotoxin increases survival in groups of lethally radiated mice; endotoxin given 24 hours before radiation produced increased recovery of hemic cells, endotoxin given after irradiation was less effective. It was remarkable in our experiments that most deaths occurred relatively early and among all monkeys radiated with 700 r only one died at a time when secondary disease would be expected. Figure 1 shows the changes in leukocyte counts and Figure 2 shows the changes in platelet counts in the group receiving $2.2-3.3 \times 10^9$ homologous bone marrow cells after radiation as compared with the values of six animals selected from the control group who received radiation only.

FIGURE 1

CHANGES IN PERIPHERAL WBC-COUNT IN 2.5-3.5 Kg.
MACACA MULATTA AFTER 700 R WHOLE BODY RADIATION
AND EFFECT OF HOMOLOGOUS BONE MARROW TRANSPLANTATION

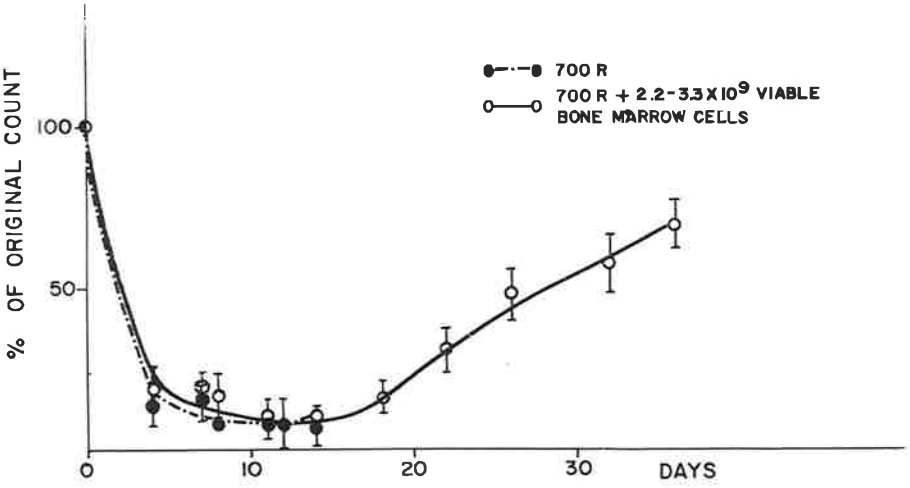
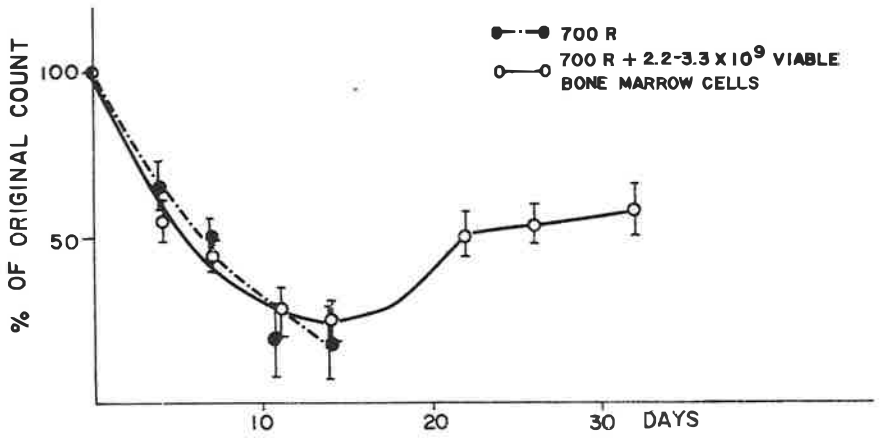


FIGURE 2

CHANGES IN PLATELET COUNT IN 2.5-3.5 Kg.
MACACA MULATTA AFTER 700 R WHOLE BODY RADIATION
AND EFFECT OF HOMOLOGOUS BONE MARROW TRANSPLANTATION



Leukocyte and platelet counts fell in both groups almost indistinguishably. The plateau of leukopenia and thrombocytopenia was reached about the 10th day. At the depth of pancytopenia, the control animals died but half of the experimental animals, whose cell counts were equally low, survived. At about the 20th day, white counts and platelet counts started to increase and reached almost normal values around the 40th day. The hematologic curves of three animals which received bone marrow but died are indistinguishable from the control animals.

Table 2 shows a similar experiment in which 800 r whole body radiation was given. In this experiment no animals survived in the group protected with homologous marrow.

TABLE 2

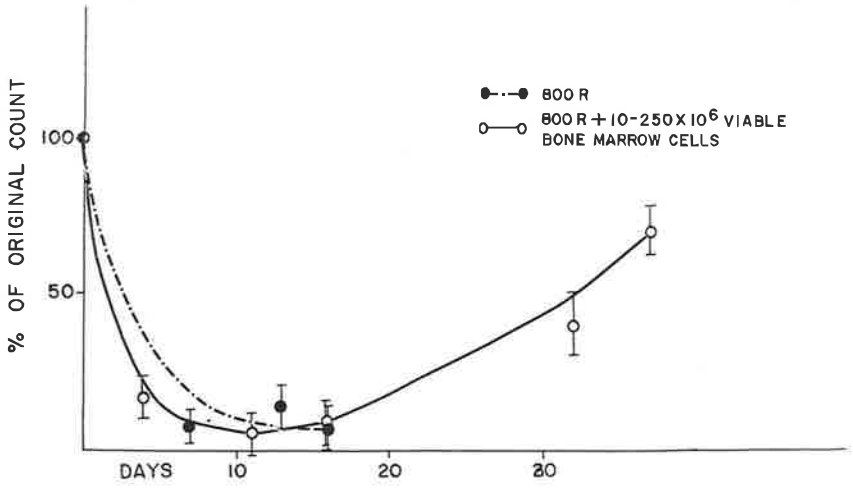
EFFECT OF BONE MARROW TRANSPLANTATION ON LETHALITY OF 800 r WHOLE BODY RADIATION IN 2.5 - 3.5 KG MACACA MULATTA

Type and No. of bone marrow cells transplanted	None	Homologous 250×10^6	Autologous $10\text{–}250 \times 10^6$
No. survived/Total No.	0/6	0/4	2/4
Mean time of death of nonsurvivors	8 days	10 days	14 days
Survivors observed for	—	—	24 months

The number of marrow cells given corresponded to the first group of homologous marrow protected animals in the previous experiment where 25 percent of the animals survived. Autologous marrow protection resulted in survival of half of the animals while in the previous experiment with 700 r, three quarters of the animals survived. Figure 3 shows white cell counts in the autologous marrow protected animals compared with the control group.

FIGURE 3

CHANGES IN PERIPHERAL WBC-COUNT IN 2·5-3.5 Kg. MACACA MULATTA AFTER 800 R WHOLE BODY RADIATION AND EFFECT OF AUTOLOGOUS BONE MARROW TRANSPLANTATION



Results were comparable to those obtained in the experiment with 700 r irradiation. Table 3 summarizes the survival data of the previous two

experiments and those from an additional experiment which employed 750 r whole body radiation.

TABLE 3

EFFECT OF BONE MARROW TRANSPLANTATION ON LETHALITY OF 2 MEV RADIATION IN 2.5-3.5 KG MACACA MULATTA NO. SURVIVED/TOTAL NO.

Radiation Dose	Type of Bone Marrow Transplanted		
	None	Homologous	Autologous
700 r	0/11	12/26	6/8
750 r	0/4	0/6	-
800 r	0/4	0/4	2/4

The question arises why bone marrow protected animals survive at such low leukocyte and platelet counts at which unprotected animals succumb. The question also arose how one could test at an early time after marrow transplantation for "takes" in an autologous transplantation study. Because of the lack of immunologic markers and the apparent absence of differences in the peripheral cell count, we thought to explore the usefulness of lipopolysaccharide stimulation which is known to cause increased release of leukocytes into the peripheral circulation. Figure 4 shows the effect of 5 gamma per kilogram of

E. coli lipopolysaccharide on the leukocyte count of a Rhesus monkey. Following a brief leukopenia, considerable leukocytosis developed and the white counts returned to about the normal level by the end of 24 hours.

FIGURE 4

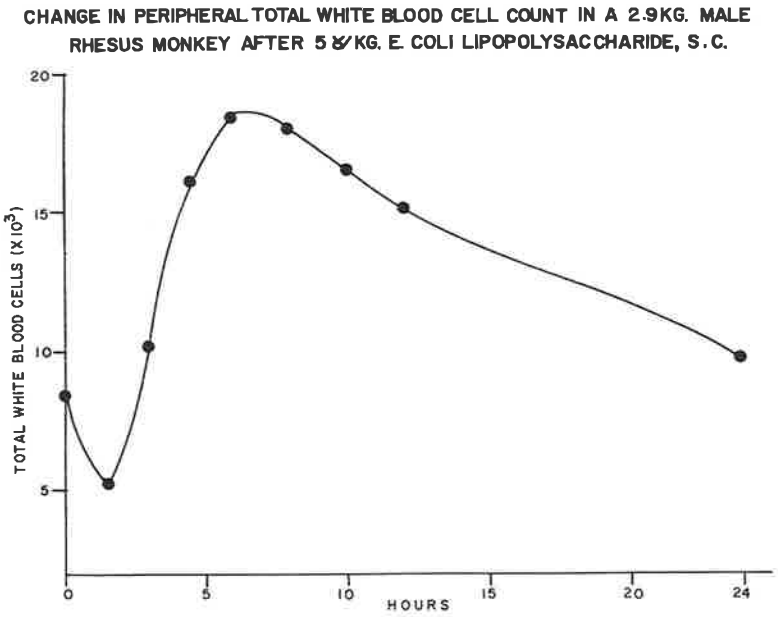
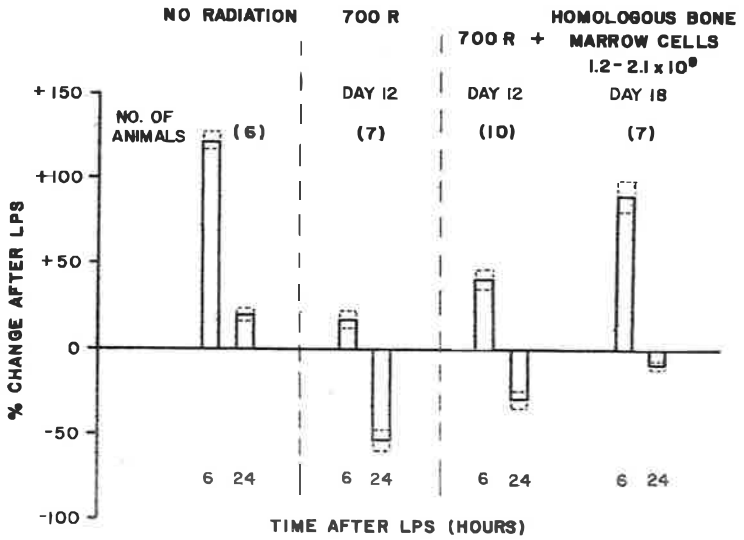


Figure 5 shows an experiment in which control animals, animals radiated with 700 r whole body radiation and animals which received homologous bone marrow transplantation after radiation were compared. Twelve days after irradiation, the leukocytosis inducing effect of the lipopolysaccharide was greatly reduced, in fact, 24 hours after injection the pre-existing leukopenia was accentuated.

FIGURE 5

EFFECT OF 5γ/KG. (S.C.) E. COLI LIPOPOLYSACCHARIDE (LPS) ON THE LEUKOCYTE COUNT OF MACACA MULATTA

MEAN VALUES AND S.E.



In the animals who received bone marrow protection, there was somewhat more leukocytosis and somewhat less leukopenia in response to lipopolysaccharide challenge. By the 18th day, the response of the latter group approximated that of the unirradiated animals. It thus appears that at the time when pancytopenia is at its depth and when bone marrow protected and control animals cannot be distinguished on the basis of peripheral blood cell counts, lipopolysaccharide stimulation will identify those animals in which bone marrow reserves have been established. It is possible that bone marrow transplantation results in active hemopoiesis, however, the number of leukocytes produced are rapidly utilized by the tissues and few are available to increase the circulating count.

Another question is whether homologous marrow protected animals against lethal doses of radiation by replacement of the donor marrow or whether it merely tided the host over a critical period of time and then host type marrow cells reappeared. We hope that studies aimed at answering these questions would also help in understanding why secondary disease was so infrequent in our series. Tables 5 - 8 show immunologic studies in a group of monkeys. Red cell types were determined by the methods of Owen and Anderson (1962). Donors were immunized against typhoid, diphtheria, and tetanus antigens. Baseline titers were determined. It was thought that the immunologically competent donor elements may continue to produce antibodies or at least will provide for characteristic anamnestic reactions. The titers given are the maximal titers obtained within 5 days of a challenging antigen injection. In monkey No. 3, donor type blood groups were noted on the 60th day after marrow transplantation but by the 136th day, the animal reverted to its original type. In monkey No. 26 no donor type red cells were demonstrable at any time but tests for typhoid antibodies indicated a positive anamnestic reaction on the 42nd day after radiation. In monkey No. 11 donor type red cells were found on the 60th day. Two days after this test was performed, the animal died. In this instance, secondary disease should be seriously considered. In monkey No. 91 no donor type red cells were demonstrable at any time. The typhoid test indicated demonstrable antibodies on the 42nd day but no significant anamnestic reaction.

TABLE 5

MONKEY NO. 3, 3.5 KG., MALE

700 r WHOLE BODY RADIATION
+ 3.1 x 10^9 FEMALE BONE MARROW CELLS

	Days after transplantation	Type of Test					
		Blood groups	Typhoid antibody titers and max. response to challenge			Diphtheria antitoxin antibodies, max. resp. to challenge	Tetanus antitoxin antibodies, max. resp. to challenge
			H	O	O-HA		
Donor		aBCd	80	40	160	> 1 U	> 8 U
Recipient	0	AbCd					
	42		<20, <20	<20, <20	20, 20		
	60	ABCd					
	136	AbCd					
	172					<0.01U, <0.01U	<0.01U, <0.01U

TABLE 6

MONKEY NO. 26, 3.2 KG., MALE

700 r WHOLE BODY RADIATION
+3.1 x 10^9 FEMALE BONE MARROW CELLS

	Days after transplantation	Type of Test					
		Blood groups	Typhoid antibody titers and maximum response to challenge			Diptheria antitoxin antibodies, max. resp. to challenge	Tetanus antitoxin antibodies, max. resp. to challenge
			H	O	O-HA		
Donor		aBCd	80	40	160	> 1 U	> 8 U
Recipient alive at 12 months	0	aBcD					
	42		< 20, < 20	< 20, 40	40, 160		
	60	aBcD					
	136	aBcD					
	172					< 0.01 U, < 0.01 U	< 0.01 U, < 0.01 U

TABLE 7

MONKEY NO. 11, 3.1 KG., MALE — 700 r WHOLE BODY RADIATION
+3.22 x 10^9 FEMALE BONE MARROW CELLS

	Days after transplantation	Type of Test			
		Blood groups	Typhoid antibody titers		
			H	O	O-HA
Donor		aBcD	< 20	40	320
Recipient	0	ABcd			
died on	42		< 20, < 20	<20, < 20	80, 80
62nd day	60	ABcD			

TABLE 8

MONKEY NO. 91, 2.2 KG., MALE — 700 r WHOLE BODY RADIATION
+3.25 x 10^9 FEMALE BONE MARROW CELLS

	Days after transplantation	Type of Test				Diptheria antitoxin antibodies, max. resp. to challenge	Tetanus antitoxin antibodies, max. resp. to challenge
		Blood groups	Typhoid antibody titers and maximum response to challenge				
			H	O	O-HA		
Donor		aBcd	20	80	320	>1U	>8 U
	0	aBCd					
Recipient	42		<20, <20	<20, < 20	40, 40		
alive at	60	aBCd					
12 months	136						
	172					<0.01 U, <0.01 U	< 0.01 U, < 0.01 U

In our entire series, only one monkey was suspected of having died from secondary disease. This animal exhibited diarrhea and anorexia before death. It lost about 25 percent of its body weight. Autopsy revealed pneumonitis and pulmonary edema, enteritis with inflammatory exudate overlying the intestinal mucosa, surface necrosis of intestinal epithelium and disappearance of lymphoid nodules from the intestines. Since red blood cell type reversal was not present at the time of death, it is possible that this animal died of secondary disease.

Further studies are required to elucidate the reasons for the relatively low incidence of secondary disease in this series.

SUMMARY

Autologous bone marrow transplantation appears to be more effective in lethally radiated monkeys than homologous bone marrow transplantation. Significantly higher number of homologous marrow cells are required than autologous marrow cells for protection. Platelet and leukocyte counts decreased equally rapidly in control and bone marrow treated animals following lethal doses of radiation. However, control animals died at the depth of pancytopenia while many of the marrow protected animals survived. Injection of a bacterial lipopolysaccharide produces leukocytosis in the marrow treated animals but little or no leukocytosis in control, radiated monkeys. The leuko-

cytosis producing effect of lipopolysaccharides may be a useful tool to determine autologous bone marrow takes. Analysis of immunologic markers indicated that donor type marrow was present in at least some of the radiated recipients. Eventually these animals reverted to the original antigenic types. Possible late disease was observed in only one monkey injected with homologous marrow.

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DISCUSSION

KURNICK I am interested in comparing the results which were obtained with several routes of injection.

C. AMBRUS The reinjection method was varied depending on the condition of the bone marrow suspension. Occasionally we had some agglutinates and particulare matter in it, which might produce emboli when injected by intravenous route. We then preferred to make a small hole in the tibia through which we reinjected the whole bone marrow. This is an easy procedure if the animal is anesthesized for a period of about 5 minutes. Otherwise the bone marrow was always given through the intravenous route. We saw no difference in effectiveness if results with the two routes of injection were compared.

DE VRIES You mentioned that some of your monkeys died before you could expect secondary disease. In our later series of experiments we observed characteristic lesions of secondary disease as early as 6 or 7 days following bone marrow transplantation.

AMBRUS Dr. de Vries, could you tell us whether this particular phenomenon occurs only if you give enormously large numbers of cells? It may be the others who have not found it, have given smaller numbers of cells than you did.

DE VRIES I think you can see this phenomenon even with the minimal number of cells, which you need for obtaining a take of the homologous graft.

MATHÉ I think we observed in man the same phenomenon as Dr. de Vries described. We lost the last patient after 17 days and he had clinical signs of secondary disease, especially erythrodermatosis.

C. AMBRUS In the monkey which died at 62 days we were considering secondary disease. Actually one of the main symptoms was an overwhel-

ming infection. We were not quite sure what contributed to the death. A possibility of course is that infection was due to the presence of secondary disease.

PITCOCK Have you seen at any time jaundice or any lesions in the liver of any of these animals? We have seen this a number of times in animals that we considered to have secondary disease.

C. AMBRUS In some of the monkeys we have seen necrotic areas in the liver. I did not notice any jaundice.

KURNICK I am very interested in the matter of the routes of administration. Congdon demonstrated far superior results after intravenous than after intra-arterial or intramedullary inoculation in mice. I note that you had 4 out of 12 animals dying after receiving autologous marrow. Can you recall whether these 4 received the bone marrow by a specific route of administration? In our own experience we have become quite brave, almost heroic in injecting particulate matter intravenously. In man particles that you can barely squeeze through a 17 gauge needle appear to be entirely innocuous in our experience. Occasionally the patient feels "marbles" running up the arm.

C. AMBRUS As you will have noticed, in the autologous series we have given much smaller numbers of bone marrow cells than in the homologous series, obviously because we had less tissue to provide the bone marrow. This was one of the reasons we tried to recover everything possible and we did not use filtration of the marrow. I am quite sure that there was an equal distribution between the numbers which received intravenous and intramedullary injections. In the homologous treated animals we mostly gave it intravenously because there we were dealing with much larger numbers of cells and we were not afraid of loosing some by filtration.

VAN BEKKUM In contrast to Congdon's results as just quoted by Dr. Kurnick, we have reported previously that in mice intrasplenic injections of bone marrow (isologous) is essentially as effective as the intravenous administration (van Bekkum et al., Revue d'Hematologie, 11, 477, 1956).

KURNICK I would like to raise a question in the matter of the temperatures at which you hold the marrow. We made some studies on the matter of survival using the stain exclusion method. If we kept the marrow at room temperature for 24 hours, we had much better apparent survival by stain exclusion than if we held it at 0-4°C.

C. AMBRUS In this series we have given the bone marrow back after 24 hours and kept it at 4°C. Irrespective from this we have another study, where we were trying to see how long we could preserve bone marrow cells at different temperatures. We kept some samples at room temperature and some at 37°C. We found by the same stain exclusion technique that most of the cells remained viable. In many instances however we had in spite of aseptic techniques an overgrowth particularly with fungi. Therefore we felt that for other types of experiments where it is not the primary aim to study survival of bone marrow cells but rather protection of the animal we should restrict ourselves to a 24 hour maintenance of the bone marrow at 4°C. This seems to be satisfactory to exclude the growth of other organisms.

VAN PUTTEN For your autologous studies you mentioned that your cell dose was lower. Did you find a lower limit, below which you did not get survival or are the mortalities distributed at random among all cell dose variations you used?

C. AMBRUS We did not find a lower limit for survival.

SCHOFIELD I was rather interested in Dr. Kurnick's suggestion, that

storage at 4°C reduced survival of the cells. We kept cells overnight, then looked at them by stain exclusion technique and found virtually no difference at all, either in the number of cells taking up the dye or in the total number of surviving cells.

The other point I would like to mention was in connection with Dr. Ambrus' observation of infection in animals which died later after irradiation. All animals we have had in the autologous system dying after 30 days have been completely sterile. I would suggest that the infection has some considerable bearing on secondary disease.

F. NEWSOME Do you consider anorexia as one of the symptoms of secondary disease in your monkeys?

C. AMBRUS One particular monkey, several days before dying, lost its appetite, started loosing weight, got a slight diarrhea and then died. All the other animals which died at approximately 2 weeks $\pm$ 2 or 3 days had a loss of appetite and loss of weight, but we did not consider this death as death due to secondary disease, we simply considered this as a hematologic death.

AMBRUS I am not a pathologist and I am having an extremely difficult time in distinguishing between secondary disease and radiation effects. If a monkey which exhibits all sorts of symptoms after radiation after which it recovers and appears to be perfectly normal for a period of time and then becomes sick again and dies, we feel comfortable to call this secondary disease. This I believe is a simple functional way of distinguishing secondary disease and radiation effects. I am certainly intrigued by the suggestion that some of the animals that have never recovered from the primary effects of radiation might have died from secondary disease and I would like to have an opinion how one can really distinguish between these conditions.

DE VRIES I will deliver a paper on the pathology of secondary disease, so

I will not give a detailed account now. After the radiation doses we used, the intestinal damage is recovered in about 4 or 5 days. After that period we see that in those monkeys which in our opinion die from secondary disease, distinct intestinal changes are found, which are specific for secondary disease.

TRANSFUSION OF HOMOLOGOUS AND AUTOLOGOUS BONE MARROW IN PRIMATES EXPOSED TO 900r WHOLE BODY X-RADIATION

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Several studies involving the transfusion of homologous and autologous bone marrow in primates have now been reported (Crouch et al. 1961; De Vries et al. 1961; Crouch and Overman 1961; McAlpine et al. 1960; Newsome and Overman, 1960; Rothberg et al. 1959). Crouch et al. (1961) were the first, however, to utilize a marker indicating the success of proliferation of the donor cells, this being the female sex chromatin of the leukocyte. Odell et al. (1957) utilized the serologic difference of donor and host erythrocytes for their studies on homotransplantation of erythroid elements. Piomelli and Brooks (1961) in similar studies with rabbits irradiated with 600 r and 500 r on successive days utilized both methods in combination.

During the course of our experiments Dr. Ray D. Owen detected five antigens on the erythrocyte of the rhesus monkey occurring in various combinations (Owen and Anderson 1962). Two of these were sufficiently different to provide a useful marker for our homotransplantation experiments. The purpose of this report is to 1) relate our experience with rhesus monkeys exposed to 900 r of x-irradiation and transfused with homologous (HBM) and autologous (ABM) marrow cells, 2) to demonstrate the proliferation of erythroid elements in animals receiving homologous bone marrow and 3) to report the serum glutamic pyruvic transaminase levels in animals receiving homologous marrow as contrasted to irradiated animals and those receiving autologous marrow transfusion. The detailed histopathology of these animals will form the basis of a subsequent report (Pitcock et al.).

MATERIALS AND METHODS:

Twenty rhesus monkeys were selected from the colony and divided into

three groups. Group I consisted of 6 animalsreceiving 900 r of x-irradiation only. Group II consisted of 8 animals which received homologous bone marrow transfusion and Group III consisted of 6 animals receiving autologous marrow transfusion. The animals ranged in age from 27 to 33 months and weighed from 5 to 7 pounds. All animals were screened for enteric organisms known to be pathogenic by means of culture and warm stool flotation. No selection was made on the basis of sex nor antigenic cell type other than to assure that the A antigen was present on the red cells of the donor animals and that the C antigen was present on the red cells of the hosts. All animals were observed for a period of two weeks prior to irradiation and baseline values for temperature, weight, and water intake recorded.

IRRADIATION:

Animals in each of the groups received 900 r whole-body irradiation delivered at the rate of 20 r per minute from a Picker X-ray machine, 250 KVP, 18 MA, with 1 mm aluminum and 0.25 mm copper filtration. Each animal was placed in a cylindrical exposure tube 20 cm. in diameter by 40 cm. in height which was rotated 3.5 times per minute. The target distance to the center of the cage was 95 cm.

COLLECTION OF DONOR MARROW:

The donor animals of homologous marrow were anesthetized and exsanguinated. Cells were obtained from the six long bones by removing the condyles and expelling the marrow under pressure with Gey's BSS containing heparin. Additional cells were obtained by slicing thin sections from the bodies of the thoracic vertebrae and gently washing the slices in a baffled tube with Gey's BSS. The marrow suspensions were then centrifuged and the supernatant fat layer removed.

The marrow for the autologous experiments were aspirated from the long bones, screened through stainless steel wire mesh and prepared as above. Marrow for transfusion was held for a period of one hour at room tempera-

ture to allow for irradiation time. The cells were suspended in 20 ml. of Gey's solution and injected into the superficial popliteal vein of the irradiated host animals. Cell counts were made immediately before injection and were performed using 0.1 NHCl acid as the diluting fluid, a standard RBC pipette and a Neubauer hemocytometer.

SAMPLE COLLECTION:

Blood samples were collected from the animals of each group at weekly intervals to minimize handling. Specimens for hematological examination were collected using ethylene diamine tetra acetic acid as the anticoagulant. Hematocrits were done by the microhematocrit method in duplicate. Erythrocyte and leukocyte counts were performed using duplicate pipettes and chambers and a standard Neubauer counting chamber. Differential counts were made on fresh blood smeared on standard acid washed microscope slides. Reticulocyte counts were performed by the method of Brecher et al. (1950) and expressed as the percent of 1000 erythrocytes. The absolute number of reticulocytes was estimated by multiplying the percentage value found by the erythrocyte counts.

Samples for differential agglutination were collected in citric acid, sodium citrate and dextrose solution and shipped under refrigeration to Dr. Ray D. Owen, California Institute of Technology, for blood group determinations (Owen and Anderson 1962).

Serum samples were also collected and the serum glutamic pyruvic transaminase determinations performed by the method of Wroblewski and LaDue (1956).

TREATMENT:

All animals were treated symptomatically. Antibiotics were chosen on the basis of results of culture and antibiotic sensitivity studies. Antidiarrheal agents were used when indicated and electrolyte replacement solutions ad-

ministered to maintain insofar as possible fluid balance. During the period of anorexia, animals were supplemented via gastric tube.

RESULTS AND DISCUSSION

TABLE I

SURVIVAL OF MONKEYS EXPCSED TO 900r WHOLE BODY IRRADIATION

Group	Day 8 Alive/ Total	Day 15 Alive/ Total	Day 22 Alive/ Total	Day 28 Alive/ Total	Day 90 Alive/ Total	Survival Time
I Irradiated Controls	6/6	2/6	1/6	1/6	0/6	12.4 d. *
II Homologous Bone Marrow	8/8	5/8	2/8	1/8	0/8	17.8 d.
III Autologous Bone Marrow	6/6	3/6	3/6	3/6	3/6	71.5 d.

* Excludes monkey 114E surviving 81 days.

MORTALITY:

Table I summarized the survival of monkeys in each of the three groups. All animals survived to the eigth day. At fifteen days two animals of Group I (irradiated controls) survived, five of Group II (HBM) and three of Group III (ABM). At twenty-two days, one animal of Group I survived. This animal lived for 81 days and is the only animal in our experience surviving 900 r x-irradiation longer than 20 days. Two animals of Group II survived to the twenty-second day and one animal to the twenty-eigth day. Three animals of Group III survived beyond 90 days.

CLINICAL OBSERVATIONS:

All animals in each group demonstrated diminished cage activity during the 24 hour period following irradiation. Transient erythema was noted in some but not all animals during the 24 to 72 hour postirradiation period. A latent period of relatively normal activity then occurred until about the

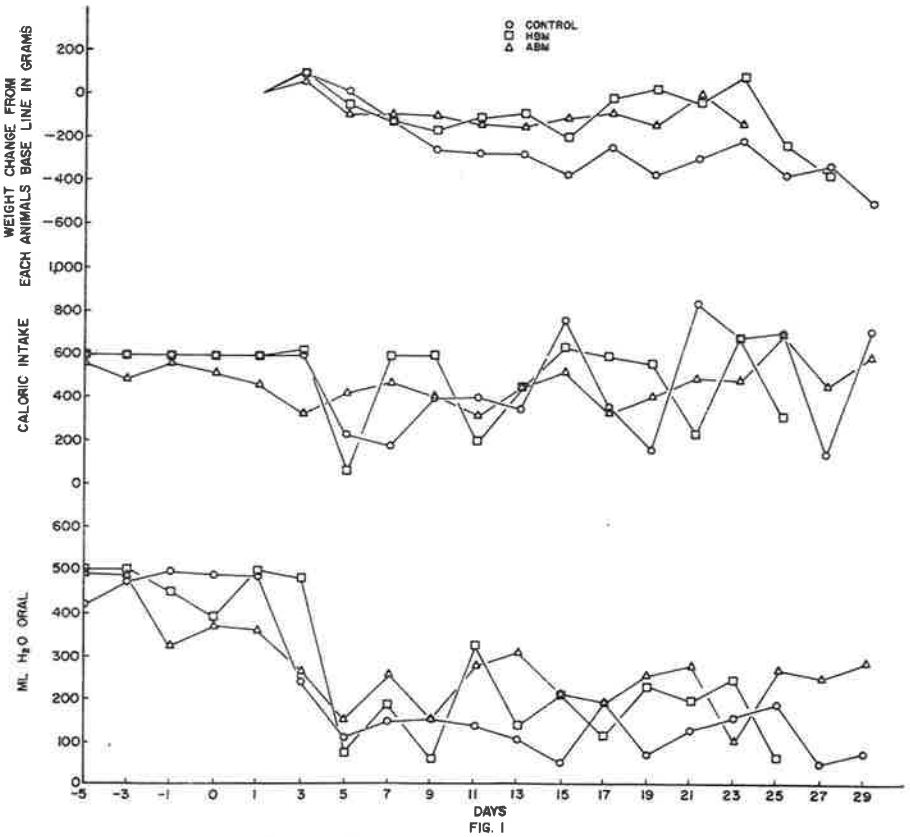

FIG. 1

WEIGHT REDUCTION, FLUID, AND CALORIC INTAKE AFTER 900r X-IRRADIATION

fifth to seventh day when they again became much less responsive, refused food and water, and exhibited diarrhea. Postirradiation weight reduction for each of the three groups is depicted in figure 1. There appears to be a 20 to 30% weight loss when all groups are combined and this is in agreement with the report of Crouch et al. (1961). The oral water intake is also shown in figure 1 and it may be seen that a close correlation exists between water intake and weight loss during the first 15 days. The rectal temperatures for each of the three groups are presented in figure 2. The values shown represent the deviation from the baseline mean for each of the groups for the first 30 days postirradiation. The temperature of this species varies from animal to animal when selected at random. We found, however, that if the animals were repeatedly studied with the same equipment a consistent baseline for each animal could be established. It may be seen that the animals receiving HBM showed a consistent temperature elevation from the sixth to about the eleventh day postirradiation (3 to 8 days post-marrow transfusion). Temperature elevations for Group I, the control animals and those receiving autologous marrow occur at a later point in time. A marked decrease in temperature often heralded the preterminal state.

A bleeding tendency manifest by petechiae at sites where pressure was exerted in restraining the animal and hypodermic injections were given was noted in some animals of each group. Our subjective impression was that this was much less pronounced in animals receiving marrow transfusion, be it autologous or homologous, than in the control animals regardless of the platelet count in the circulating blood.

Diarrhea manifest by liquid stools was observed in all animals. There was no apparent correlation in this study between the occurrence or severity of diarrhea and the presence of enteric pathogens and/or parasites when the diarrhea was first noticed at 5-8 days postirradiation. The influence of normal flora in the irradiated animal and the presence of possible latent

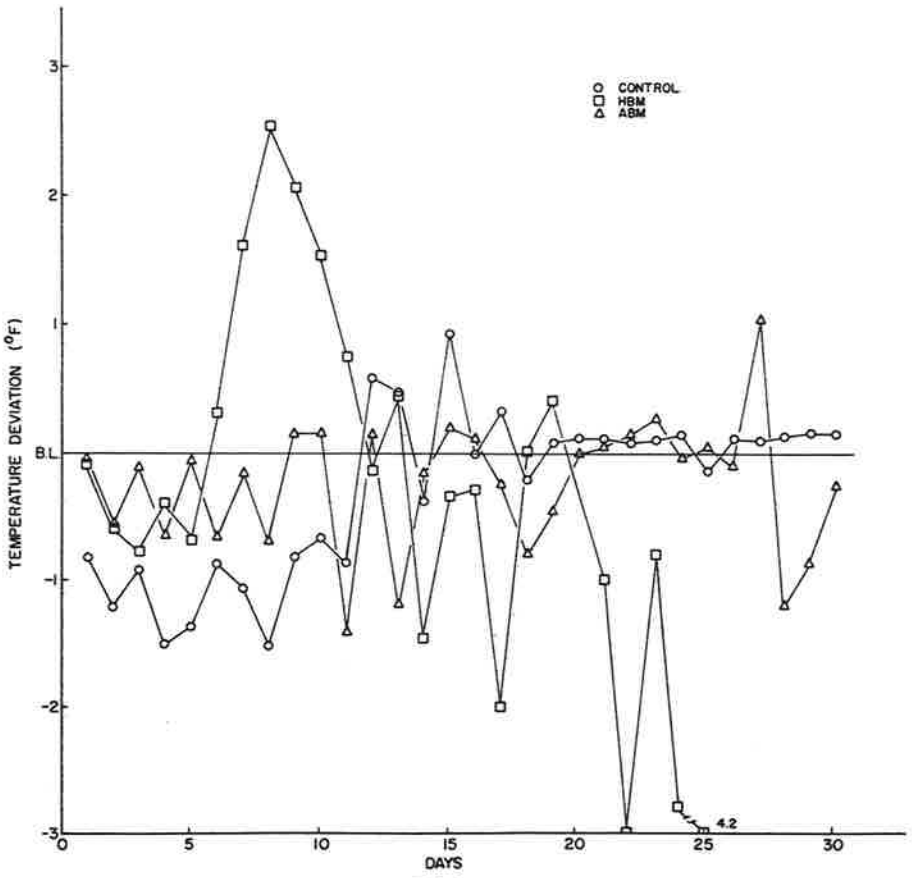

FIG 2

CHANGES IN TEMPERATURE OF THREE GROUPS OF MONKEYS FOLLOWING 900r X-IRRADIATION

virus infections cannot be excluded in these animals.

PROLIFERATION OF DONOR ERYTHROCYTES AND HEMATOLOGY:

Figure 3 illustrates the percent of donor cells found in the blood of the host on subsequent days following the homologous marrow transfusion. All animals with the exception of 59E received homologous marrow. This animal received a chemical protection mixture of AET-cysteine prior to irradiation and is therefore not included in the remaining discussion. Animals 51H and 30H were sacrificed on the day of sampling for a study to be reported subsequently and have also been excluded from further discussion.

Donor cells were detected as early as the seventh day posttransfusion. There was then a progressive increase in the number of cells reaching a maximum of forty-eight percent on the twenty-ninth day. Further observations were precluded by the demise of the animals. In the earlier studies of Odell et al. (1957) utilizing rats irradiated with 700 r x-ray (55 r/min), donor cells were found to first appear 7 days posttransfusion and to reach a maximum of from 0 to 95% fifty days posttransfusion. Piomelli and Brooke (1961) working with rabbit exposed to 600 r and 500 r x-radiation on successive days (20 r/min) found approximately 50% of donor cells present in the blood of the host at 30 days and in animals with successful "takes" complete repopulation occurring at about 10 weeks.

Figure 4 depicts the erythrocyte counts $X10^6$ and the reticulocyte counts $X10^5$ for the irradiated control animals. Also shown are values for the animals transfused with MBM and ABM cells. It can be seen that the erythrocyte counts of the HBM hosts reach a low point on the twenty-second day sample but are greater than the control animal and less than the ABM hosts. The reticulocyte counts of the HBM and ABM hosts appear to increase from one to two weeks ahead of the control animal. The reticulocyte count of the HBM recipients are worthy of note being greater than those of the ABM recipients.

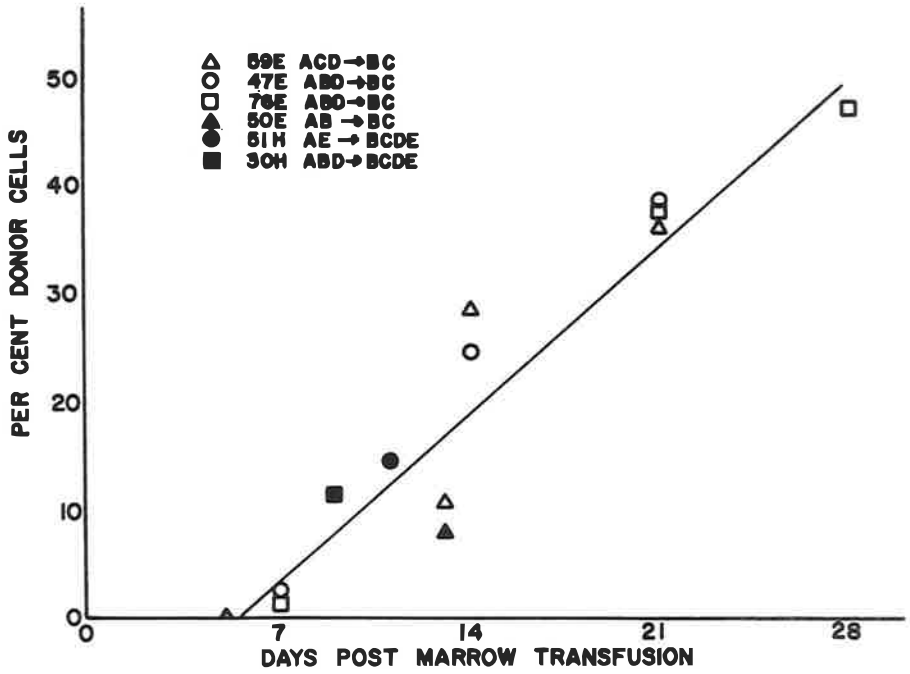

FIG. 3

EVIDENCE OF BONE MARROW PROLIFERATION IN RECIPIENT ANIMALS

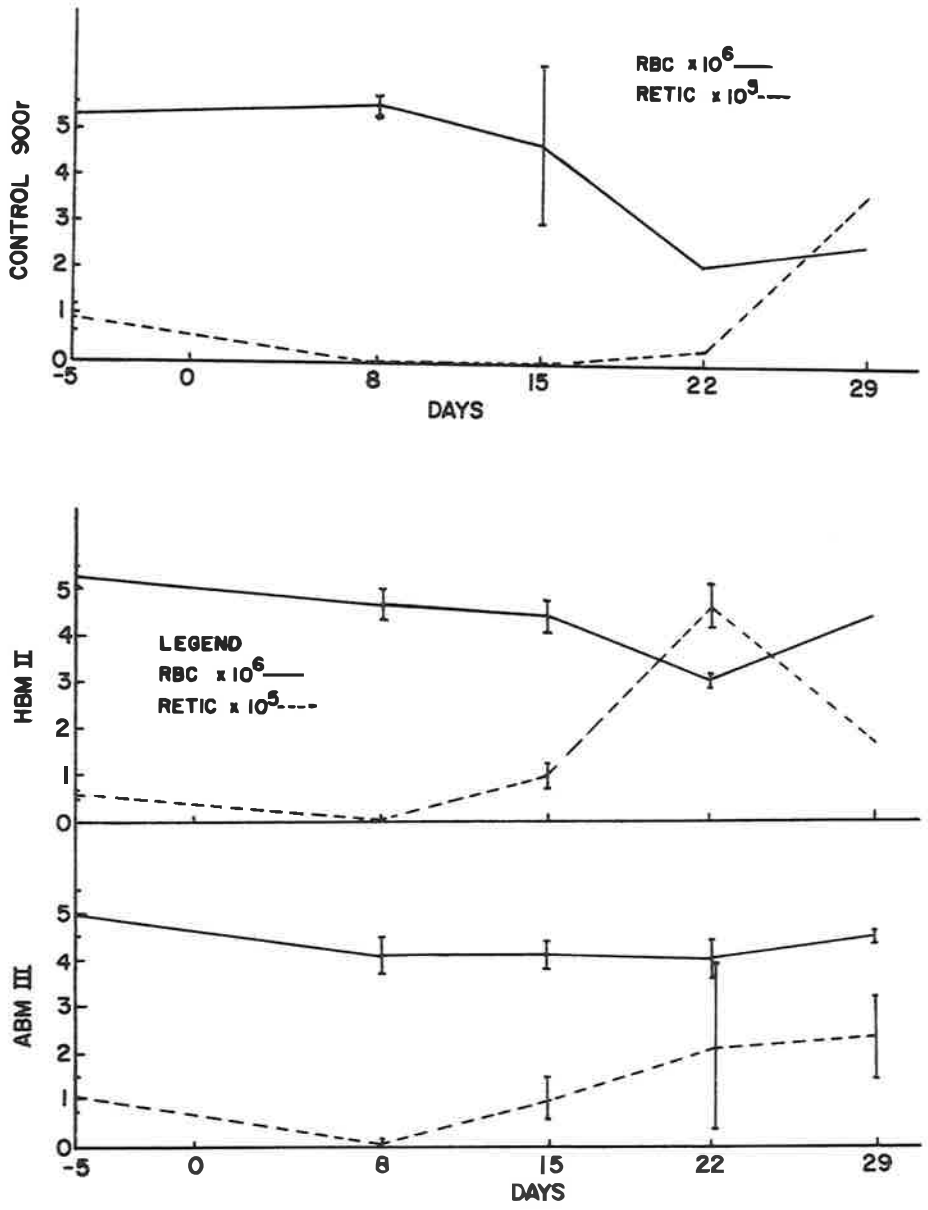

FIG. 4

ERYTHROCYTE AND RETICULOCYTE COUNTS FOLLOWING 900 r X-IRRADIATION

This response suggests either an abnormal release of reticulocytes or the presence of an hemolytic process occurring at this time. Odell et al. (1957) reported that the number of host-derived erythrocytes progressively decreased in successful homotransplants when compared to unsuccessful homotransplants and suggested that the irradiated host marrow was unable to compete succesfully with the implanted marrow. We are unable to detect any significant difference in the total erythrocyte counts of animals in which donor cells are demonstrable when compared to HBM recipients in which donor cells are not demonstrable on the 15th day, although we find that 25% of the total erythrocytes on day 15 are of donor origin in the former group. Piomelli and Brooke (1961) have shown that the life span of host erythrocytes is decreased when injected into the chimera while the life span of donor erythrocytes is normal. If one assumes that the factors controlling the differentiation of erythroid marrow are the same in both instances, viz., successful take and unsuccessful take, and that the erythroid marrow cells of the host are capable of responding to this stimulus, then hemolysis would appear to be the mechanism of greatest importance to account for the decrease of host erythrocytes. Further studies are required to elucidate this point.

Figure 5 similarly illustrates the total leukocyte and total lymphocyte counts of the control animals as well as a group of chemically protected animals. Figure 6 illustrates these values for the animals receiving HBM and ABM cells. Again, it appears that these values reach significant levels about one week before the control animals. The proportion of lymphocytes contributing to the total count can be seen to be greater in the ABM recipients than in the HBM recipients. In the control animal (114E) surviving to 81 days, the proportion of lymphocytes did not return to normal until 60 days postirradiation.

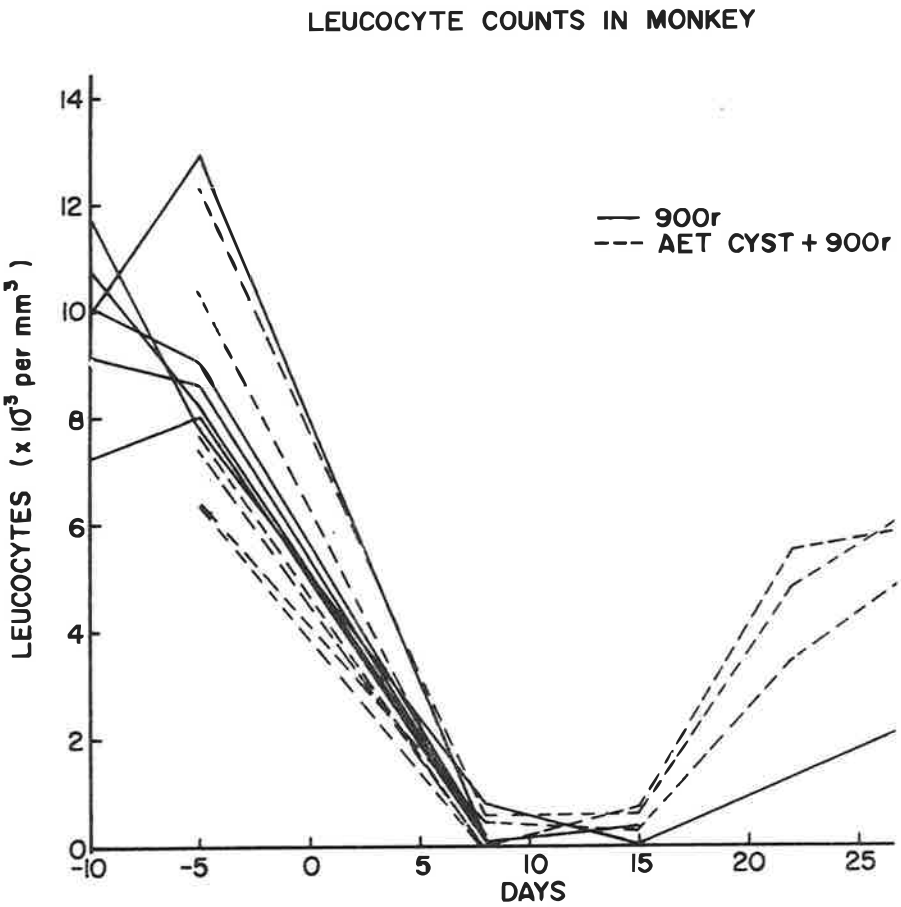

FIG. 5

TOTAL LEUCOCYTE AND LYMPHOCYTE COUNTS FOR CONTROL AND CHEMICALLY-PROTECTED ANIMALS IRRADIATED WITH 900 r X-IRRADIATION

TOTAL LEUKOCYTE AND LYMPHOCYTE COUNTS IN THE ABM AND HBM GROUPS

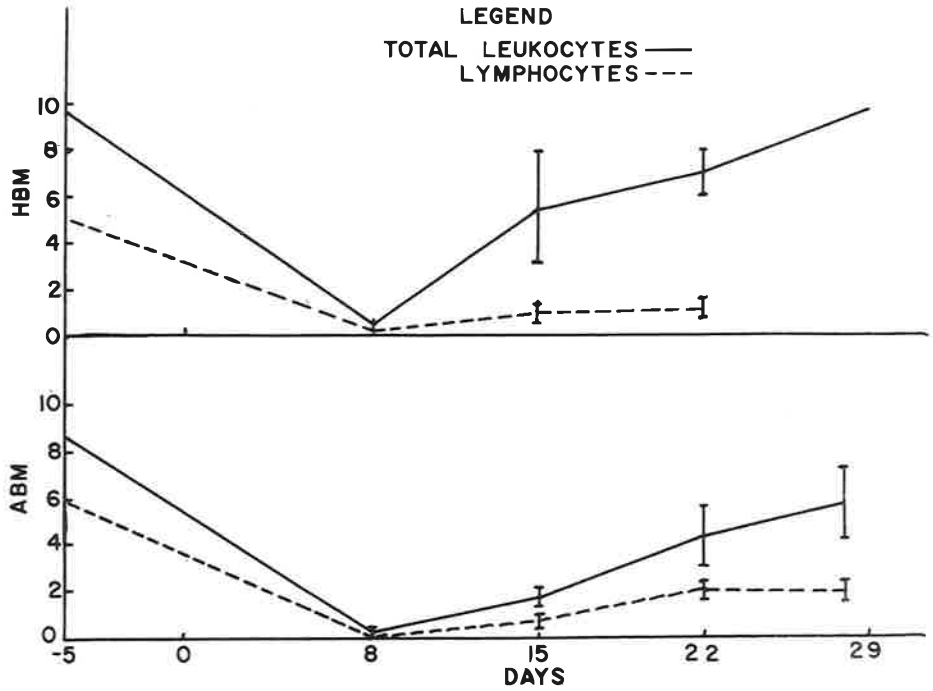

FIG. 6

TOTAL LEUCOCYTE AND LYMPHOCYTE COUNTS IN X-IRRADIATED TRANSPLANTED MONKEYS

SERUM GLUTAMIC PYRUBIC TRANSAMINASE:

The values for SGPT, expressed in Wroblewski units, for the HBM and ABM recipients are presented in figure 7. We selected this enzyme for measurement because of reports indicating that serum levels reflected liver cell necrosis and we had observed necrotic areas in the livers of animals receiving HBM as have De Vries et al. (1961). Recent work by Balaza et al. (1962) utilizing rats exposed to allyl alcohol for the purpose of inducing hepatic necrosis, has shown that indeed a close correlation exists between SGPT levels and areas of necrosis of the rat liver. In those animals receiving HBM and surviving to the 15th postirradiation day, a marked increase in SGPT concentration was observed. This increase was not observed in those animals receiving ABM. The possibility existed that by coincidence these few animals receiving HBM had some other illness to account for the observations such as viral hepatitis. That this is unlikely is indicated by the fact that we have not observed these SGPT levels in 100 animals sampled at random from the colony. We have duplicated these results in animals receiving the chemical protection mixture of AET cysteine plus HBM (to be reported). This elevation is not observed in irradiated control animals nor in animals receiving the chemical protection mixture. Sass and Spear have shown that serum glutamic oxaloacetic acid is increased in hemolytic states. We have been unable to find a comparable reference relating to SGPT; however, Wroblewski and LaDue (1956) report that SGPT is not elevated in "hemolytic jaundice" in man. While this is a nonspecific test for the homograft reaction, being in all likelihood related only to hepatic parenchymal cell necrosis, it may be useful as an indicator of the homograft reaction in the intact animal.

In Table II are presented a breakdown of the data presented for each animal in regard to a) antigenic blood groups of donor and recipient, b) number of cells transfused, c) survival time in days, d) indication of donor

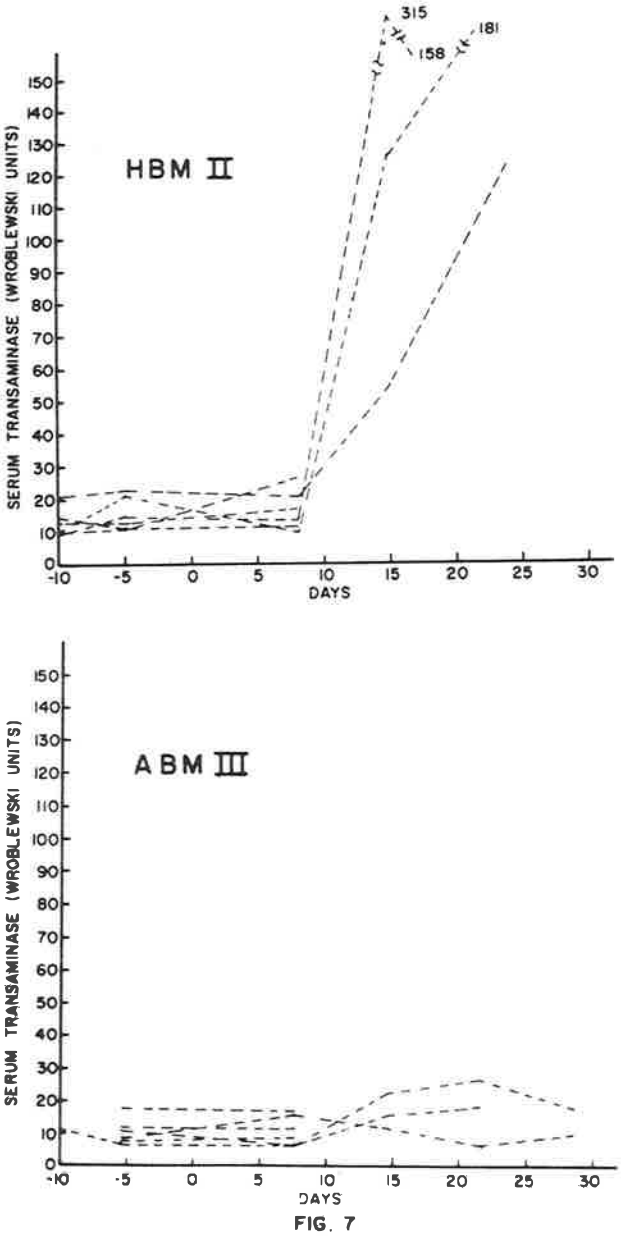

FIG. 7

MEAN SERUM GLUTAMIC PYRUVIC TRANSAMINASE LEVELS IN TWO GROUPS OF MONKEYS AFTER EXPOSURE TO 900r OF X-IRRADIATION

TABLE II

MARROW TRANSFUSION, PRIMATES, 900r WHOLE BODY

	Homologous Bone Marrow								Autologous Bone Marrow					
Animal Number	76E	47E	50E*	103E	34E	85E	104E	96E	127F	98F	106F	116F	30F	84F
Donor Type	ABD	ABD	AB	AB	AB	AB	AB	AB						
Recip. Type	BC	BC	BC	BC	BC	BC	BC	BC						
No. Cells x10^9	1.8	2.2		4.3		3.3	3.9	3.1	†47	218	68	32	353	57†
Survival	29	24	18	18	15	14	13	12	153	124	117	14	11	10
Take	T	T	T	PT	PT	-	-	-						
WBC>10^3	+	+	+	+	+	-	-	-	+	+	+	-	-	-
Retic>5x10^4	+	+	+	+		-	-	-	-	+	+	-	-	-
SGPT > 60	+	+	0	+	0	-	-	-	-	-	-	-	-	-

*650r
†These figures are x10^6

erythrocyte proliferation in the host (T), e) possible takes (PT) based on criteria similar to that published by Crouch et al. (1961) consisting of leukocyte counts in excess of 1000 per mm^3 on day 15 and reticulocyte counts in excess of 50,000 per mm^3 on day 15 and f) SGPT concentrations above 60 units. The data are arranged in order of decreasing survival time of the animals. It is apparent that HBM hosts which survived the longest show donor-erythrocyte proliferation. That this is not an artifact is suggested by the data shown in figure 3 in which it may be seen that each of these animals had donor cells present as early as the 12th day posttransfusion. The donor cells progressively increased to 37% on the 21st day and to 48% on the 29th day in one animal surviving to that time. These three animals, 76E, 47E, and 50E are the only animals of this group in which a take is confirmed. Animals 103E and 134E, however, differed in no way other than the absence of demonstrable donor erythrocytes and are comparable to animals reported by Crouch et al. (1961) in which a take was confirmed. Animals 85E and 104E died with cellular marrows. A similar pattern has been observed in 6 animals receiving AET-cysteine plus HBM cells. In regard to the HBM recipients dying before the 15th day, De Vries et al. (1961) have reported histologic evidence of homograft disease in animals with no evidence of a take in the circulating blood and suggest that a spectrum exists in regard to the homograft reaction in animals receiving a similar dose of irradiation. Piomelli and Brooke (1961) reported data indicating that donor erythrocyte and donor leukocyte proliferation can occur in combination or independently. We would postulate that a similar phenomenon may be exhibited here, viz., that some of the recipients of HBM demonstrate erythrocyte regeneration, some may have had leukocyte regeneration, and some of the animals expire having demonstrated neither.

The recipients of ABM expiring before the 15th day showed no evidence of marrow regeneration. Those animals surviving beyond the 90 day period

died with ulcerative colitis associated with fecal cultures showing Salmonella B.

SUMMARY AND CONCLUSIONS:

Twenty rhesus monkeys were irradiated with 900 r x-irradiation. Six animals served as irradiated controls, eight were transfused with homologous bone marrow (HBM), and six were transfused with autologous bone marrow (ABM). The serologic difference of the donor and host erythrocytes was used as an indicator to establish definite takes in the HBM hosts. Three of the HBM hosts demonstrated donor erythrocytes in the circulating blood which first appeared seven days posttransfusion. Two of the HBM hosts demonstrated leukocyte and reticulocyte concentrations equal to the above but with no evidence of donor erythrocytes. Three of the HBM hosts expired early, two of which demonstrated hypercellular marrows. Three of the ABM hosts expired without evidence of marrow regeneration and three expired after the 90 day observation period with ulcerative colitis associated with Salmonella B infestations. Animals receiving HBM and ABM had significant leukocyte and reticulocyte concentrations approximately one week before the irradiated control animal. The serum glutamic pyruvic transaminase values were markedly elevated in all HBM hosts surviving to the fifteenth day and beyond. A correlation exists between HBM hosts showing donor erythrocytes and their survival time. The data suggest, when compared to that of Crouch et al. (1961) and Piomelli and Brooke (1961), that a spectrum of the degree of graft rejection exists for this species and that hemolysis of host erythrocytes occurs in HBM hosts exhibiting donor type erythrocytes.

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DISCUSSION

SCHOFIELD I wonder if Dr. Young could tell me whether animals dying with autologous transplants at 15 days had any evidence in the marrow of islets of regeneration. I ask this because in looking at these marrows histologically at autopsy we find islets of recognizable marrow cells indicating an early regeneration in the marrow cavity.

YOUNG Yes, for the most part these animals do show marrow regeneration. The animals dying at this early date seem to be dying from an ulcerative colitis of some sort, and I would presume that they are probably overwhelmed by this stress and are unable to compensate for it.

KURNICK In regard to this situation in man, we have also had the experience that those who die of sepsis by the 14th day show very definite islets of active bone marrow regeneration. On the other hand individuals who had been irradiated and not infused with marrow die of sepsis in a relatively short time with persistent atrophy of the marrow.

CROUCH Dr. Young, what do you find the LD_{50} to be in your colony of monkeys? I ask this question in relation to the fact that you chose 900 r in your bone marrow experiments.

YOUNG The LD_{50} seems to range from about 550 r to a possible 625 r. The LD_{100} seems to range up around 650 r to 725 r.

SCHOFIELD Our previously published data on the monkey show the LD_{50} to lie between 500 and 600 r. We have found along this range however, some individual animals who have survived 650 r. Continuing the probit curve upward we would expect perhaps one in 200 survivals at 800 r, providing no other mechanisms of death are involved.

VAN PUTTEN Dr. Young, would you comment on the one animal which survived at 900 r without treatment. Do you think this is the result of sta-

tistical distribution or do you think something unaccounted for happened here.

YOUNG This particular animal was actually comatose and lying in the cage prostrate, which all of us know usually heralds the end. Although we have not developed our capabilities for electrolyte determination, evidently we happened to hit the desirable combination of electrolyte balance in this animal. We gave him 15 ml of a balanced salt solution, and this animal was up and eating the same day and continued to live for 81 days.

VAN LANCKER I would like to comment on the LD_{50} work of Dr. Wolf in the Primate Center of the University of Wisconsin. He has found the 50% lethal dose for rhesus monkeys to be around 600 r; however, he has had animals surviving with doses up to 800 r. Those that survived these doses seemed to be in a heavier weight range than the others.

SCHOFIELD Dr. van Lancker was there any supporting therapy used in these animals that survived 800 r?

VAN LANCKER None whatever; they just survived. If I remember correctly 16% of these animals survived. The animals in our colony are reasonably healthy and I suppose this might be a factor here.

PITCOCK Some years ago we did an LD_{50} experiment in the rhesus monkey in which the animals were irradiated and no further treatment was given. The LD_{50} was approximately 500 r and the LD_{100} was about 650 r. More recently in doing the bone marrow experiments we have noticed an increasing number of untreated animals surviving at higher dose levels. There seems to have been a change in the LD_{50} but I have no idea what it is at this time in our colony.

CROUCH I am sure we all agree that the state of health of these animals makes a considerable difference in the LD_{50} and that for this reason it

might change from time to time. In our laboratory in San Francisco we have seen such a change over a two year period in LD_{50} studies on cinomolgus monkeys. We have seen the LD_{50} on a rather large group of animals shift from approximately 525 r to approximately 625 r over a two year period. The animals used in these experiments however were from various shipments into the laboratory and I am sure the variation in degree of health or sickness plays an important role in the LD_{50} determination.

AMBRUS Dr. de Vries, I should like to ask a question regarding the following situation: let us say we have a bone marrow treated animal dying at around 2 weeks following irradiation and homologous bone marrow with evidence of infection. Would you think that this infection is primarily a radiation consequence or could secondary disease have something to do with the infection? Could both be involved and both together contribute to the animal's demise? If such were the case, do you think that one could differentiate between the various phenomena?

DE VRIES We have noted infections in about half of the monkeys which died after treatment with homologous marrow, some with bacterial diseases, helminthic diseases and in 2 animals we have some indication of viral disease. A few animals are seen which do not show bone marrow regeneration but in which there is evidence of septicemia similar to that seen in irradiated non-treated controls. The death of a number of these animals could be explained on the basis of infections whereas others were not infected to such a great extent. Therefore, I think that the differences in this situation between monkeys and mice are as follows: in those host-donor combinations of mice in which you see sever septic disease most of the animals evidently die of infection. In contrast, only some of the monkeys treated with homologous marrow die directly of infectious disease, and in more than half of these animals death must be attributed to causes other than infections; for example, severe lesions of the intestinal tract and in a few cases le-

sions in the liver.

WHITCOMB I should like to re-emphasize our findings in relation to infection in monkeys. For the irradiated control animals, for those animals receiving homologous bone marrow and for those animals receiving chemical protection + homologous marrow, we have found that all of them had positive cultures in the heart blood at the time of post-mortem. This totalled approximately 18 animals, but we were unable with this number of animals to determine any definite pattern of infection among these three groups.

AMBRUS I should like to ask if anyone has information on what the gut injury dose is in monkeys?

DE VRIES We saw only one case in which the typical intestinal radiation death in monkeys, as described by Wilson, seemed apparent. This animal received 1065 r and died 6 days following irradiation. The animal was extremely dehydrated and at autopsy showed intestinal changes which are well known to be typical of the radiation induced intestinal syndrome.

AMBRUS Do you think therefore Dr. de Vries, that if you found intestinal injury at autopsy in an animal treated with homologous bone marrow after 700 r this would indicate secondary disease rather than primary intestinal injury?

DE VRIES Yes, I think so, because these intestinal lesions are not found in monkeys treated with autologous bone marrow after the same radiation dose. I think that after radiation in this range the morphological damage to the intestine is repaired within the first week following irradiation.

PITCOCK If I may comment on Dr. Ambrus' question it seems to me that we have 3 forms of intestinal injury here. With relatively low doses of radiation for example 600 to 700 r, the intestinal effects are probably not due to a direct effect of irradiation on the gut. This is the sort of thing we see in monkeys that are irradiated with this dose without receiving any

bone marrow transplantation. This is a manifestation of infection in the monkey. Unirradiated control animals will often die with a dysentery-like syndrome showing ulcerations in the gastrointestinal tract, but we see this more commonly with these low irradiation levels. The type of lesion that one sees in secondary disease is somewhat different histologically. The third type of lesion is that which one sees at much higher levels of irradiation for example consistently with about 1500 r and occasionally between 900 and 1500 r and this is the direct effect of irradiation on the gastrointestinal tract.

VAN LANCKER In regard to the intestinal lesions I think we have to distinguish these types in another way at least at 800 r. In our animals which were irradiated at 800 r and received no treatment, death occurred between the 12th and 16th day and the gut epithelium was completely regenerated if any injury had occurred. However, there are various foci of hemorrhage and the hemorrhage is important enough to distend the epithelium and to provoke degeneration. Therefore, I think it is clear that this is a different mechanism than the direct effect of irradiation upon the gut. In the 800 r animals hemorrhage seems to be one of the main factors in the cause of death as far as we have been able to determine.

DE VRIES I agree that in irradiated non-treated monkeys one of the most important lesions is, in fact, found in the colon and these hemorrhages cause necrosis and quite possibly secondary infections. In animals treated with homologous bone marrow we see during the first 9 days and before the 14th day after irradiation and treatment signs of hemorrhage, possibly because of a rather late regeneration of the megakaryocytes and thrombocyte levels in the blood. However, we can clearly distinguish these lesions from those found in typical secondary disease because in the latter case hemorrhages are not found in the mucosa.

VAN PUTTEN Dr Young, you mentioned forced feeding as a way of treatment of your monkeys. We have had some doubt about the efficacy of forced feeding in our own experience. Would you comment on this subject in view of the fact that frequent handling may be traumatic to irradiated animals.

YOUNG I quite agree that one can certainly traumatize these animals with too frequent handling. Originally, we were force-feeding these animals with Sustagen or with some other sustaining ration, and as you, we have just about given this up now, in the subsequent groups. We are relying heavily, however, on electrolyte supportive therapy which necessitates tying the animal down sometime for longer than 30 or 40minutes. However, we make every effort to accustom the animal to this regime which may reduce the trauma somewhat. But I still agree that there is some degree of disturbance or trauma, with such treatment. We must make every effort to minimize this trauma.

VAN PUTTEN I have had some discussions with Dr. de Vries about the cause of jaundice in homologous treated animals. Dr Young mentioned two possible causes of jaundice in his animals, i. e. liver damage and increased hemolysis. Would Dr. Pitcock comment on these two possibilities?

PITCOCK We don't know the cause of the jaundice. We have not actually demonstrated the presence of immune hemolytic anemia in these homologous animals but we suspect that such a thing may be present.

SCHOFIELD Regarding hemolysis in these monkeys, a paper from our laboratory by Gilbert shows that there is some loss of red cells in monkeys irradiated at 600 r without any treatment whatsoever and this loss is over and above the daily average loss one would expect from suppression of hemopoiesis. This is something like 2. 5% per day in addition to the expected 1% per day.

BONE MARROW TRANSPLANTATION IN IRRADIATED MONKEYS.*

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This study includes homologous and heterologous bone marrow transplantations in the rhesus (Macaca mulatta) and cynomolgus (Macaca irus) monkeys. The term transplantation as used here means that the marrow was injected and does not necessarily indicate that it became functional. We have no evidence indicating that the transplants became permanently functional. Our data indicate, however, that the transplanted marrow functioned temporarily in some of the animals. The study includes a group of homologous transplants in the rhesus monkey in which the bone marrow was injected either 30 or 48 hours after sublethal X-irradiation. This work has been reported previously, but will be reviewed here briefly. Two heterologous transplants were done between rhesus and cynomolgus monkeys after 700 r and a larger but still incomplete group of homologous transplants were performed in the cynomolgus monkey after various lethal doses of X-irradiation.

The monkeys were irradiated with a Westinghouse deep therapy unit operated at 240 Kv and 15 ma. The hvl was 2 mm Cu. The rate of roentgen output ranged from 13 to 17 r/min. during the course of these experiments. The animals were irradiated 1 meter from the scource to mid-body. In the groups involving rhesus monkeys the machine was metered monthly with a Victoreen rate meter set by a standardized Co^{60}

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source. For the last group however, the machine was metered just prior to the radiation of each animal. The animals were placed facing the beam for half the period and in reverse for the second half.

In the homologous transplantation following 550 r, marrow was given intravenously mixed with 20 cc of blood, 30 or 48 hours after radiation. No attempt was made to achieve sterile conditions. Out of 7 controls given 550 r only, one died. The monkeys given marrow 30 hours after radiation showed severe radiation damage. Six of seven died within 15 days. The effects of the 48 hour injection were similar to the effects of 550 r only. It was assumed that the marrow was rejected. If given 30 hours after radiation the rejection involved disturbances which added to the radiation damage. If given later, the monkey seemed better able to withstand rejection.

In the experiments to be considered next the function of the donor marrow with respect to erythrogenisis was followed by paper electrophoretic analyses of the recipient's hemoglobin. Some cynomolgus monkeys used had 2 hemaglobin components. These animals were designated as hemoglobin type AB. Other cynomolgus monkeys and all rhesus monkeys had only one type of hemoglobin and were designated as type AA.[2]

In the heterologous experiments donor marrow from a cynomolgus with hemoglobin type AB was given to a rhesus monkey 48 hours after 700 r X-irradiation. The donor hemoglobin did not appear in the peripheral blood. The bone marrow showed only a slight erythrocytic regeneration by the 11th day after radiation. We have observed this, however, after 700 r only. Significant findings at autopsy were pulmonary and intestinal hemorrhages. In another experiment rhesus

marrow was given to a cynomolgus monkey after 700 r (fig. 1). An increase was observed in the proportion of type A hemoglobin to type B when compared to the control proportion and was assumed to be due to the production of donor type A hemoglobin. The results indicated that donor marrow functioned for about 30 days and was then rejected. The animal died shortly after rejection at a time when his own marrow was beginning to function. The animal lost considerable weight and was hyporexic and weak. The skin was somewhat dry and rough but this condition was not pronounced. It seems likely that here as in the sublethally irradiated rhesus transplants the animal died in the process of graft rejection. It is difficult to determine the causes of the long illness during the functioning of the graft. The donor marrow was unable to adequately rehabilitate the peripheral blood. A graft versus host reaction was apparently not the major lethal factor but such a reaction may have possibly added to the basic radiation damage.

A similar situation occurred in the homologous transplants using only the cynomolgus monkey. The same hemoglobin tagging system was used. The results of transplants in this species should be interpreted with due regard to the possible existence of considerable genetic variability. The proportions of monkeys with hemoglobin types AA and AB differed considerably between shipments. A similar situation was also encountered by Owen and Anderson[3] who have used erthrocyte antigen tags in the rhesus monkey. The cynomolgus monkey inhabits a long U-shaped disconnected geographical area. Some of the transplants in this group were between animals from widely separate areas while in other experiments monkeys from the same area were used.

The donor marrow was mixed with Hank's saline under sterile conditions and injected intravenously 24 hours after various lethal doses of X-irradiation. Tests for viability with Eosin Y suggested a cellular mortality from 13 to 57% just prior to injection. Sections of lymph nodes obtained by biopsy 4 and 9 days after radiation were stained with Hemotoxylin and Eosin. All of the transplants involved donors with hemoglobin type AB and recipients with type AA. The control animals which did not receive marrow showed considerable variation in their reaction to radiation (Table I). Even though one sensitive animal died after 500 r, a few survived 900 r.

Control monkeys suffered severe bone marrow and lymph node damage. Slight if any recovery was noted in the animals which died. The peripheral blood of all monkeys reflects this damage. The leucocyte count dropped below $1000/mm^3$ within about a week. The hemoglobin dropped steadily. Few if any reticulocytes appeared. All suffered hyporexia, diarrhea and weight loss. Those which died within 7 days did not show noticeable hemorrhage at autopsy. The monkeys which died later had scattered pulmonary and intestinal hemorrhages. Some had petichiae on the surface of the ventricles. The tissues were usually very pale. A few animals had some pulmonary edema. Infection was not obvious. Some had moderate worm infestations. The worms were encased in the walls of the large intestine and caecum. Histological analyses indicated some liver necrosis and slight necrosis and sloughing of the intestinal mucosa. The spleen and the lymph nodes were mostly inactive. The follicles when visible were very small and probably not very active.

In the animals which received bone marrow the peripheral blood picture was very similar to that in the controls. Donor hemoglobin

was not detected. The bone marrow, however, showed some regeneration beginning about day 7 after radiation. The appearance of the different elements was extremely variable. The lymph nodes showed very little recovery. In a few animals slight nodular activity was indicated. The condition of the animals at death differed little from that of the controls. Hemorrhage, however, was less extensive or absent in those animals which had some megakaryocyte regeneration. Some of these monkeys had a functional bone marrow as indicated by histological study and peripheral blood findings. It is difficult to say if the early regeneration is of donor origin. If so the reaction of the host against the graft was not intensive. Erythrophagocytic histiocytes were present in the recipient animals which lived longer than 7 days as well as in a few of the controls. Their somewhat greater prevalence in the animals with transplants however, may be an indication of a reaction of the host against the graft. The findings are not consistent with a graft versus host reaction. It would seem then that the early bone marrow proliferation was not effective enough to prevent death from radiation.

In this work we have been very fortunate to have the helpful cooperation of the Department of Radiology of the University of Tennessee Medical Units. We also appreciate the assistance given in the analysis of the autopsied tissues by Mr. D. H. Knott, Dr. A. C. Upton and Dr. G. E. Cosgrove.

Figure 1

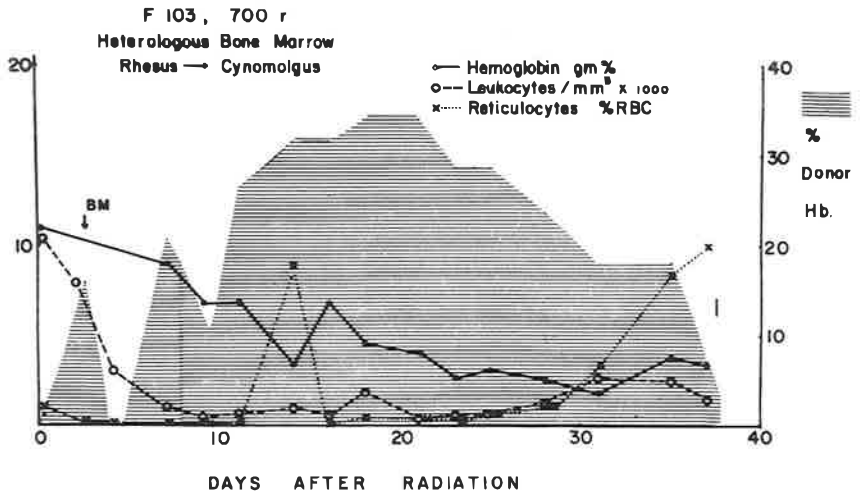

Table I

HOMOLOGOUS BONE MARROW TRANSPLANTATION
IN THE CYNOMOLGUS MONKEY

	Dose r.	Number of Animals	Survival Time Days
Control	500	2	17, 1 survived
	550	2	19, 20
	700	2	14, 17
	750	3	7, 13, 12
	900	4	12, __, 90, 1 survived
	1000	4	7, 13, 13, 16
Experimental	700	3	9, 11, 12
	750	5	9, 10, 15, 16, 1 survived
	900	1	12
	1000	2	8, 20

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DISCUSSION

CROUCH With regard to the location from which the animals came the possibility must be contemplated that Dr. Newsome's transplants were heterologous rather than homologous since two well-known species of cynomolgus monkeys, Macaca irus and Macaca philippinensis exist with a distinct geographical pattern, though there is a great deal of crossover.

NEWSOME There would be no objection to change homologous in the title into "heterologous". The monkeys from the Philippines look somewhat different but Philippine type monkeys had sometimes been obtained from Pakistan. Distribution of the monkeys seems to be patchy, small colonies live together they don't mix too much. A genetic variability can be expected.

CROUCH The importer cannot always tell the exact area from which the monkeys originally came and the subtle differences of appearance make a distinction between the two types (Macaca irus and Macaca philippinensis) difficult. Therefore we would welcome any suggestion as to how to differentiate between them.

NEWSOME No great difference in the number of "takes" was noticed between monkeys from the same region or from different regions.

CROUCH Had not differences in the hemoglobin-pattern been observed?

NEWSOME Such differences were indeed seen but were not a consistent finding.

OVERMAN What is the actual basis for the distinction between the two species.

RIOPELLE did not know this but a useful basis for a distinction might be Dr. Morris Goodman's (Wayne State University) finding that the Philippine

Macaque showed one single plasma protein pattern whereas 15 plasma protein patterns were found for the Malayan type.

VAN LANCKER Had there been an opportunity to investigate the hemoglobins of monkeys from India electrophoretically?

NEWSOME Some of the cynomolgus were from Pakistan. Details of the hemoglobin electrophoretic analysis can be found in a publication by Dr. Tuttle et al. (Science, 133, 578, 1961).

VAN LANCKER Dr. L. Gottlieb had studied the hemoglobin electrophoretically (by an identical method) and in a rhesus colony of about 500 monkeys only one type of hemoglobin was found. Electrophoretically this hemoglobin moved similarly to human hemoglobin A. A few pig-tail monkeys (Macaca nemestrina) had hemoglobin moving much faster than rhesus hemoglobin. These pig-tail monkeys were crossed with rhesus and their offspring showed both types of hemoglobin.

KAY If the hemoglobin marker is not always a sensitive indicator, one could improve on this by centrifugation to get a reticulocyte-rich layer at the top so that in the case of regeneration of donor marrow a high concentration of donor hemoglobin should be obtained.

KURNICK What method of cooling the marrow suspension had been used?

NEWSOME The suspension was kept in an ice water bath for a short time only as it was attempted to inject the suspension as soon as possible after preparation.

VAN PUTTEN Was the assumption that proliferation of the graft occurred as long as there was donor hemoglobin quite correct? Would it not be better to presume proliferation of the graft only during the presence of reticulocytes and only during the increase of the percentage of donor hemoglobin since this donor type hemoglobin naturally does not disappear immediately?

NEWSOME This was indeed quite likely and the graft was probably being rejected while the percentage of donor hemoglobin was high.

CROUCH Is this evidence of graft rejection then the reason to assume that in the case of the heterologous combination there was no graft-versus-host reaction and no evidence of secondary disease?

NEWSOME No definite conclusion was drawn. There may have been some reaction though the animal did not die solely due to the graft reacting against the host. It was assumed that at the time the animal died the graft was being rejected.

VAN BEKKUM Should it be concluded from the data presented today and those published in a previous paper (Blood, 16, 1762, 1960) that the midlethal dose effect (MLD effect) does actually occur in monkeys? Should the radiation dose be lethal or supralethal in order to obtain survival when primates are treated with foreign bone marrow?

NEWSOME No definite answer can be given though the one experiment where 700 r was used indicates that chances of a graft take were better at a lethal dose than at 550 r when the graft was presumably rejected.

OVERMAN In view of the frequent lack of knowledge of the amount of radiation which a patient has received it seems important to know what the effect of marrow would be after sublethal exposure. If it is dangerous, then dosimetry data would be extremely important before marrow therapy is attempted. Our experiments were undertaken not so much to produce survival of the animals but to see whether marrow under these circumstances might be worse than no marrow at all.

VAN BEKKUM Was there a definite conclusion?

OVERMAN There is no clear indication from these experiments on monkeys that early use of marrow following sublethal radiation is worse than no

marrow at al.

NEWSOME If a dose of irradiation is not known but suspected to be sublethal it might be better, in humans, to postpone giving the bone marrow until one knows more about the clinical course of the case.

MATHÉ It should be recalled that Uphoff demonstrated that in mice there are only two strain combinations where bone marrow administration after sublethal doses of irradiation is detrimental whereas into other strain combinations it is not. There is no evidence of a detrimental effect of homologous marrow in sublethally irradiated humans. In the case of the Yugoslaves, who received something like a 75% lethal dose of irradiation, administration of bone marrow seemed to have had a good effect. The clinical aspect of the patient seems to be a better indicator for bone marrow therapy than dosimetry.

VAN BEKKUM Similar arguments were put forward by Dr. Loutit about 2 years ago at the Geneva Conference on Diagnosis and Treatment of Radiation Injury (Proceedings published by the WHO, Geneva 1961) and he concluded that homologous bone marrow in the treatment of sublethal irradiation was probably not too harmful. The results that were obtained since. however, might make us change this attitude and consider the possibility that, in primates, early administration of homologous marrow to sublethally irradiated subjects may be dangerous. Could anyone of those present add to the available information on this subject?

AMBRUS Referring to some of our preliminary experiments we feel that in the sublethal situation where homologous marrow is rejected one has not protected this animal against irradiation but one is not worse off than if no marrow had been given at all. If, however the graft takes and develops antibodies against the host, as happens in mice, one is clearly worse off. Pretreatment of donors with radiation, alkylating agents or steroids to suppress

antibody forming elements selectively seem to have reduced the graft-versus-host activity somewhat. The effect, however, was too small, to have any practical significance.

VAN PUTTEN would like to add that in his experiments one animal treated with homologous marrow after 550 r survived after what was probably a temporary take and reversal to host type cells.

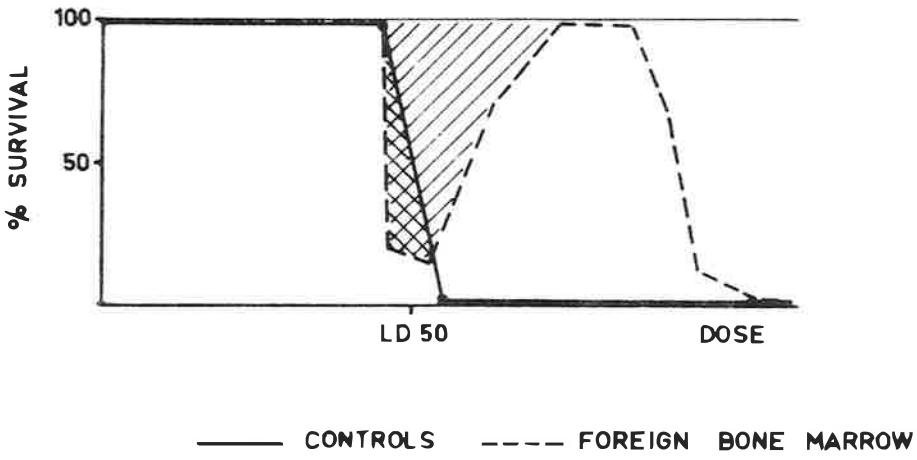
CROUCH asked Mathé whether in the Yugoslaves too, there might have been a temporary take similar to the case just mentioned by van Putten.

MATHÉ Yes indeed. As indicated by the red cells there was a temporary graft and the radiation dose received accidentally was estimated to have been 75% lethal (see for further details: general discussion on human applications of bone marrow transplantation).

VAN BEKKUM It has been proposed to use the term MLD effect only when foreign bone marrow increases the 30 day mortality as compared to no treatment at all - and this terminology was used for mice where secondary disease does not influence the picture much before 30 days. In slide 1

MLD EFFECT

Slide 1



the doubly hatched area represents the MLD effect. In mice it has also been demonstrated that the MLD effect is the result of the rejection of the graft. From previous results, especially the experience with the Yugoslaves, one was inclined to think that there was no deleterious effect of homologous marrow after sublethal irradiation. The data that have been presented at this meeting make one feel less sure.

AMBRUS We will have an opportunity later on in this conference, to review data on homologous bone marrow protection after LD_{50} and LD_{100} doses of nitrogen mustard. No increased mortality could be contributed to the homologous bone marrow transfusion after an LD_{50} as compared to the effect after an LD_{100}.

SOME ASPECTS OF PROTECTION OF RHESUS MONKEYS AGAINST LETHAL IRRADIATION WITH AUTOLOGOUS CELLS

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In common with other workers who have carried out bone marrow transplantation in monkeys we did so because we felt that some bridge should be built between experimental transplantation in rodents and therapeutic marrow transfer in humans. We also thought that we should initially attempt to sepcify conditions under which survival could be maintained using autologous marrow before proceeding to the infinitely more complicated system of homologous transplants or other modifications. The autologous system provided information which still occupies our attention.

METHODS

The monkeys (Macaca mulatta) weighed between 3 and 5 kg. at the time of irradiation and all except one were imported animals. The one exception was born in the colony. Before inclusion in an experiment each animal was screened by tuberculin testing and X-radiograph of thorax, and normal weight increase was confirmed. A series of normal and consistent blood counts was also obtained from each monkey before use.

Bone marrow was obtained from the shaft of the humerus by cutting through the tissues and exposing the bone, drilling into the marrow cavity with a dental drill and replacing the drill by a needle which fitted tightly into the hole. A mixture of marrow and blood was withdrawn into a succession of syringes containing heparin and injected into a known

volume of Hanks' solution. In later experiments the yield of cells was increased by injecting a few ml. of Hanks' solution into the marrow cavity and immediately withdrawing it. The cells were prepared for re-injection by straining through diminishing grades of stainless steel gauze in order to obtain a discrete cell suspension; centrigugation and removal of the supernatant fluid and fat by suction; and dilution in fresh Hanks' solution ready for administration. A total, and also a viable, nucleated cell count was made at this stage; there was invariably little difference between the two.

In one experiment lymph node cells were injected in addition to the bone marrow cells. The nodes were obtained from the axilla by blunt dissection and, after being chopped with scissors, were processed in a similar manner to the bone marrow.

The cells were re-injected intravenously within 12 hours of irradiation, usually about 3 hours later. When lymphoid cells were given these were injected as a mixed suspension with marrow cells. The numbers of nucleated cells injected varied between 2×10^7 and 5×10^8.

The animals were irradiated as described by Haigh and Paterson (1956) but most of the irradiations were carried out with a 300 kvp machine using the modifications described by Gilbert, Paterson and Haigh (1962). Briefly, the animals, lightly anaesthetised with thiopentone sodium are placed in oblong boxes and are packed with bags containing tissue equivalent bolus. The anaesthesia is brief and the monkeys are conscious during irradiation. The dose rate at the centre of the animals was between 3.2 and 5.4 rads per minute and the total doses given were either 800 rads or 850 rads. In one experiment three monkeys had

to be irradiated by means of a 250 kvp machine (HVL 1.6 mm.Cu.,8mA) due to failure of the 300 kvp apparatus. The dose rate in this experiment was only 1.4 rads per minute. From data collected by Daquisto and Blackburn (1960) on LD 50 (30) in monkeys no dose rate effect is seen between 3 rads / minute and 23 rads / minute. Probably below 3 rads / minute there is a dose-reducing effect and we have assumed that 890 rads at 1.4 rads / minute is similar in biological effect to 800 rads at 3-5 rads / minute, a 10 per cent reduction.

For several days before, and for up to several weeks after the irradiation the experimental animals were isolated from the main colony and admission was restricted to the necessary minimum. Blood was obtained by intravenous puncture for blood counts and bacteriological culture at various times.

RESULTS

In this series 19 monkeys were irradiated. Table I shows the X-ray dose, the number of cells administered and the length of survival. Of the 19 animals irradiated 6 survived beyond 30 days, i.e. had haemopoietic recovery, and 5 of these are still alive. The remainder died between 7 and 23 days after irradiation, but it is obvious that the length of survival cannot be equated in any way with the number of cells administered.

Table 2 indicates the antibiotic treatment given and also the results of blood cultures taken at, or near, the time of death. In the event of a monkey dying during the night when a blood sample could not be obtained without considerable delay no culture was performed. The monkeys can be conveniently divided into two groups: 1 - 7 in which anti-

biotic treatment was given haphazardly and only in response to symptons which were considered to indicate the possibility of infection; 8 - 19 were submitted to a routine course of antibiotics consisting of penicillin and tetracyclin (Archromysin, Lederle), the latter being considered especially important in view of the type of bacteria found in blood cultures. In group I there were no survivors whereas 6/12 survived in the second group.

Blood counts were performed at various times before and after irradiation; the results can be seen in figures 1 - 5. The curves are on semi-log scale and constructed from mean values of all counts at a given point, calculated as percentages of pre-irradiation counts. Counts from both survivors and non-survivors are included, since there was no difference between the appearance of the curves for the two. For comparison, the corresponding curves for monkeys given 500 - 550 rads X-rays, without bone marrow treatment, are also shown.

Each limb of the reticulocyte curve in the present series is advanced upon the 550 rads group by 3 or 4 days, i.e. disappearance, recovery and return to normal are all somewhat faster. This is not significantly reflected in the erythrocyte counts but considering the relatively small decrease in red cells and the factors affecting these counts, this is not surprising.

The disappearance of neutrophils also occurs more rapidly in the present series and return to normal values precedes that in the 500 - 550 rad group by about 5 days. It appears that the steady state after 800 - 850 rads is only about 80 per cent of the pre-irradiation level but the later part of this curve represents only 5 or 6 monkeys

whereas the 500 - 550 rad curve represent approximately 50 animals.

The platelet count falls to lower levels after 800 - 850 rads and marrow transplantation than after 550 rads only, but the return to pre-irradiation levels occurs considerably earlier - 30 days as compared with 80 days.

In contrast to the earlier recovery, after 800 - 850 rads and bone marrow, of the blood counts of circulating elements produced by the marrow, the return to pre-irradiation levels of lymphocyte counts is retarded as compared with the 500 - 550 rad counts.

DISCUSSION

Since all these transplants were with autologous marrow there was no marker system which we could employ and, therefore, we have no proof positive that the injected cells have, in fact, acted as a graft. But the evidence from the blood counts indicates that repopulation of the marrow was accelerated by these cells. Release of circulating cells derived from bone marrow was earlier, despite the higher dose of radiation, in the replanted group; re-appearance of cells of extra-medullary origin was retarded in agreement with the effect one might expect from a tissue to which no assistance toward re-population had been given.

It would be simple to say, then that a graft had taken and that therefore the animals had survived, but the explanation cannot be so facile. In the first place re-population of the marrow does not guarantee survival and the cause of so-called "haemopoietic death" is not inability of the marrow to show histological recovery from these doses of radiation. In this series only two of the marrows examined histologically from autopsy showed no islets of regeneration. Of these two, one received

only 2 x 10^7 cells and died 7 days after being irradiated; the second was given, in addition to 10^8 marrow cells, 2 x 10^8 homologous lymphoid cells and suffered the consequent disasters. The remainder all showed evidence of recovery to various degrees. At autopsy, 23 days after the irradiation, monkey 2 had a largely repopulated marrow, but he also had an E.Coli bacteraemia. The repopulation of the others was less than this but there were at least small islets of repopulation with recognisable marrow cells. With the two exceptions already mentioned it is obvious that the marrow was capable of repopulation and it appears that the answer to the question of whether the marrow will recover or not depends on whether the monkey survives rather than the more popular concept of survival depending upon ability of the marrow to recover.

"Haemopoietic death" has a number of causes, prime among which are generalised infection and haemorrhage, both of which can arise as a result of depletion of circulating blood elements. Access of gut flora into the circulation is normally prevented by an intact gut wall and possibly also by antigenic defences by the gut lymphoid tissue. Suppression of invading bacteria could normally be effected by circulating phagocytes and by anti-bodies produced in functional lymphoid tissue. All these defence mechanisms suffer damage by ionising radiation. Bone marrow transplantation of sufficiently large numbers of cells will replace the phagocytes and may allow the animal to survive until the other normal mechanisms for preventing invasion by bacteria have had time to recover. But I question whether bone marrow transplantation does much more than this and, therefore, whether bone marrow grafting is the only, or an essential, means of preventing death from the radiation

doses being considered here.

The fact that an irradiated animal can acquire a systemic infection from its own intestinal tract has been shown by various workers with mice, rats and dogs, and experiments have been conducted to evaluate antibiotic treatment of the radiation syndrome. Sorensen et al (1960) conducted an experiment in dogs in which, by use of antibiotics and blood transfusion, mortality after 400 rads was reduced from 9/10 to 2/10. They observed that the need for platelets was reduced in the absence of infection. Our observations in monkeys support the idea that haemorrhage often accompanies infection but is much less common alone. In the absence of bacteraemia severe haemorrhage is rare, the occurrence of skin petechiae is greatly reduced, and it is significant that haemorrhage in the small intestine (which is bacteria-free) is not seen, but massive haemorrhages arise in the caecum and colon.

In all the monkeys in this series which died in less than 30 days and at a time when terminal blood culture could be performed immediately, a bacteraemia was detected, whereas in all 30-day survivors the blood remained sterile throughout. It is probable that generalised infection plays a greater part in death or survival from radiation (at dose-levels below those causing early gut death) than the question of whether or not the bone marrow will function again. Two important considerations must, however, be observed: (a) antibiotic treatment must be instituted before symptons of bacteraemia emerge, i.e. before large numbers of bacteria are circulating; (b) even though an antibiotic having the required spectrum is used resistance is sometimes encountered and death of an animal treated in this way does not necessarily occur without bacteraemia.

Dr. J. W. Byron, working in this laboratory, is accumulating information, which will be published shortly, that monkeys can survive 800 rads whole body irradiation without the use of bone marrow and even without the use of any viable cells. He employs a strict antibiotic regime and this is now being applied to monkeys as the only form of treatment. He also has evidence to show that in monkeys given 800 rads the loss of platelets is considerably more rapid in the absence of antibiotic treatment than when treatment is carried out. Even when the platelet count has fallen to almost zero there is a very low incidence of skin petechiae and haemorrhage when bacteraemia is prevented.

It is important to determine exactly how bone marrow, when injected after irradiation, prevents death of the animal. If cells are performing a function which could be undertaken by some non-cullular substances "haemopoietic death" can be specified in terms we can more readily understand and the problem of secondary disease becomes one of academic interest.

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No.	X-ray Dose	Cell numbers	Survival (days)	No.	X-ray Dose	Cell numbers	Survival (days)
1	800	2×10^7	7	10	800	5×10^8	9.5
2	800	5×10^7	23	11	850	1.06×10^8	49
3	800	3.1×10^7	14.5	12	850	2.07×10^8	14
4	800	5.3×10^7	13.5	13	850	2.71×10^8	12.5
5	800	10^8 plus 2×10^8 homologous lymphoid cells	10.5	14	850	3×10^8	13
				15	850	3×10^8	13.5
6	800	10^8	14	16	850	3×10^8	500
7	800	10^8 plus $\times 10^8$ autologous lymphoid cells	18	17	890	4.75×10^8	8.5
8	800	5×10^8	450	18	890	1.63×10^8	600
9	800	5×10^8	450	19	890	2.02×10^8	600

Table I. Details of irradiation, cells injected and survival in Rhesus Monkeys

No.	A/b	Days after radiation	Route	Blood culture
1	T	6, 7	Oral	E.Coli
2	T	19,23	Oral	
	C	6, 19-22	Oral	E.Coli
3	T	13-15	Oral	Strep. Viridans
4	T	13,14	Oral	Not cultured
5	None			E.Coli
6	None			E.Coli
7	None			
8	P	1 - 20	I.M.	No
	T	4 - 25	Oral	growth
9	P	1 - 20	I.M.	No
	T	4 - 25	Oral	growth
10	P	1 - 9	I.M.	Not
	T	4 - 9	Oral	cultured
11	P	1 - 20	I.M.	No
	T	4 - 26	Oral	growth
	S	7 - 11	I.M.	
12	P	1 - 14	I.M.	
	T	4 - 14	Oral	E.Coli
	S	7 - 11	I.M.	
13	P	1 - 12	I.M.	
	T	6 - 12	Oral	E.Coli
		9 - 12	I.M.	
	S	6 - 12	I.M.	
14	P	1 - 13	I.M.	
	T	4 - 13	Oral	E.Coli
15	P	1 - 9	I.M.	Not
	T	4 - 9	Oral	cultured
16	P	1 - 20	I.M.	No
	T	4 - 26	Oral	growth
17	P	1 - 9	I.M.	B.Pyo-
		6 - 9	I.M.	cyaneus
18	P	1 - 20	I.M.	
	T	9 - 13	I.M.	No
		13 - 26	Oral	growth
19	P	1 - 20	I.M.	
	T	8 - 13	I.M.	No
		13 - 26	Oral	growth

Table 2. Details of antibiotic treatment and blood culture in irradiated, marrow-treated Rhesus Monkeys.

Key P = Penicillin C = Chloramphenicol T = Tetracycline S = Streptomycin

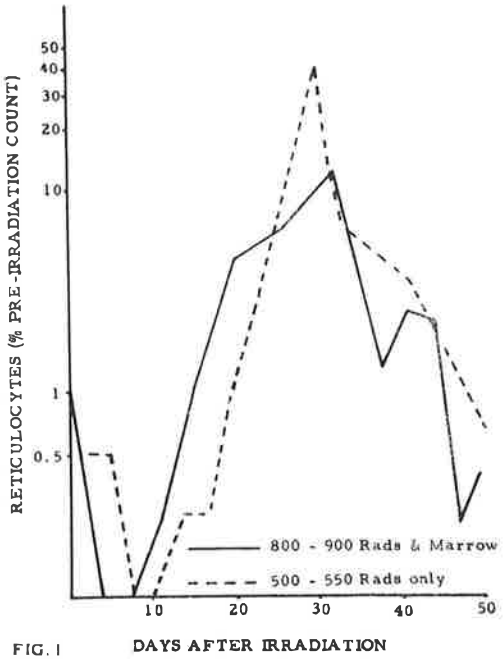

FIG. 1

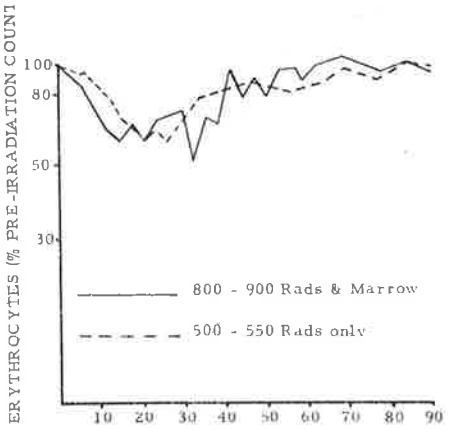

FIG. 2

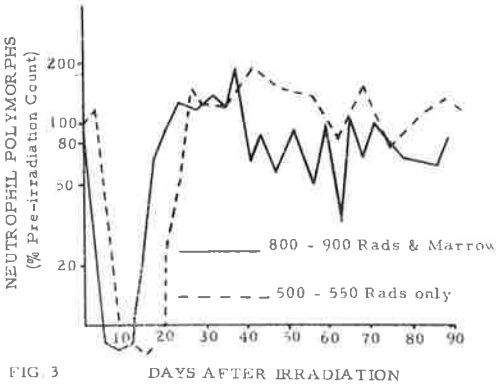

FIG. 3

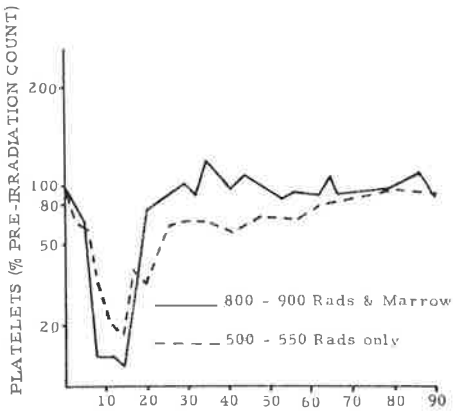

FIG. 4

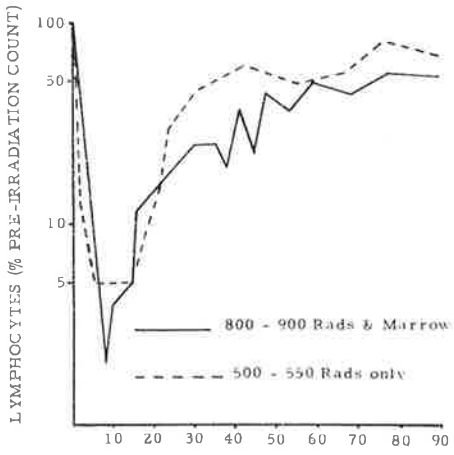

FIG. 5

DISCUSSION

AMBRUS would like to know what kind of cells were used in experiments, where an attempt was made to isolate stem cells.

SCHOFIELD Circulating blood was taken, the red cells were removed, the white cells were put in culture with phyto-hemagglutinin, a substance extracted from red beans. In this situation the mononuclear cells begin to synthesize RNA, then DNA and eventually start dividing. The question was whether these cells had stem cell potential and whether they could be a substitute for bone marrow transplants.

AMBRUS We have thought about similar problems and injected mice with various tissue culture lines. First an established culture derived from mouse bone marrow with cells of an epitheloid character was used, later a number of other cultured cells from different species. There was a significant effect as long as the radiation dose was not too high. Experiments by other groups made us realize eventually that these cells most likely acted as particulate matter, stimulating surviving stem cell elements in one way or another. If the radiation dose is sufficiently high to wipe out the stem cell potential of the irradiated animal, then this kind of non-specific stimulation will have little effect.

CROUCH Goodman, at Oak Ridge, quite clearly showed the transplantability of various peripheral blood elements.

KURNICK It should be explained that all this happens in the mouse. Almost any trauma at sublethal or near lethal radiation doses will have a protective effect in the mouse. The effect of anaesthesia has been ascribed to a low oxygen tension, but if surgery is super-imposed one gets an additional protective effect. In the latter part of Dr. Schofield's paper autologous bone marrow was used and the early rise of the peripheral blood count would in-

dicate a take of the marrow graft. Does Dr. Schofield seriously suggest that this has nothing to do with the survival of the animals because the cause of death in the first place is bacteriemia and in the second place hemorrhage due to thrombocytopenia? But if marrow repopulation is not achieved would not all of your animals have died of those two causes since you could not perpetuate a bacteria-free and "hemorrhage free" state?

SCHOFIELD A number of animals with nicely repopulating marrow died of bacteriemia when no antibiotics were given. Others, after 800 r and no marrow treatment succumbed with E. coli bacteriemia, insensitive to the antibiotics that were used.

KURNICK Does this not indicate that though an autologous marrow graft will not guarantee the animal's survival, the failure of a graft to take would nearly guarantee the animal's death, since sooner or later there would be an invasion by an organism resistant to antibiotics?

SCHOFIELD We are not depleting the animals completely of stem cells. Assuming that cell survival curves can be applied to the stem cell population, aplasia will not persist indefinitely. At these doses of radiation, given time, the marrow will come back and the defences will recover. Secondly, I would think that invasion of bacteria from the gut is a great danger only during the first week. Suppressing the bacteriemia up to the time when the lining of the gut has recovered, may be sufficient. With the antibiotic regime we are using at the moment, the antibiotics are stopped at 7 days, in order to avoid hemorrhage at the site of i. m. injection during the following period.

VAN PUTTEN stresses the difference between the data of his own monkey experiments and those reported by Dr. Schofield. No positive blood cultures were found in the former monkeys except shortly before death. In terminal septicemia the bacteria are also found in cultures of the spleen and the lungs.

This may be due to different treatment of the animals. The different results may also be due to the number of bone marrow cells injected. Does Dr. Schofield correct the number of injected marrow cells by subtracting the number of cells present in the peripheral blood which is inevitably injected with the marrow suspension?

SCHOFIELD No, this is not done. Practically all the cells we inject in small doses are almost certainly peripheral blood cells and yet we can get survivors.

VAN PUTTEN But 100% survival is obtained with higher numbers of autologous marrow which makes us believe that autologous marrow works. Increasing the number of injected cells causes earlier recovery of the peripheral blood.

SCHOFIELD No doubt this is true. But is it the only effective treatment? And what is the exact mechanism? Which component of the injected cell suspension is in fact keeping the animal alive?

VAN PUTTEN Since recovery of the peripheral blood may start as early as the 7th day, marrow treatment may exert its effect by just shortening the time interval during which there is an absence of necessary blood elements. Admittedly, the presence or absence of infection makes a big difference, even though it may look as if the untreated animal was dying from hemorrhage. As soon as leucocytes return to normal levels though the platelet count may still be very low, one does indeed find less hemorrhage. Though we agree on this point I still think that we talk about different things when referring to marrow transplantation.

SCHOFIELD This is not quite so. True enough you are getting early repopu lation but which of the cells that are repopulating the blood are in fact preventing the animal from dying? You postulate that when getting freedom from infection you also have a lower platelet requirement and less bleeding.

Would you agree then that early repopulation is suppressing the infection?

VAN PUTTEN Yes, but I got the impression that, in addition, you think that proliferation of the autologous marrow transplant is not essential since dead cells also do the trick.

SCHOFIELD No, this is not so. The point I'm trying to get at is what in fact we are doing by giving bone marrow. And if we can keep the animal alive without giving marrow we would get around the question of homologous disease.

KURNICK would entirely agree with Dr. van Putten's view. At dose levels of 900 r and higher we reduce the time of pancytopenia from years, maybe 10 years, to 20 or 30 days. It is questionable whether recovery occurs as long as there is one stem cell left. In man, unlike the situationin the mouse, no local recovery takes place in heavily irradiated areas although the non-irradiated parts of the hemopoietic system keep the level of thrombocytes and leucocytes compatible with life. The irradiated areas remain severely hypoplastic for years. Had anyone present ever given 900-1000 r to a monkey while protecting one extremity and if so, what had been found?

SCHOFIELD states that monkeys were given 800 r and nothing else except antibiotics and marrow repopulation was observed. Since the whole body had been irradiated it is tempting to assume a Puck-type survival curve. If $\frac{1}{2}$ % of the stem cells survived and a generation time of approximately 24 hours is assumed, the stem cell population would be restored within 10 to 12 days.

KURNICK It is questionable however, whether irradiated stem cells behave like unirradiated cells. There is in fact evidence that they do not: 2 or 3 years after local irradiation one can see little islands of repopulation in these areas; they are not filling up the marrow cavity as they would after chemotherapeutic agents for example.

PATHOLOGY OF SECONDARY DISEASE IN PRIMATES

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Secondary disease of radiation chimeras was extensively studied primarily in mice. The etiology of the disease was analysed by various experimental approaches and found to be related to an immunological reaction of grafted immunologically competent cells against the host (the evidence has been reviewed several years ago by van Bekkum et al. (1959). Although a variety of characteristic lesions have been described, death in these mice appears to be almost uniformly due to infectious disease (van Bekkum et al., 1959; de Vries and Vos, 1959). The susceptibility to infections seems to be indirectly related to the graft anti-host reaction, since it is most readily explained by the generalized atrophy of the lymphatic tissues in these animals. As will be discussed later, this lymphatic atrophy may be considered to be a direct consequence of the interaction between reacting donor cells and host antigen.

Other characteristics of secondary disease in such bone marrow treated mice are its late appearance and chronic course and the fact that, depending on host-donor combination, a certain proportion of these mice may ultimately survive in the presence of permanent chimerism (an extensive discussion of the changes in these old chimeras and the development of immunological tolerance of the graft towards the host in these mice will be published else-

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where by van Bekkum et al., 1962). Lastly, an important feature is, that the survival may be greatly enhanced by treatment with antibiotics (van Bekkum and Vos, 1961).

In contrast to the results obtained with mice, it was found from limited experience in man and more extensive experience in monkeys, that grafting of homologous bone marrow in irradiated primates is uniformly lethal if permanent chimerism is successfully obtained (Mathé et al., 1960; Crouch et al., 1961). The existence of chimerism was determined by serological typing of erythrocytes or sex chromatin counts of neutrophils. This lethality may also be attributed to secondary disease mainly because of the following two observations. Treatment with autologous or monozygotic twin bone marrow can be successful; secondly, the lesions seen after homologous marrow transplantation in particular those of the skin and the liver parenchyma, are highly similar to those found in mice with secondary disease (de Vries et al., 1961).

In primates, however, the disease has a much more acute course and is apparantly of a very severe character. Moreover, infectious disease cannot explain death in a substantial proportion of cases and antibiotics are not effective in preventing death (Crouch et al., 1961).

It has been put forward, that the pattern of secondary disease in bone marrow treated primates is highly reminiscent of the acute mortality obtained in irradiated mice treated with lymphoid cell suspensions in addition to bone marrow (de Vries et al., 1961). The similarity was stressed when a number of lesions present in primate chimeras were seemingly duplicated in these mice (de Vries, to be published). These observations led to the provisional conclusion that mortality caused by secondary disease in primates is pathogenetically more directly related to graft anti-host activity than in bone marrow treated mice and secondly, that lymphoid cells are of prime importance in its production. In the following paragraphs morphologic-

al evidence in favour of these assumptions will be presented.

The most important lesions found in primate chimeras are briefly reviewed in table I. These lesions are accompanied by bone marrow regeneration (plate 1), which clearly distinguishes the syndrome from graft rejection.

Table I

PATHOLOGY OF SECONDARY DISEASE

Bone marrow regeneration.
Regeneration, necrosis and atrophy of lymphatic tissues.
Dermatosis: acanthosis, follicular hyperkeratosis, parakeratosis, dyskeratosis, vacuolar degeneration, liquefaction degeneration of basal layer, metaplasia of excretory ducts of sweet glands, atrophy.
Loss of crypt and surface epithelium in the intestines.
Liver necrosis and isolated cellular degeneration in other epithelial tissues.
Infectious disease.

A dermatosis is found, characterized by either acanthosis (plate 2 and 3) or atrophy of epidermis and hair follicles (plate 4). A number of degenerative changes of the epithelium are also apparent: premature keratinization of cells (dyskeratosis, plate 5, 6 and 17), liquefaction degeneration of basal cells (plate 6) and vacuolar degeneration in the malpighian layer, sometimes leading to reticular degeneration and the formation of multilocular vesicles (plate 4). Finally, follicular hyperkeratosis and parakeratosis are characteristic (plate 2 - 4).

In almost all cases examined, severe intestinal changes are present. Extensive disintegration of cells in the glandular crypts of the intestinal mu-

cosa occurs (plate 7), leading to the loss of all epithelial elements, i. e. intestinal denudation (plate 8 and 9). Although the colon and ileum are most severely affected, lesions may be present in the entire intestinal tract, including the stomach (plate 10). The intestinal lesions to a certain extent do resemble those known to be caused by radiation alone, but several arguments may be put forward, which plead strongly against such an interpretation (de Vries et al., 1961). The most important of these are that the extent of the lesions is out of proportion with respect to the radiation dose, the lesions occur later than would be expected if the radiation were responsible and they are not found in irradiated non-treated monkeys neither in monkeys treated with autologous bone marrow after similar radiation doses (table II).

Table 2

PATHOLOGY OF IRRADIATED MONKEYS TREATED WITH BONE MARROW

	Irradiated non-treated	Autologous bone marrow	Homologous bone marrow		
			T*	ID*	NT*
Bone marrow regeneration	0/7	8/11	11/12	2/2	0/3
Hemorrhagic necrosis colon	6/7	0/11	0/12	0/2	2/3
Septichemia	4/7	0/11	0/12	0/2	0/3
Other infecteous diseases	1/7	3/11	8/12	1/2	0/3
Denudation intestines	0/7	1/11**	10/12	0/2	0/3
Massive liver necrosis	0/7	0/11	3/12	0/2	0/3
Jaundice	0/7	0/11	5/12	2/2	0/3
Dermatitis	0/7	0/11	8/10	-	-
Regeneration lymphatic tissues	1/7	7/11	6/12	0/2	0/3
Necrosis lymphatic tissues	0/7	0/11	3/12	0/2	0/3

* T = Take
ID = Insufficient data as determined by sex chromatin counts of neutrophils
NT = No take

** Monkey irradiated with 1065 r

Dissociation and necrosis of liver parenchyma and a variety of bacterial, mycotic, helminthic and possibly also viral infections have been found in a number of chimeras.

Other lesions than the above mentioned may be found in secondary disease, but will not be further digressed upon at this moment. For the present discussion it is only of importance to summarize the essential features which all lesions have in common.

1. Degeneration of cells, mainly in epithelial tissues, as already indicated in the previous paragraph. This may be massive in the intestines, the skin and the liver. Diffuse isolated degeneration of cells has been found, however, in a great number of other sites: the epithelium of the oesophagus (plate 12), renal pelvis, salivary glands, exocrine and endocrine parts of the pancreas, adrenals, Fallopian tube (plate 13) and bile ducts (plate 14 and 15).

   Cell death is indicated by a number of changes: karyorrhexxis (plate 7, 3, 14 and 15) and pyknosis of nuclei (plate 7), vacuolar degeneration (plate 4, 6, 12 and 17) and increased eosinophilia of the cytoplasm (plate 14). In the skin premature keratinization of epidermal cells has been mentioned (plate 5, 6 and 17) which may also be interpreted as a mode of cell death. Atrophic changes may supervene in the most severely affected tissues (plate 4 and 7).

2. Concurrently with the regressive changes, regeneration usually occurs, which, depending on the site, may or may not be able to restore the integrety of the tissues affected. The regeneration is indicated by an increase of mitotic frequency, which is most conspicious in tissues in which normally mitoses are not easily found (plate 13). The regenerative activity often gives rise to a hyperplastic appearance of glands or other epithelial tissues (plate 2, 3, 6, 7 and 10).

3. The affected tissues generally are more or less heavily infiltrated with lymphoid cells (plate 4, 6, 7, 10, 12, 15 and 16). Sometimes small numbers of these cells are seen to have penetrated into the epithelium while the epithelial cells adjacent to the invading cells display the various degenerative changes described before (plate 6, 7, 10, 12 and 17). In addition one often gets the impression that the intra-epithelial lymphoid cells also disintegrate, although this is difficult to verify.

4. A most significant sequence of changes is seen in the lymphatic tissues proper, i. e. the cortex of the lymph nodes and the splenic follicles. Following the radiation atrophy (plate 18), extensive regeneration occurs in a number of animals, mainly at the end of the first week (plate 19 and 20). Many mitotic figures are noted and in addition large numbers of reticular cells, stem cells and immature lymphoid cells distend the available tissue space.

   At the end of the first week and in the second week the regenerated lymphatic tissues of a number of monkeys examined in this period, show massive disintegration of lymphoid cells (plate 21). The characteristic feature is, that the necrosis does not involve the stromal supporting tissues and the vascular endothelium.

   The lymphatic tissues of chimeras which survived beyond the first 2 weeks are almost universally severely depleted of lymphoid cells. The atrophic lymph nodes and splenic follicles are made up of an empty reticular stroma and distended sinusoids, in which only few lymphocytes and histiocytic cells and a variable number of plasma cells are distributed (plate 22).

Before we attempt to interprete the given set of morphological changes, it is necessary to review a few relevant facts concerning the function of lymphoid cells and their fate in chimeras.

Pertinent to the observation of lymphoid cell infiltration in the diseased tissues of primate chimeras, is the evidence that a skin homograft is rejected by a cell mediated immunological reaction (Brent, 1958). The immunologically active cell is believed to be the small lymphocyte (Gowans et al., 1961). Of similar significance is the finding, that the pathological changes in homologous skin grafts, which are being rejected, resemble in many details those occurring in the skin of radiation chimeras, as described earlier in this paper (de Vries, to be published). The assumption seems to be justified, that in the primate radiation chimera, certain host tissues are being "rejected" in a somewhat similar way by the homologous transplant of lymphoid cells. With the necessary reservations these assumptions are supported by the following observations in rodent chimeras. The lymphatic tissues of mice treated with rat bone marrow appear to be repopulated by lymphoid donor cells (Brocades Zaalberg and van Bekkum, 1959). Moreover, autoradiography has shown, that when donor lymphoid cells labeled with tritiated thymidine are injected into new-born rats (Porter and Cooper, 1962) or into 5-week old F_1 hybrid rats, respectively, (Gowans et al., 1961) labeled cells are found to migrate not only into the lymphatic tissues proper, but also into the intestinal mucosa. Experiments in which similarly labeled cells were used have suggested that the same occurs in irradiated F_1 mice treated with parental spleen cells (Balner, 1962). In this context it should be recalled that the mucosal epithelium belongs to the most severely affected tissues in primate chimeras.

Gorer and Boyse (1959) have described a destruction of the lymphoid tissues of the host in (non-irradiated) (C57BL x A)F_1 mice treated with isoimmune parental strain cells. They postulated that the rapid disappearance of C57BL lymphoid cells, as noted in their experiments, is due to damage by the excess of antigen in the host environment to which these cells are exposed. This would indicate that, in the graft anti-host reaction not only the target

cells but also the antibody-forming cells are being destroyed.

With these data in mind, we may now attempt a tentative reconstruction of the set of events leading to secondary disease and death in primate radiation chimeras.

In the available space in the lymphatic tissues, caused by the radiation induced cellular depletion, a repopulation by lymphoid donor cells, derived from the injected bone marrow, occurs. In the absence of sensitization the proliferation at first takes place unimpeded and is possibly even promoted by antigenic stimulation, leading to the formation of a pool of potentially immunologically competent cells. Subsequently cells from these lymphatic organs migrate to peripheral tissues.

During the second part of the first week sensitization occurs. The sensitized cells penetrate the epithelium of a variety of organs and tissues and severely damage their epithelial cells, either by the secretion of anti-body or by non-specific cytotoxic products liberated during the desintegration of donor lymphoid cells after interaction with host cells (so-called enzymatic destruction as suggested by Amos, 1960). Similarly because of the interaction between host antigen and lymphoid donor cells massive destruction of the latter ultimately occurs in the lymphatic tissues.

The loss of epithelial cells induces a process of repair, which seems to be at least partially successful in some tissues, but apparently cannot compete with cell destruction in others such as the intestinal crypts, where extensive denudation of the mucosa occurs.

The death of the host may be explained by the consequences of intestinal denudation and possibly by toxic factors due to the wide-spread destruction of both host and donor cells.

One of the facts that still demand an explanation is, why the intestinal crypt cells are so particularly sensitive to the graft anti-host reaction. One factor could be the combination of radiation induced and immunological

damage, as has been discussed in a previous paper (de Vries et al., 1961). Another may be the circumstance that the intestinal tract is in fact a lymphatic organ, and moreover, a lymphatic organ in which epithelial tissue is intimately associated with lymphoid cells. In the chimeric intestine, the epithelial target cells are obviously surrounded by the pool of immunologically active donor cells.

If our assumptions are correct the conclusion must be drawn that the whole problem of the early death of the primate chimera centres around the lymphoid donor cells. In primates treatment with homologous bone marrow results in an early unrestrained proliferation and accumulation of donor lymphoid cells, presumably accounting for the inevitable early lethality. In those homologous host-donor combinations in mice treated with marrow only, in which severe secondary disease occurs, this early repopulation is not apparent to the extent that it is seen in primates. As will be recalled secondary disease in these mice develops in a much later phase. Evidently not the primary effects of the graft anti-host reaction, but the secondary atrophy of the lymphatic tissues endangers the life of these mice by the increased risk of infection. It seems therefore, that this late form of secondary disease may be self-limiting, due to the fact that lymphoid cells newly produced by the graft, which has been sensitized by that time, are continuously eliminated due to the excess of host antigens.

As will be discussed in the next paper, one solution of the dilemma would be, to search for methods to eliminate selectively lymphoid cells from bone marrow suspensions of primates. It might be expected that monkeys treated with such suspensions would still develop the late form of secondary disease, as it occurs in bone marrow treated mice. One could hope, however, that primates treated in that way, survive the early dangerous phase and be effectively treated during the late phase of secondary disease which might ensue.

Another more attractive approach would be an in vitro induction of immunological tolerance of donor lymphoid cells towards the prospective host. Results of experiments by van Putten with mice (van Putten, 1962) give rise to the hope that large amounts of tolerant lymphoid cells may successfully compete with similar non-tolerant cells present in primate bone marrow suspension.

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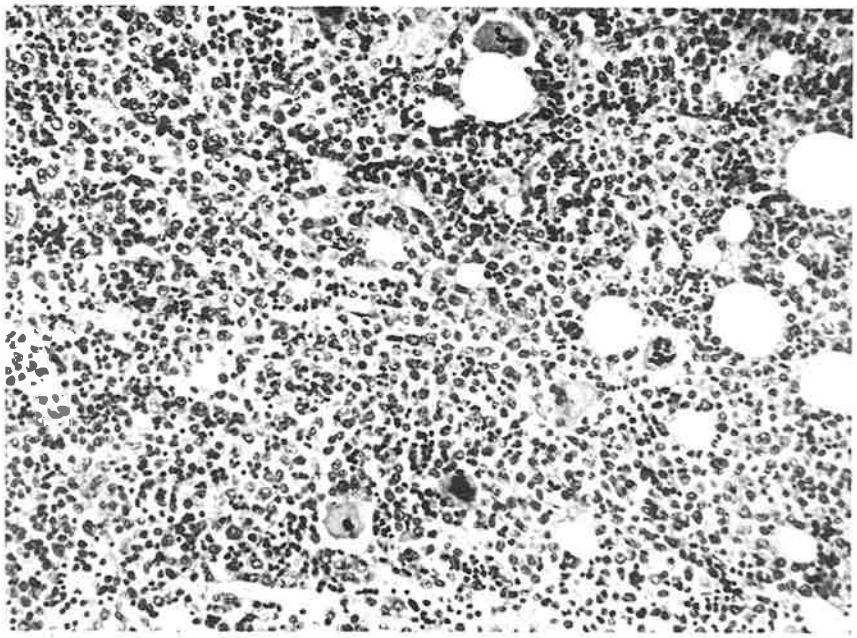
Plate 1 Bone marrow regeneration in monkey, 26 days after irradiation (700 r) and treatment with homologous bone marrow (11.2×10^8 cells).
Hematoxylin and eosin x 190

Plate 2 Dermatosis in same monkey as in plate 1. Note acanthosis, severe follicular hyperkeratosis and lymphoid cell infiltration of dermis in general and surrounding the hair follicles.
Hematoxylin and eosin x 30



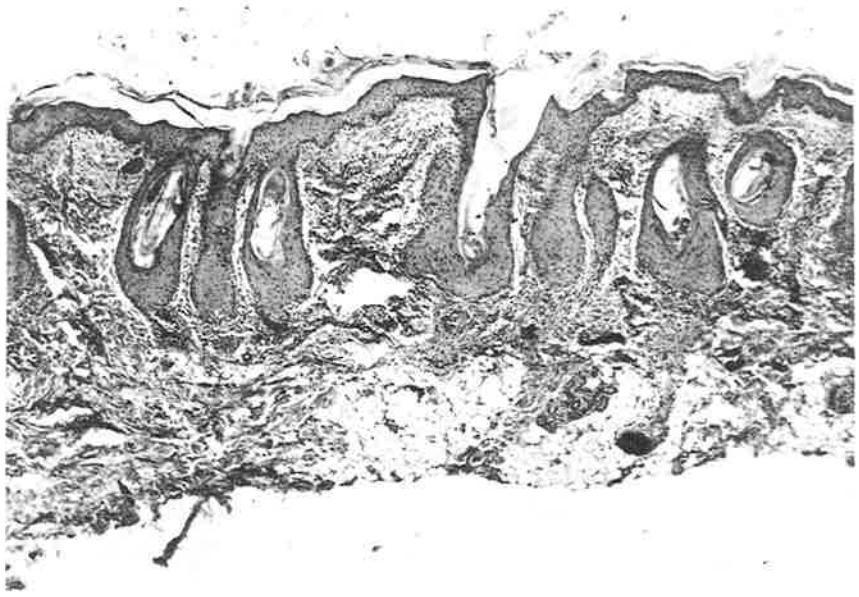
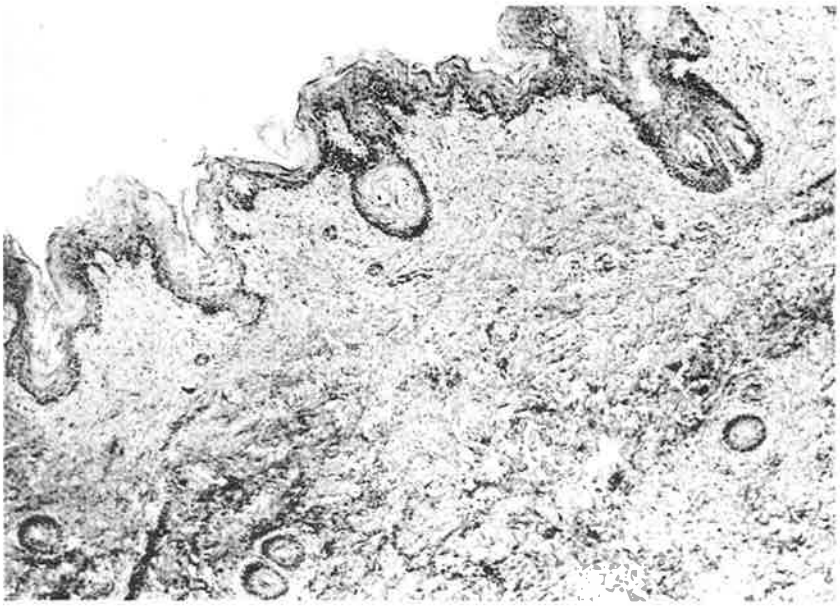


Plate 3 Dermatosis in a leukemic child, 29 days after 880 r Co^{60} γ-irradiation and 19 days after treatment with 11.5 x 10^9 bone marrow cells from its mother. Acanthosis and follicular hyperkeratosis.
Hematoxylin and eosin x 40
(courtesy of G. Mathé)

Plate 4 Dermatosis in monkey, 19 days after irradiation (600 r) and treatment with homologous bone marrow (15.2 x 10^8 cells). Parakeratosis. An atrophic hair follicle is present to the right. Vacuolar degeneration in stratum malpighii, leading to formation of clefts and small vesicles. Lymphoid cell infiltration of dermis, several of these cells have penetrated the epithelium.
Hematoxylin and eosin 190 x



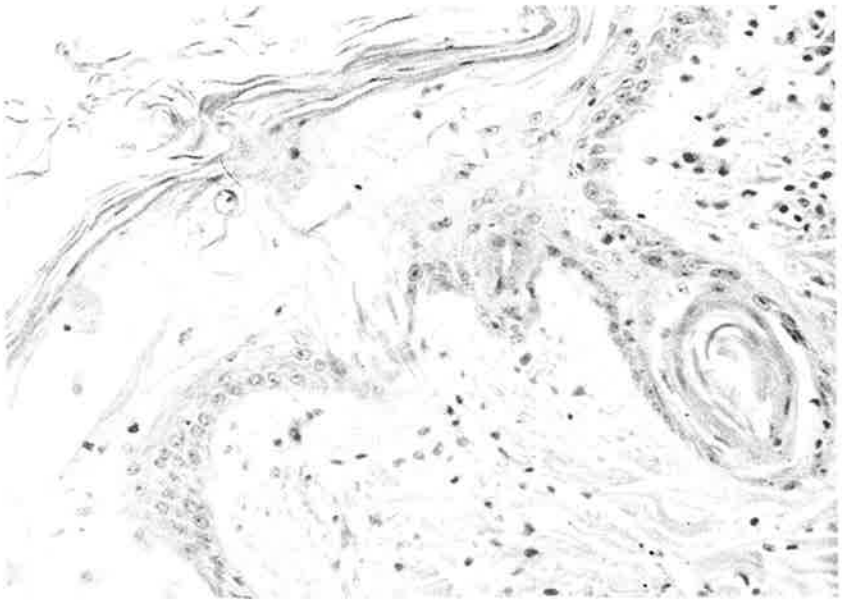
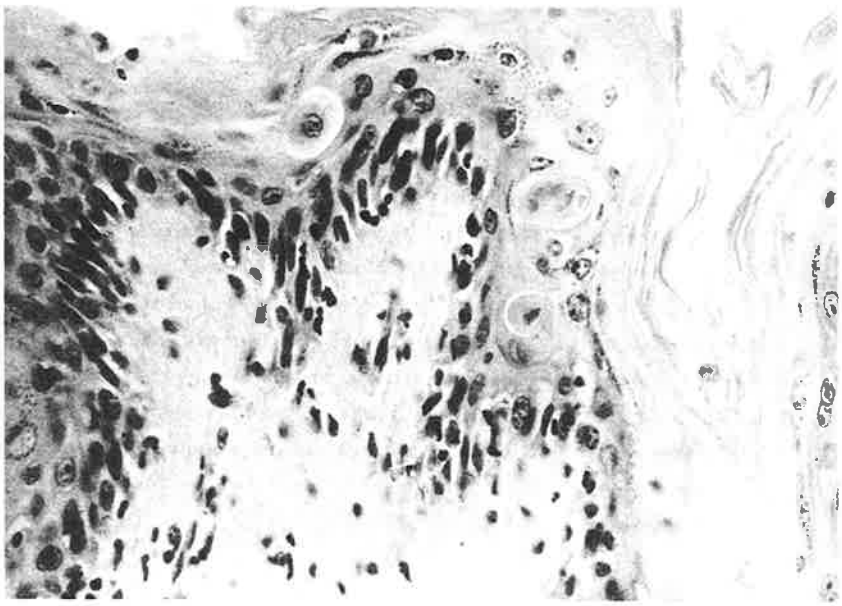


Plate 5 Dermatosis in same child as in plate 3. In the epidermis 3 dyskeratotic cells are present.
Hematoxylin and eosin x 300

Plate 6 Dermatosis in same child as in plate 3. Lymphoid cell infiltration of dermis accompanied by extensive liquefaction degeneration of basal layer. Acanthosis. A number of dyskeratotic cells may also be seen.
Hematoxylin and eosin x 190



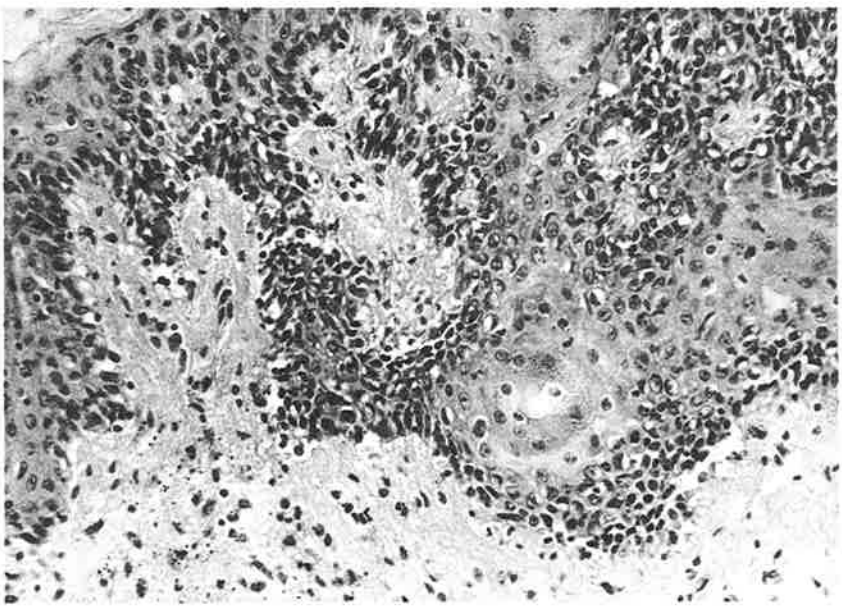
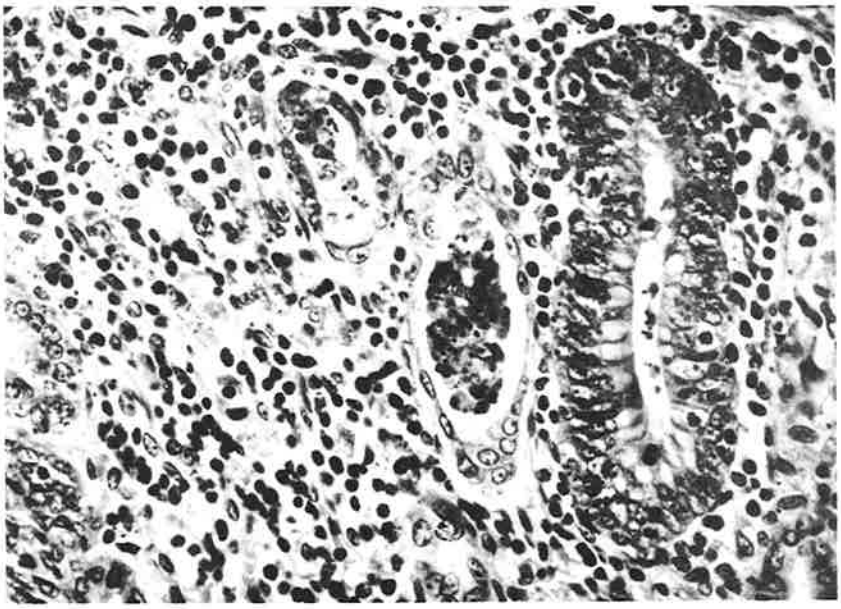


Plate 7 Wide-spread disintegration of crypts in the colon of monkey 7 days following irradiation (700 r) and treatment with homologous bone marrow (28 x 10^8 cells). Note heavy lymphoid cell infiltration of lamina propria and invasion by several of these cells of the epithelium of a hyperplastic crypt to the right. In this crypt several mitoses as well as diffuse pyknosis and karyorrhexxis of epithelial cell nuclei may be seen. Two other crypts are atrophic and show accumulation of degenerated and desquamated cells in the lumen.
Hematoxylin and eosin x 300

Plate 8 Complete loss of crypt and surface epithelium in the ileum of monkey 7 days after irradiation (750 r) and 6 days after treatment with homologous bone marrow (35 x 10^8 cells) which had been stored in an ice-box at 4^o C for 48 hours.
Hematoxylin and eosin x 30



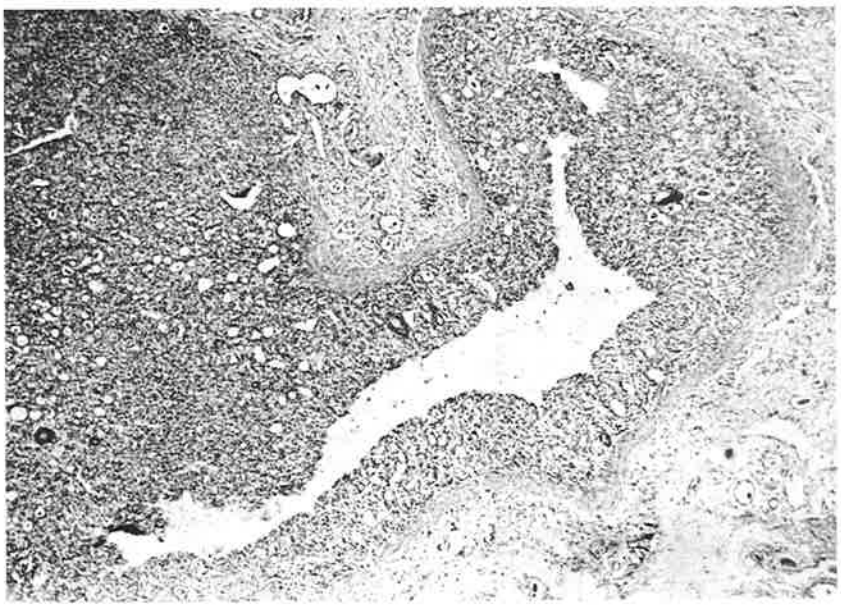
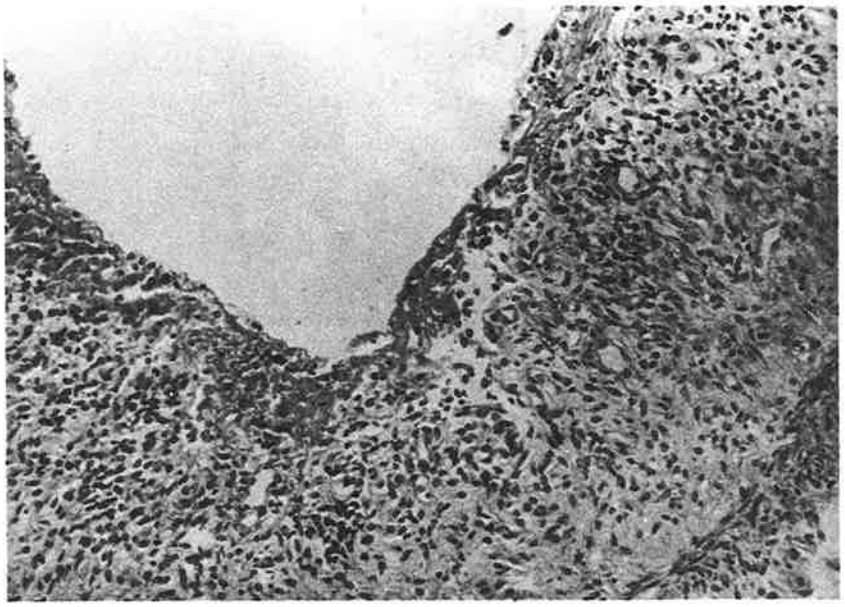


Plate 9 Complete loss of crypt and surface epithelium in ileum of leukemic child, 43 days after 950 r Co^{60} γ-irradiation and treatment with its mother's bone marrow, 12 and 15 days later (total of 14.5×10^9 cells). Surface of denuded mucosa is covered with fibrin and a few exudate cells.
Hematoxylin and eosin x 120
(courtesy of G. Mathé)

Plate 10 Cystic degeneration of mucosal glands of stomach in same monkey as in plate 8. Note desquamation of disintegrated cells and a completely atrophic gland in a centre of a focus of lymphoid cell infiltration to the right. Other glands have a hyperplastic appearance. Normal glands may be seen at left margin.
Hematoxylin and eosin x 120



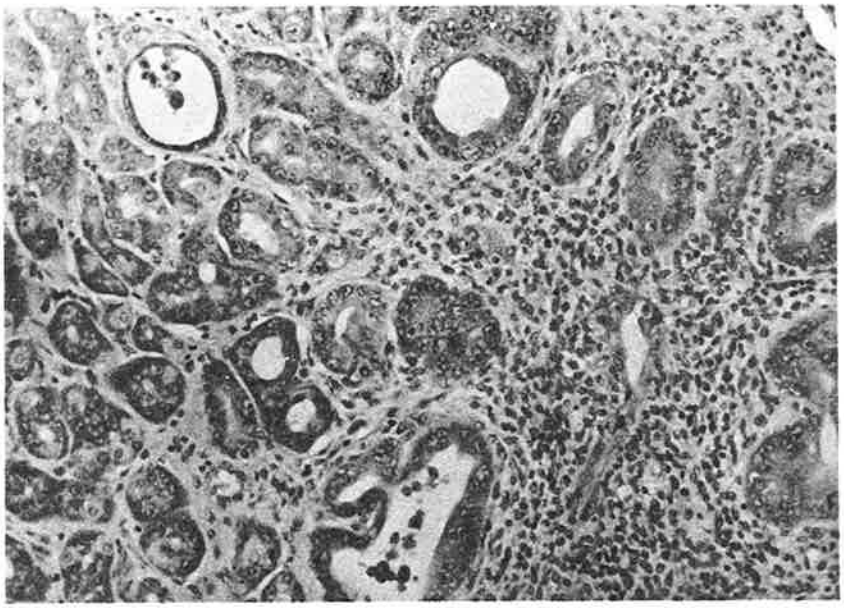
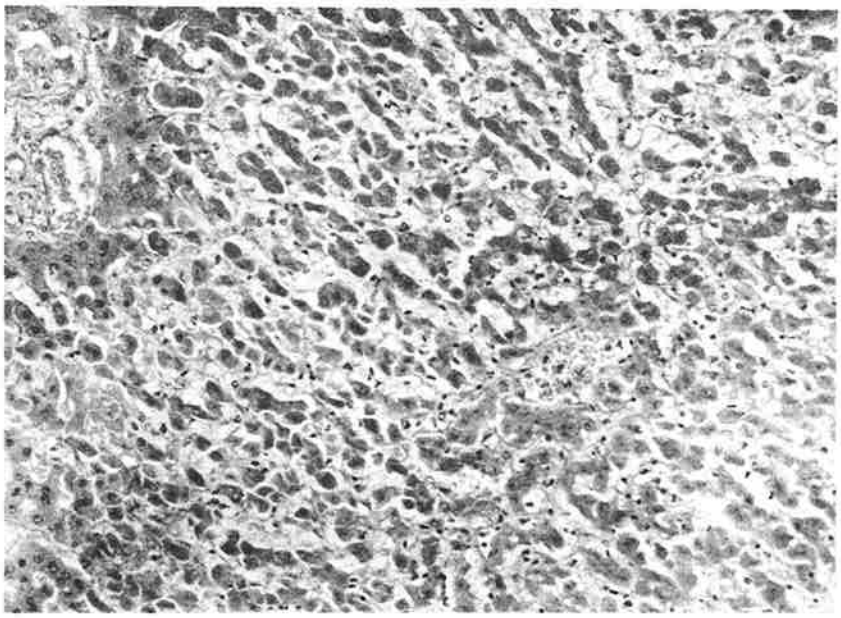


Plate 11 Dissociation and necrosis of hepatic parenchyma in monkey 23 days after irradiation (740 r) and treatment with homologous bone marrow (24×10^8 cells).
Hematoxylin and eosin x 120

Plate 12 Diffuse vacuolar degeneration of cells in esophageal epithelium of same monkey as in plate 7. A small vesicle containing degenerated cells is seen in the centre. Note lymphoid cell infiltration in lamina propria with diffuse extension into epithelial layer.
Hematoxylin and eosin x 120



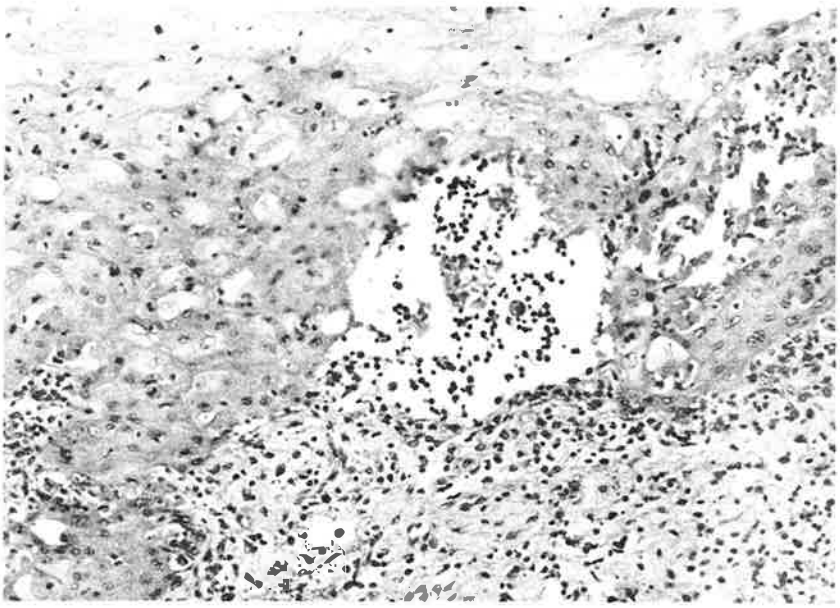
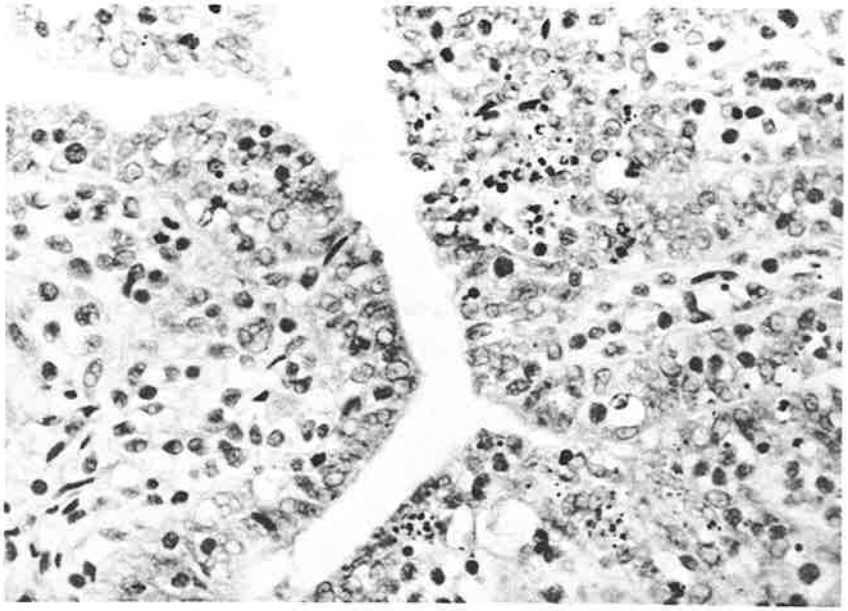


Plate 13 Karyorrhexxis and nuclear pyknosis in tubal epithelium of same monkey as in plate 8. A mitotic figure is seen in upper left corner.
Hematoxylin and eosin x 300

Plate 14 Karyorrhexxis in the epithelium of a medium-sized interlobular bile duct of same monkey as in plate 8. At the right margin a few necrotic liver cells with increased eosinophilia of cytoplasm are seen.
Hematoxylin and eosin x 190



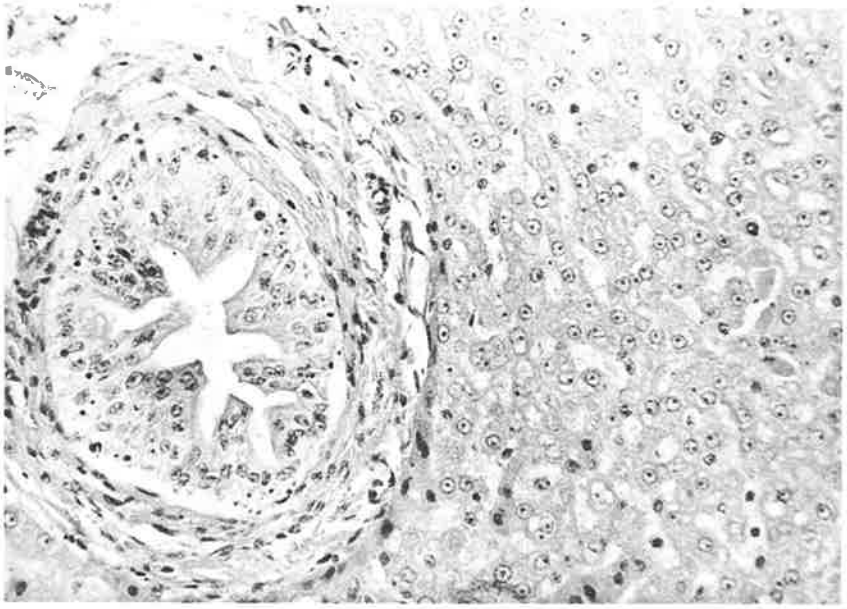
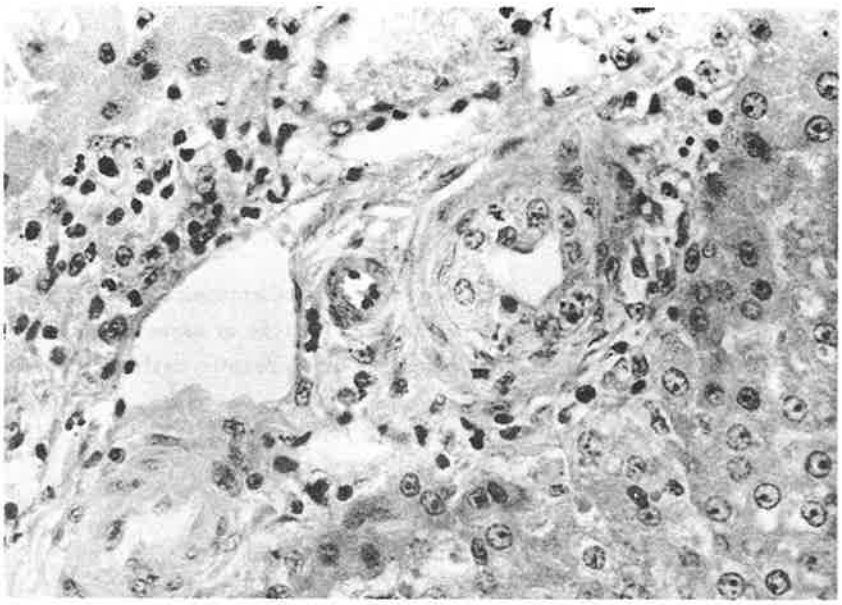


Plate 15 Periportal lymphoid cell infiltration in liver of same monkey as in plate 8. A few disintegrating epithelial cells are present in a small bile duct.
Hematoxylin and eosin x 300

Plate 16 Invasion of lymphoid cells into epithelium of hair follicles of same monkey as in plate 4.
Hematoxylin and eosin x 300



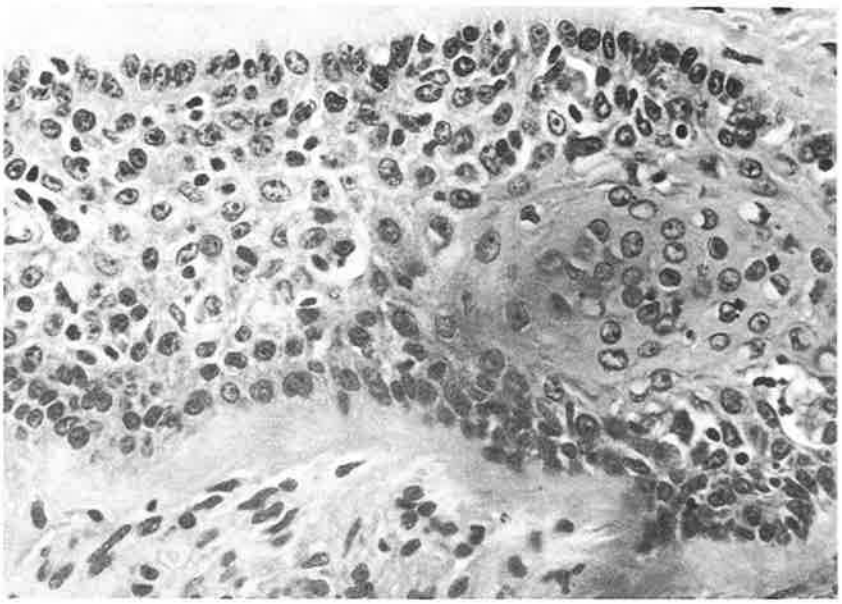
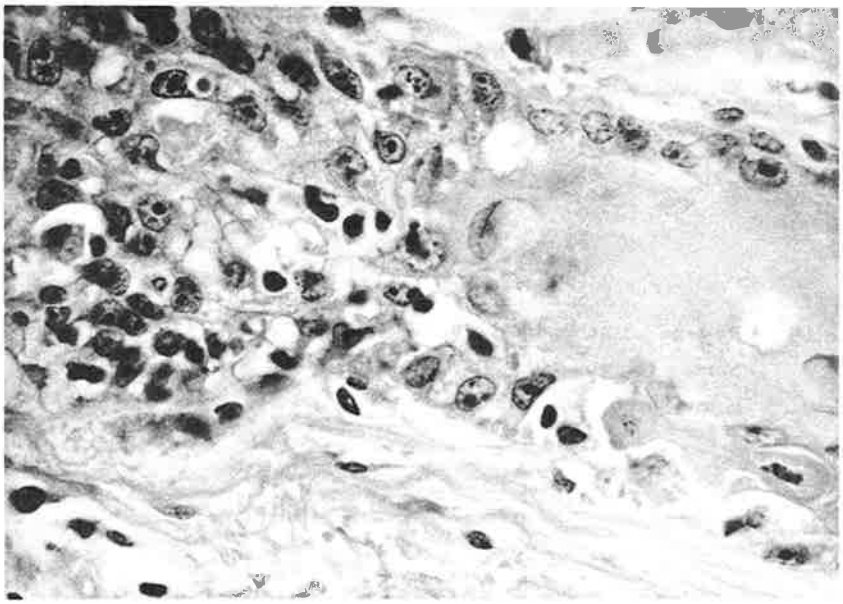


Plate 17 Pyknosis and vacuolar degeneration of epithelial cells adjacent to invaded lymphoid cells in hair follicle of same monkey as in plate 4. In lower right corner 2 dyskeratotic cells are seen. Hematoxylin and eosin. x 480

Plate 18 Radiation induced atrophy of lymph node of nontreated monkey 15 days after irradiation (810 r). A few collections of lymphocytes are still present in the depleted reticular stroma. Hematoxylin and eosin. x 120



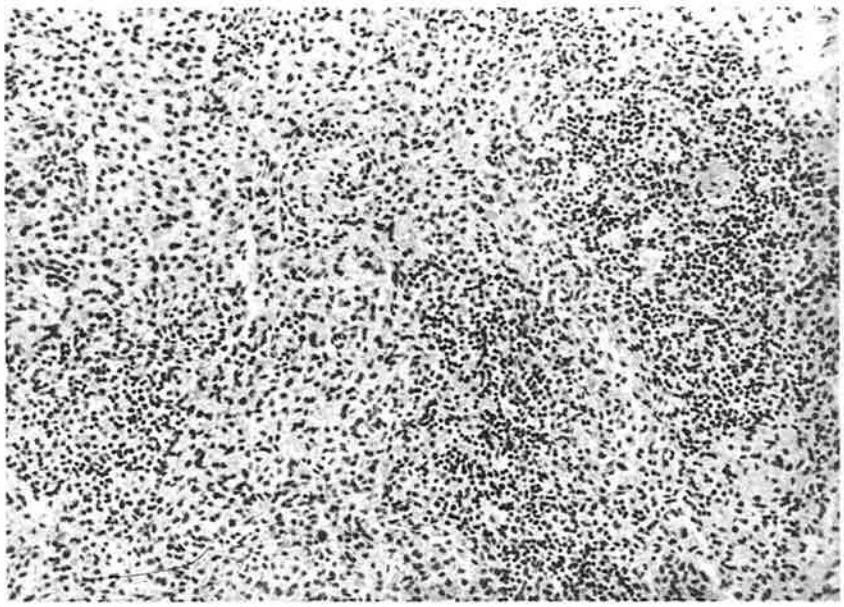
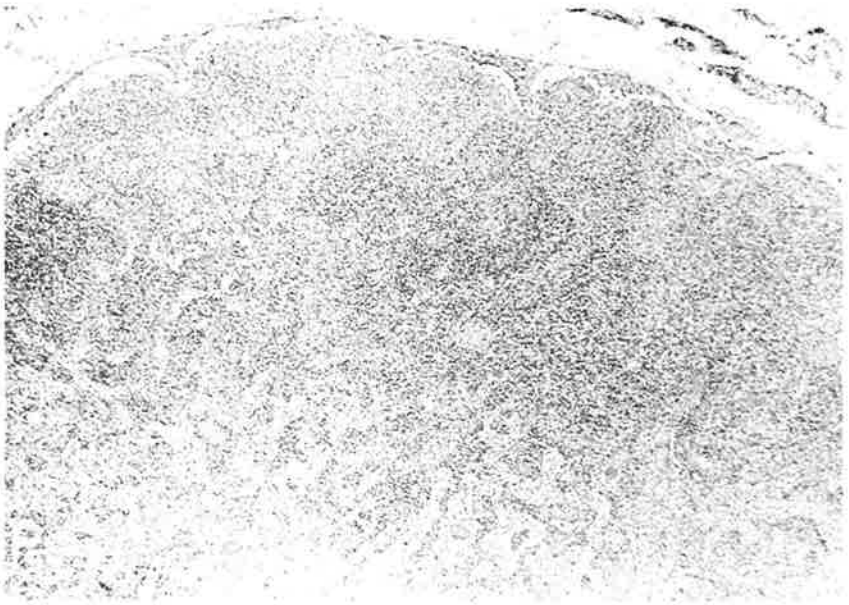


Plate 19 Regeneration in lymph node of same monkey as in plate 8. The cortex is crowded with lymphoid cells (see next plate).
Hematoxylin and eosin. x 30

Plate 20 Same lymph node as in plate 19. Extensive proliferation of reticular cells, stem cells and immature as well as mature lymphoid cells is apparent. Note several mitotic figures.
Hematoxylin and eosin. x 190



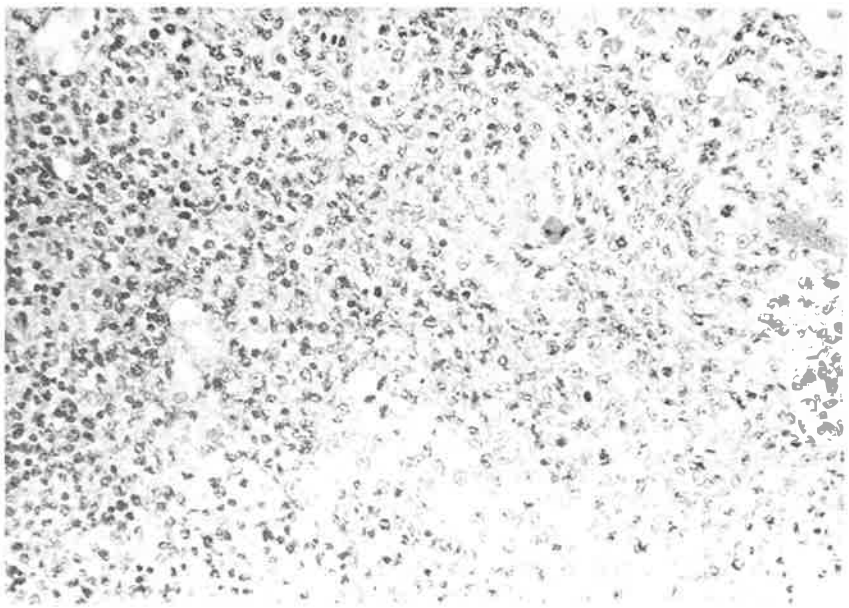
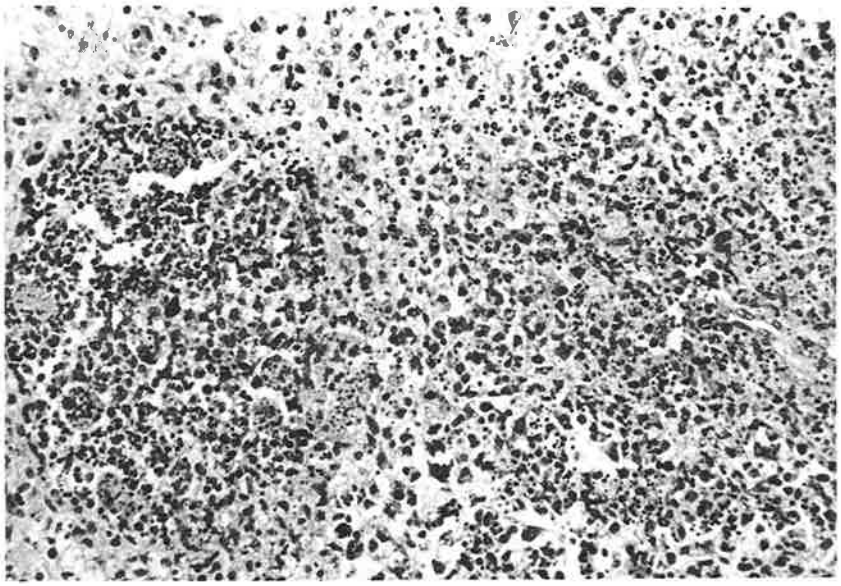
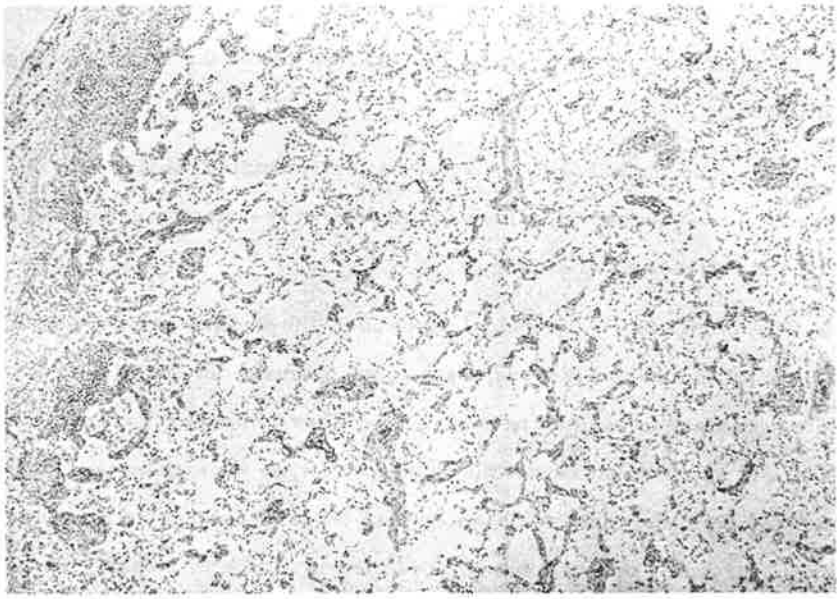


Plate 21 Wide-spread disintegration of lymphoid cells in lymphatic follicles and medullary tissue of lymph node of monkey 14 days after irradiation (650 r) and treatment with homologous bone marrow (22×10^8 cells).
Hematoxylin and eosin. x 190

Plate 22 Extreme atrophy of lymph node of monkey 47 days after irradiation and treatment with homologous bone marrow (22×10^8 cells). The cortex is more severely depleted of lymphoid cells than in control monkeys (compare with plate 18). The sinusoids are dilated.
Hematoxylin and eosin. x 30





DISCUSSION

BROCADES ZAALBERG observes that a striking similarity exists between the primate skin lesions just demonstrated by Dr. de Vries and the picture seen during rejection of homologous skin in mice and also during the rejection of isologous male skin by female mice of certain strains.

DE VRIES recalls some of van Rood's slides of human homologous skin grafts during rejection and again notes a striking similarity with the skin lesions seen in primates with secondary disease.

KROHN In view of the lesser sensitivity of endocrine glands to homograft reactions, have such glands also been examined by Dr. de Vries and were any lesions found, particularly in the ovaries?

DE VRIES Similar changes were not seen in the ovaries though they were seen in the cortex of the adrenal glands and the island tissue of the pancreas.

KROHN asks whether the thymus behaves like any of the lymphatic organs or differently.

DE VRIES The thymus was not systematically studied, besides there already is a considerable spontaneous atrophy of the thymus at the age at which the animals were examined.

PITCOCK confirms Dr. de Vries' statement, the thymus being almost impossible to find at spontaneous death of the animals. In serial sacrifice studies the thymus seemed to behave like other lymphatic tissues. He would like to add an observation not mentioned by Dr. de Vries namely that spleens of treated animals were 50% heavier than those of nontreated irradiated animals, probably due to congestion of the organ. On this basis the possibility of some immune aspects of hemolysis was considered though this is still unproven. In serial killings of treated animals, pyronin positive cells with a very prominent nucleolus, presumably plasmoblasts, were seen before

the animals got sick. Massive degeneration of lymphoid tissue was not seen except when it could be tentatively attributed to infection. Finally, the role of the small lymphocyte as the immunologically active cell is by no means certain.

MATHÉ asks whether secondary disease should be regarded as a good experimental model to study auto-immune diseases such as lupus erythematodes.

DE VRIES From a purely morphological point of view the demonstrated skin lesions have several features in common with the skin lesions in L. E. though other, equally important, histological changes of the skin are missing. A comparison of the 2 diseases, purely on the basis of the skin lesions, is highly hypothetical.

KURNICK observes that systemic L. E. is very commonly seen without any skin lesions. Could therefore discoid L. E. be the disease in question.

DE VRIES Clinically, there is the acute, subacute and the chronic form of systemic L. E. and to distinguish the latter from discoid lupus one would like to see a positive L. E. cell phenomenon; skin lesions highly reminiscent of those seen in discoid lupus are known to occur in the subacute and the chronic form of systemic L. E. Some of these skin lesions are indeed seen in homologously treated primates e. g. liquefaction degeneration of the basal layer; dyskeratosis however, does not occur in systemic lupus. On the other hand primates with secondary disease do not display general changes of the collagen, neither vascular changes or endocarditis, all features of systemic L. E.

VOS stresses the dissimilarity between mouse bone marrow and primate bone marrow as far as the killing effect is concerned. Two hundred and fifty times the minimal amount of bone marrow necessary for recovery in a homologous or parent to F_1 situation has no killing effect in mice. Is

there a hematological difference to explain this dissimilarity between mouse and monkey bone marrow?

DE VRIES thinks a greater content of lymphoid cells in primate bone marrow to be likely. Accurate differential counts of the injected marrow suspensions are difficult because of the variable admixture of peripheral blood. With both the primate marrow suspension and Mathé's human bone marrow suspension large amounts of blood with a considerable number of lymphoid cells are concomitantly injected. On the other hand, accurate differential counts of the injected primate bone marrow suspensions were not obtained, so that other factors responsible for the observed differences between mouse and primate bone marrow cannot be excluded.

BONE MARROW TRANSPLANTATION IN THE RHESUS MONKEY

Progress Report

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Studies on the protection of rhesus monkeys after lethal irradiation doses with bone marrow suspensions have been reported from our institute (Crouch et al., 1961; de Vries et al., 1961). Summarizing these data it may be concluded that:

1) With fresh autologous marrow, protection against doses up to about 1000 r is routinely obtained with 1.2×10^8 cells per kg body-weight or more.

2) With homologous marrow, takes of donor material could be demonstrated after $2.5\text{-}3 \times 10^8$ cells per kg upward; however, long survival was never seen (max. 9 weeks). The animals died from "secondary disease" with the following clinical symptoms: anorexia, diarrhea, skin lesions, jaundice, infections (the post mortem findings were summarized by de Vries in the previous paper).

3) Secondary disease was seen only when a take of the donor cells occurred. It occurred usually very early after bone marrow transplantation; there was some variation in the severity of the disease but it was generally much more severe than usually seen in rodents and it proved uniformly lethal.

* This work was performed under contract with Euratom (European Atomic Energy Community) 51-53 rue Belliard, Brussels, Belgium.

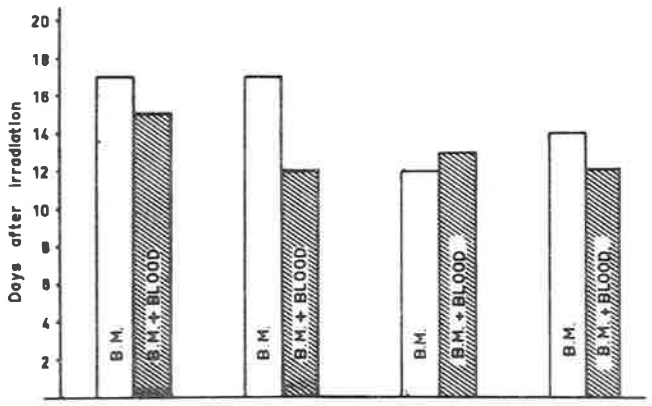
The present report is mainly concerned with studies on the cause of the severity of this secondary disease and with means to prevent it.

Since most of the earlier studies were performed with cells obtained by puncture and aspiration of bone marrow from living donors, the cell suspensions were inevitably contaminated with relatively large quantities of blood.

In rodents it has been established that admixture of the foreign marrow suspensions with donor blood markedly enhances secondary disease or even has an acute killing effect on the 6th or 7th day after administration (Cole and Garver, 1961; Goodman and Congdon, 1961).

To establish whether in monkeys a similar effect of peripheral blood was responsible for the severe secondary disease, the following experiments were performed.

Figure 1



Survival times of X-irradiated monkeys (650 r) after treatment with bone marrow alone or bone marrow + 30 ml blood.

Donor monkeys were bled to death under anaesthesia and their bone marrow was collected. One part of the suspensions was mixed with 30 ml of blood and administered to one lethally irradiated monkey, another part was mixed with Tyrode's solution and injected into another similarly irradiated monkey. No marked differences in survival were seen. Autopsy showed severe secondary disease in all animals (fig. 1).

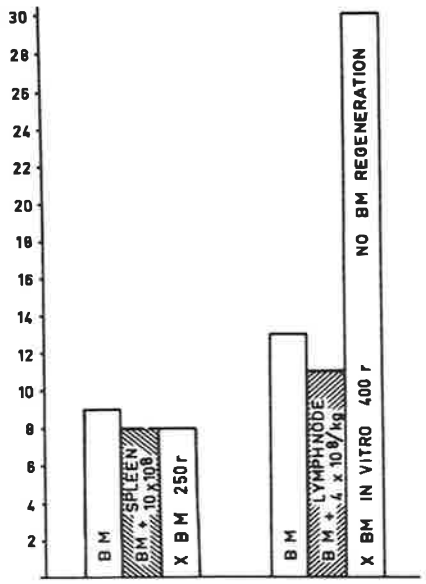
Another experiment was designed to elucidate whether the acute killing effect as seen in rodents after injection of peripheral blood, or large numbers of lymph node or spleen cells, could be reproduced in the monkeys.

Large numbers of immunologically competent cells (10×10^8 spleen cells or 4×10^8 lymph node cells per kg) were injected in addition to 5×10^8 bone marrow cells per kg body-weight of the X-irradiated recipient. This caused neither a markedly shorter survival time (fig. 2) nor any special clinical symptoms or autopsy findings in these monkeys in comparison with the controls, that received bone marrow alone from the same suspensions.

This result may be explained in two ways. Either lymphoid cells have nothing to do with secondary disease and with the mortality of these animals - a thesis which is contradicted by the pathological studies of de Vries (see previous paper) - or the bone marrow suspensions alone contain already a maximally effective number of immunologically competent cells. This latter supposition would be in agreement with the finding that lymph node repopulation usually seems to precede bone marrow repopulation after administration of bone marrow.

The next step in our studies is a logical consequence of the finding of an excessive immunological activity of the bone marrow suspensions: Attempts were made to reduce selectively the number of immunologically active cells

Figure 2



Survival times of X-irradiated monkeys (850 r) after treatment with bone marrow (5×10^8/kg) or with bone marrow (5×10^8/kg) + lymphoid cells from lymph nodes or spleen or with X-irradiated bone marrow (20×10^8/kg).

in the suspensions by various methods.

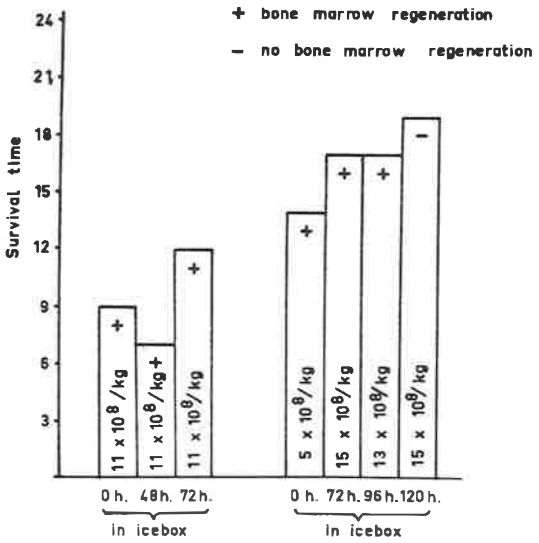
a) Irradiation might favourably influence the relative immunological and hemopoietic potencies of the cell suspensions. Irradiation was tested with an increased number of cells administered, to compensate for a partial loss of hemopoietic cells.

A fourfold increase in cell number combined with in vitro irradiation of the suspension with 250 r of X-rays did not change survival time or severity of secondary disease. A similar experiment with a fourfold increase in cell number and 400 r of X-rays resulted in a longer survival time with evidence

of temporary and incomplete regeneration of bone marrow (fig. 2) but with at the same time definite evidence of a minor degree of secondary disease.

b) Another method was found to be much more successful in mice in preventing homograft reactivity from mouse spleen suspensions without equivalent loss of radiation protection potency (van Bekkum, 1962). This method consists simply of storage at + 4°C for 3 or 4 days. The results in monkeys are shown in figure 3. It is evident again that the longest survival time is obtained where no bone marrow regeneration occurs. There

Figure 3

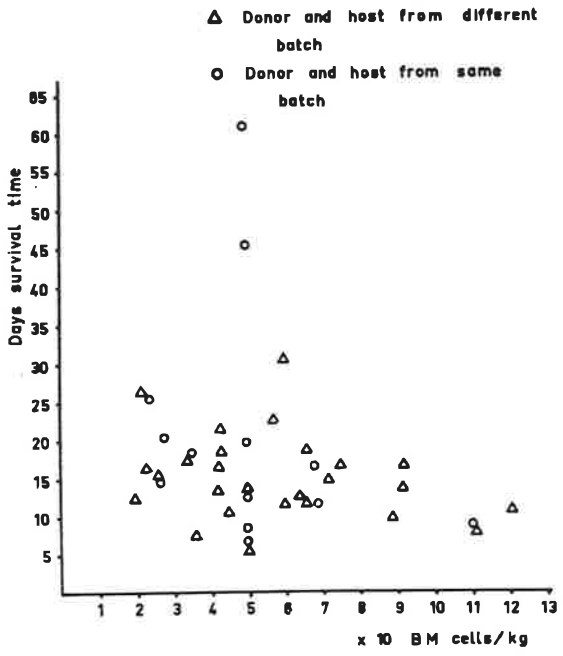


Survival times of monkeys treated 24 hours after 850 r total body irradiation with bone marrow stored at + 4°C for various periods.

may be a slight effect of storage but this has certainly no clinical importance. These observations suggest that methods which are effective in

decreasing the secondary disease in rodents are not necessarily effective in the monkey. This could be due to resistance of monkey lymphoid cells but it could also be due to an excess of immunological potency of the bone marrow suspension. If the latter is true it is rather surprising that we ever observed survival times of over 30 days and it might be worth-while to reconsider these results. If these long survivors were not obtained by low immunological activity of the suspension, they could perhaps be a result of variations in the degree of "compatibility" between host and donor.

Figure 4



Survival times of monkeys after irradiation and homologous transplantation plotted against bone marrow cell dose. Donor-host combinations from possibly related monkeys, arriving in the same batch are presented as circles; the others as triangles.

With this in mind we have tabulated all our data in a search for compatible and incompatible donor-host combinations. Most of the studies have been done with monkeys which arrived in this country in different transport batches. Only in a few instances donor and host were selected from the same batch. Among these instances were our two longest survivors but the total average of this group is not significantly better (fig. 4). We are thus unable to conclude whether selection of "compatible" or related monkeys may improve the results.

Finally we have started work with fetal donor material. Satisfactory suspensions from fetal monkey liver and spleen could be obtained from fetuses of an estimated age of 100 days (after the last menstrual bleeding). The number of cells obtainable from one fetus was at best only slightly more than the minimal number of adult homologous cells (8×10^8 for a 3 kg recipient) necessary to obtain a "take". Nevertheless in two trials no effect of these numbers of hemopoietic cells was observed in irradiated recipients and several attempts with lower numbers of cells have also been unsuccessful.

A mixture of suspensions from two fetal donors was tried once, supplying 7.2×10^8 cells per kg recipient. This produced temporary incomplete bone marrow recovery and incomplete lymph node recovery and survival for 21 days, histologically there was no evidence of secondary disease.

It seems that just as in mice (Crouch, 1960) more donor cells may be needed from fetal than from adult sources to produce a similar degree of hemopoietic recovery. For monkeys this seems to imply that more than one fetal donor is certainly needed and we plan to test large numbers of donors. We do not know what are the effects of administering suspensions made up from a number of donors. For practical application it will almost certainly be necessary to have a dependable freezing method, but we believe the treatment should first be worked out using pooled fetal cell suspensions.

As regards the freezing method, preliminary data have indicated that the storage of monkey bone marrow suspensions causes in our hands a much greater loss of protection ability than when the same storage techniques were applied to mouse bone marrow.

Recent experiments in our institute demonstrated that the standard slow freezing in 30% glycerol-tyrode with rapid thawing and Sloviter procedure gives for mice about 60% preservation of the fresh protective effect. For monkeys the preservation percentage seems in preliminary studies to be below 10 and we have as yet not been able to protect a single animal with autologous marrow after freeze-storage.

In summary we feel that notwithstanding a large number of negative results, the subject has not been covered exhaustively and there is still a lot of work to go through before all the possibilities of preventing secondary mortality have been explored.

ACKNOWLEDGEMENTS

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DISCUSSION

KURNICK When exactly did Dr. van Putten determine the viability of his foetal cells? In our limited experience with human foetal cells we found that within an hour the viability, as measured by trypan blue exclusion, had fallen to almost zero, both for foetal spleen and bone marrow. Concerning the use of frozen stored bone marrow we found that the viability is much improved by avoiding dilution by the Sloviter method (H. A. Sloviter. Am. J. Med. Sci., 231, 437, 1956). We found no harm in injecting it in the presence of glycerol without filtration and without removing fat. Slow thawing at zero degrees C in air seemed to have an advantage over fast thawing.

VAN PUTTEN As to the first question, we do find a reasonable viability of the monkey foetal material using the eosin-test. Some of the foetuses are obtained from an institute where great numbers of monkeys are sacrificed for polio investigations. If a pregnancy is found, the foetus is put on ice and received by us within 3 hours. Roughly 7% of the cells of our foetal marrow suspensions are dead. Foetal livers of a suitable age yield suspensions with up to 60% non-viable cells, the dead cells being mainly parenchymal, non-hemopoietic cells. I must admit however that our only case of repopulation by foetal material, was when the suspensions were rapidly obtained from 2 foetuses who were delivered by cesarian section in our laboratory and were injected soon afterwards. In answer to the second question I would like to mention that in a statistical study in mice, undiluted glycerol-stored suspensions are much less effective in saving irradiated animals than the same suspensions if diluted by the Sloviter method. However if dimethyl sulfoxyde is used in a 15% concentration for storage, the undiluted suspension is very effective but the Sloviter dilution, in this case, completely abolishes the protective effect.

KAY would like to confirm the difficulties with eosin, when testing foetal

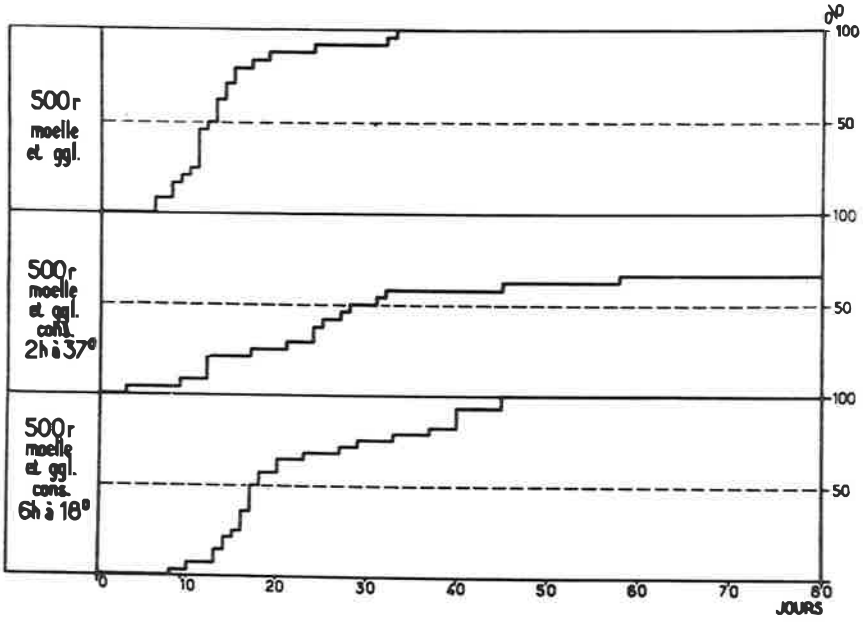
material, since all parenchymal cells take up the dye. He uses acridine orange which produces an orange fluorescence in viable cells. This correlates well with the biological activity of cell suspensions after freezing with dimethyl sulfoxide (which is preferred to freezing with glycerol).

VAN PUTTEN Could a rough estimate be given of the percentage viable cells recovered after freezing?

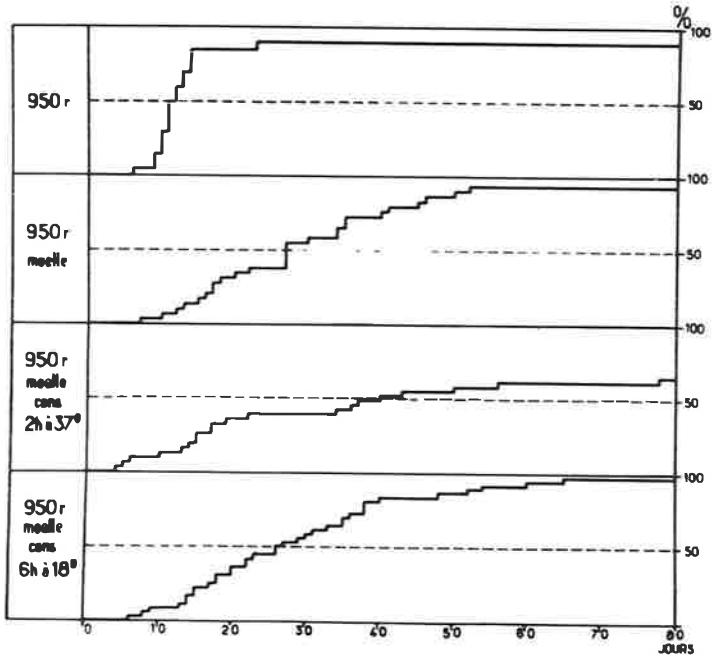
KAY Yes, with the optimum concentration of dimethyl sulfoxide (12.5%) one gets about 40% of the original number of viable cells. With human HeLa or lung culture cells one can get 80% recovery by a Puck plating technique so that the results are not only species-specific but also depending on the cell type.

VOS As has been mentioned by Dr. van Putten, attempts have been made to eliminate selectively lymphoid cells from bone marrow in order to reduce secondary disease. One of the methods is based on X-irradiation, but when Smith and I tried to estimate the radiosensitivity of lymph node cells and marrow, the LD_{37} was not significantly different for the two types of cells in mice. There seems to be little hope to kill either of these two cell types selectively.

MATHÉ would like to mention another way of conservation which seems to affect the immunologically competent cells somewhat more than the myeloid cells. Conservation of cells for 2 hours at 37°C or 6 hours at 18° reduces the number of eosin-resistant cells to about 50%. The three tests used are depicted in the slides. The top row shows the mortality when we inject C57BL marrow and lymphoid cells into sublethally irradiated F_1 (C57 x DBA2) and look for a killing effect and one can see that only the conservation for 2 hours at 37° (second row) decreases mortality significantly (slide 1). In the second experiment only homologous marrow was used and one can see that conservation for 2 hours at 37°C decreases lethality significantly. Though

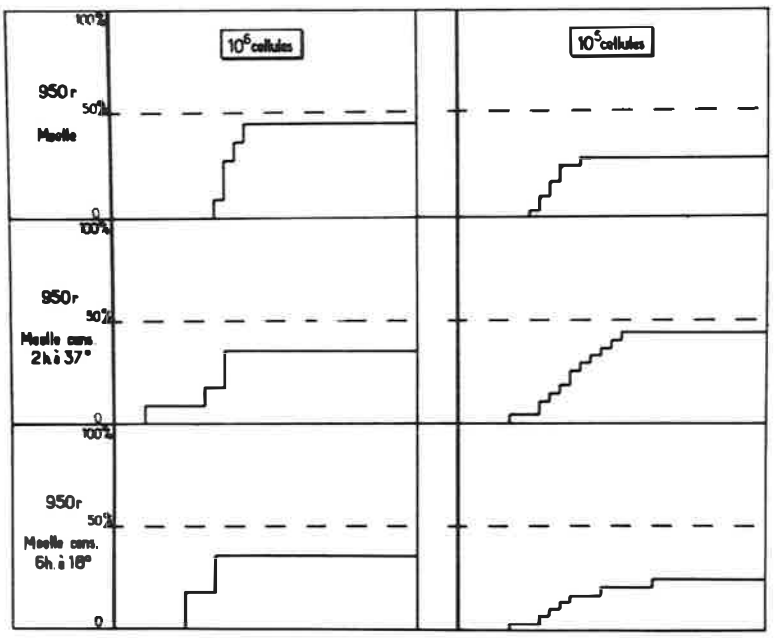


Slide 1



Slide 2

myeloid restoration is not decreased, storage for 6 hours at 18° does not work (slide 2). Then, in the third experiment where restoration was obtained by minimal numbers of isologous marrow cells (10^5 and 10^6) no significant differences are observed between the three groups (slide 3). But



Slide 3

one loses 60% of the cells and to inject the same number of viable cells one would have to start with twice that number of cells. If we want to apply this to humans we would need more cells than can be obtained from one individual so we expect to use several donors per recipient to test this.

VAN BEKKUM Unless minimal effective numbers of cells were used in the first two tests your results do not prove a selective effect on either of the two cell types. From my experiments there seems to be a slight selective effect at last and the only real proof for this is the following. In certain mouse host-donor combinations, parent spleen suspensions will not provide

EFFECT OF STORAGE AT 4°C ON THE KILLING EFFECT OF LYMPHOID CELLS

donor cells CBA ♂		irradiated recipients ♀ F_1 ♀♀ percentage mortality	
5×10^6 marrow + 2×10^5 lymph node cells		30 days	60 days
fresh	(14%)*	90	90
3 days	(27%)	25	100
4 days	(33%)	25	40
5 days	(42%)	100	-
20×10^6 spleen cells			
fresh	(34%)	100	-
3 days	(40%)	45	56
4 days	(71%)	56	66

* percentage of eosin positive cells in suspension

Slide 4

protection to irradiated F_1 hybrids regardless of the number of cells injected, the mice die either from direct killing or from bone marrow aplasia. If however the suspension is stored in the refrigerator for a few days before injection we do get a certain percentage of survivors, though the protective effect is rather small (see slide 4). Therefore, I would like to ask you whether you have done cell number titriations in your tests and whether you found the effect when a minimal effective number of cells was used.

MATHÉ In the case of isologous restoration (slide 3) we worked with 10^5 and 10^6 cells and found the same protection in both. We do not have 100% protection and we are not down to the minimum level. But it seems that we can decrease the secondary syndrome in practice by this method. That is, if we can get more donor cells.

VAN BEKKUM agrees that it is very difficult to prove a selective effect on immunologically active cells and that one must keep in mind that any decrease of the number of cells of a suspension capable of causing a graft-

versus-host reaction, will indeed reduce the chances for this graft-versus-host reaction to become manifest.

AMBRUS Did Dr. Mathé actually propose to give irradiated patients marrow from several donors at a time? Would this not complicate matters by adding a multiplicity of graft-versus-graft reactions to the already existing immunological reactions of graft-versus-host and vice versa?

MATHÉ With this in mind we have restored irradiated mice with marrow from 4 different strains, including C57 marrow causing a high percentage of secondary disease and DBA2 marrow which rarely provokes a secondary syndrome. When these 4 marrow suspensions are mixed we observe less secondary syndromes than when the strain with maximal anti-host activity (C57) is used alone.

AMBRUS wonders how the experiments are actually set up since one must consider the possibility that putting inother grafts next to the C57 marrow, which is so damaging to the host, the additional grafts may simply destroy part of the primary C57 marrow graft. One would only get a seemingly better result.

MATHÉ The experiment is now in its 50th day and apparently less secondary disease is seen than when only C57 cells were used.

COHEN would like some more comments on the use of marrow pools. Though the problem of graft-versus-host reactions as mentioned by Dr. Ambrus must be kept in mind, Lengerova of the Immunological Institute at Prague, gets quite favourable results by using large pools of some 40 types of cells. This might be due to a "self-clearance effect" of lymphoid cells. Something like a "clonal selection mechanism" might be operative, by which the antigenically remote cells perish while those antigenically acceptable to the host might be saved. Maybe it would be useful to continue the kind of work Lengerova has been doing with pooled cells, in the hope of

saving selectively those immunologically competent cells that would be antigenically most related and most acceptable to the host.

CROUCH Had anyone in the group ever done this in monkeys?

LOEB mentions the use of 3 donors for the treatment of patients. The different marrows were not injected simultaneously but successively within a few days. Death in these children occurred 28 to 30 days after total body irradiation. Secondary disease may have played a role in the death of these children: the lymphoid tissue was definitely depleted in 4 out of 5 at autopsy. The marrow of both parents and of other close relatives was used in these cases.

GENERAL DISCUSSION
ON BONE MARROW TRANSPLANTATION IN MONKEYS

Chairman: B. G. Crouch

CROUCH We should start with the problem of the results of blood cultures, of determining whether animals treated with autologous or homologous marrow are killed by a septicemia or by a generaiized infection. Can blood cultures really tell us anything about the cause of death in these animals.

SCHOFIELD All the organisms that were cultured at the time of death were organisms which are normally present in the gastrointestinal tract. We start off with treating the animals with antibiotics quite early after irradiation and our big problem is development of resistance to the antibiotics. We think about changing the antibiotic after about 7 days and continue with another antibiotic which is still active against the bacteria. Furadantine seems to be a good second best because practically all tetracycline-resistant organisms were still sensitive to furadantine. The other thing which would be very nive to have is a completely bacteriocidal antibiotic because all we do with tetracycline is keeping the organisms down to a very low level.

VAN PUTTEN Two years ago Dr. Wensinck, at that time the bacteriologist of our team, studied this and found that an animal which showed a positive blood culture would not live longer than 24 hours which means that positive blood cultures were a terminal event. We have never been able to treat an animal succesfully with antibiotics after finding a certain micro-organism in its blood. Do you have the same experience that once you can obtain a positive blood culture, the animal will not live very long whatever you do.

SCHOFIELD Yes, I quite agree with this.

DE VRIES One of the methods by which one can judge whether the bacteremia has anything to do with the death of the animal is to look for lesions

caused by septicemia. These lesions are regularly found in irradiated non-treated controls, for example septic necrosis in the liver and other organs. Such lesions which are evidently caused by the bacteria found in these centres are not found in animals treated with homologous bone marrow dying weeks following the bone marrow transplantation. Although one cannot say with certainty that septicemia may cause death without causing any lesions. The absence of such lesions could be an indication that bacteremia, especially of E. coli which is so often seen in monkeys at autopsy may not be the actual cause of death.

VAN LANCKER I would like to go even further than Dr. de Vries. Close to 20 animals died after the administration of 800 r, we have not been able to do bacterial cultures of the blood while they were alive nor after death but the lesions that Dr. de Vries described which would result from bacterial infection were not present at autopsy, not even in the lungs. We have done bacterial stains in many of these animals and it seems they died practically aseptic. We are still wondering what killed them.

PITCOCK We have seen micro-abscesses in the liver in some animals, particularly those dying after 15 days, when the marrow will begin to re-generate spontaneously. This is in animals who have been irradiated and were not treated with marrow. I would agree with Dr. de Vries that we have not seen this very often in those that have received bone marrow.

VAN LANCKER I only say that animals that had received total body irradiation sometimes die without septicemia. As to the micro-abscesses in the liver, did you culture them? Are they just the result of focal necrosis which might very well be due to anoxemia or are they typical micro-abscesses.

PITCOCK These are typical micro-abscesses. We did not culture the liver but we cultured the blood from the right heart and whenever we found micro-abscesses we did obtain positive blood cultures. On the other hand we have

found positive blood cultures without finding micro-abscesses so I am not sure that it is always significant.

SCHOFIELD We have done blood cultures and also stains for bacterial organisms and find the blood cultures positive on cases where we can find no bacterial organisms in sections.

KURNICK There seems to be little doubt that animals that are rendered pancytopenic by irradiation are more susceptible to infection and that some of them, perhaps even the majority of them die with infection. However, the work on the germ-free animals at Notre Dame reported at Harrogate by R. Wilson indicated that in the midlethal range the germ-free animals die at about the same time and still their death cannot be due to infection.

SCHOFIELD These findings reported at Harrogate are quite surprising. The LD_{50} in these mice was only about 50 rads higher whereas at a dose level where acute intestinal death occurs, the survival time was prolonged as compared to conventional animals. This seems the wrong way around to me and I wonder if anybody could throw any light upon this problem.

KURNICK To me it does not seem really surprising because I think that gut death in bacteria-carrying animals is due to the fact that the epithelial destruction readily admits the bacteria into the blood stream whereas in the germ-free animal this will not happen, so therefore intestinal death will not occur until you have really enough destruction to get hemorrhagic death from gut damage.

DE VRIES I think we are now concentrating too much on septicemia as a cause of death. In the bone marrow syndrome there is another important cause of death and this is hemorrhage due to loss of platelets in the peripheral blood, and this may cause excessive, massive hemorrhage in the colon. These hemorrhages, through pressure, may interfere with the circulation thereby causing hemorrhagic necrosis of the colon and this is an

important cause of death in irradiated nontreated monkeys. So there are two modes of death: firstly, hemorrhages complicated by ulceration in the intestine which can be clearly distinguished from the intestinal lesions found in secondary disease and from the irradiation-induced intestinal lesions after much higher doses of irradiation and secondly, septicemia. In the case of septicemia because there are no leukocytes to give abscess formation, we often find necrosis, with in the center of the necrotic areas accumulations of bacteria. The blood cultures are usually positive in these cases.

PITCOCK I will fully agree that the hemorrhage is important in the pathogenesis of the colitis that is seen at the lower dose levels. However, we have generally not seen massive hemorrhages from the gut with the animal dying from shock. I wonder if the mechanism is not hemorrhage into the colon leading to ulceration and then invasion of bacterial organisms.

OVERMAN We have made a number of measurements of blood volume and circulating red cell mass in animals serially killed up to the time of spontaneous death and certainly the amount of blood lost was insufficient to be very important from the cardiovascular stand-point. I agree that hemorrhage in these animals must have another importance than the simple loss of fluid.

VAN LANCKER I have always been impressed by the importance of the hemorrhage in the heart and wonder to what extent the hemorrhages may play a role in causing death. I think there is also quite a bit of blood lost in the intestines, the feces containing large amounts of blood in most of the animals which received a lethal dose of X-radiation. It is true that otherwise hemorrhage is rather limited. Hemorrhage in the heart however is not without significance because it may be large enough to induce focal necrosis around these zones of hemorrhage and the same thing occasionally occurs in the brain.

DE VRIES Whatever the exact direct cause of death in these animals, it is primarily the hemorrhage in the intestines and whether the animals die ultimately of blood loss or by necrosis of the affected segments of the bowel or by secondary bacterial invasion, death is always somehow related to hemorrhage.

CROUCH I think we can summarize that hemorrhage plays an important role whether it is depletion of the vascular system which is not very likely or whether it causes lesions in the colon and promotes infection.

C. AMBRUS If I could again bring up the figures I have shown this morning: out of 26 monkeys which had been irradiated with 700 r we had 12 animals surviving which is approximately 50%. These animals were around for 2 years apparently in good health. In those who died (except for one, all of them died around the 15th day) we found in most of the animals petechiae in the intestine, the pleura, intraperitoneally and there was also blood in the feces, so obviously hemorrhage was implicated to a certain extent in death. In three of the homologous treated animals we were able to prove a take of the donor marrow. Two of these animals survived after reversal without any apparent signs of illness. The one monkey with a proven take who died on the 62nd day - I believe after listening to Dr. de Vries' description of secondary disease - may have died of a combination of secondary disease and infection, the infection probably superimposed on the pathological changes of secondary disease. I wanted to point out the differences in techniques between our group and the Rijswijk group, because obviously we are the only ones who seem to have long-term recovery of irradiated animals after homologous marrow transplantation. We would like to know what this may be due to.

VAN PUTTEN I have been wondering about the RBE of the 2 MeV X-rays you use. Maybe we can collect our techniques in a table (see end of this discussion).

CROUCH Mrs. Ambrus, your data and the methods you used agree closely with the work that Dr. Overman and I did some years ago in Memphis and we had about the same percentage survival (see table: Memphis 1955-1957).

BALNER The much higher survival rate, the absence of secondary disease and the absence of MLD effect that was observed in the homologous bone marrow treated monkeys by Dr. C. Ambrus might be explained by a higher degree of histocompatibility between members of the Roswell Park monkey colony. Has Dr. Ambrus any specific information on this point?

C. AMBRUS The skin grafting experiments are being done right now. We have not done it in the former group of animals but in the ones we use now, who are from the same batch, we are presently doing it.

VAN BEKKUM I think the dilemma of the different results may be clarified if you look at the animals that did not survive in your group. You have a group of animals that received 700 r and 4-6 x 10^8 cells/kg. Did these eight animals die with or without bone marrow regeneration? If without, I would think that your results suggest that you are working under minimal conditions favouring a take of the graft, while we work under optimal conditions for a successful graft.

C. AMBRUS The animals that died had aplastic marrow.

CROUCH I would like to pursue the question that was brought up before about the rationale for treating sublethally irradiated animals. In other words, the so-called MLD effect and the graphs which Dr. van Bekkum put on the board. I think we still have some disagreement that was not thoroughly talked out. There are Dr. Mathé's experiences with the Yugoslaves with a 75% lethal dose of radiation and there are some other experiences. Would anyone like to comment on this?

MATHÉ I think one must come back to Uphoff's experiments. She tried many strain combinations and in two of them, the strain combinations which also

Dr. van Bekkum had studied, mortality was increased when marrow was given after an LD_{50} dose of irradiation but in the others, maybe ten others, mortality was decreased. I think under these conditions the histocompatibility relationship between host and donor is the most important thing.

VAN BEKKUM How would you transfer this information from mouse to man Dr. Mathé?

MATHÉ I would not refuse to give bone marrow to sublethally irradiated patients. This is my conclusion. I would not indicate the bone marrow treatment by dosimetry, especially not by physical dosimetry. First I would give symptomatic treatment, this means asepsis, antibiotics and platelet transfusions and if this treatment is sufficient, I would not give bone marrow. If this treatment seems to be insufficient I would inject bone marrow.

OVERMAN I wonder if anyone else has got any evidence that the time of introducing marrow in relation to the time that the irradiation is given is an important point. For instance, I've got the notion that the Yugoslaves were given bone marrow quite a long time after the damage was originally done and not within 24 hours or anything like that. Is that correct?

MATHÉ I think this is a very important question. If one needs a transitory graft I would inject the bone marrow late, as late as possible, when there is erythropoietin and maybe some leucopoietin etc. If I would try to have a permanent graft I would inject it sooner. But one must remember in man that the spleen is not myeloid. In mice the spleen is myeloid and most of the graft at the beginning is in the spleen, not in the bone marrow. In human beings the spleen is not myeloid, the grafts must go to the bone marrow, but just after the irradiation there is a very serious congestion of the bone marrow, possibly due to lysis of the cells and for this reason I don't like to inject bone marrow very early. Did someone try to inject marrow at a late stage into irradiated monkeys who may be in the same position, as far as

the spleen is concerned, as man?

OVERMAN The only thing we know about, would be the difference between 24 hours and 48 hours, where it looked as though 48 hours was a better time to do this than 24, and possibly if we had waited longer, which we never did, it would have been even better.

SCHOFIELD There has been a certain amount of discussion today suggesting that repopulation with circulating cells is the important thing in keeping the animal alive. In monkeys anyway, delaying the bone marrow transplantation would also delay the repopulation by circulating cells and would appear to be contra-indicated.

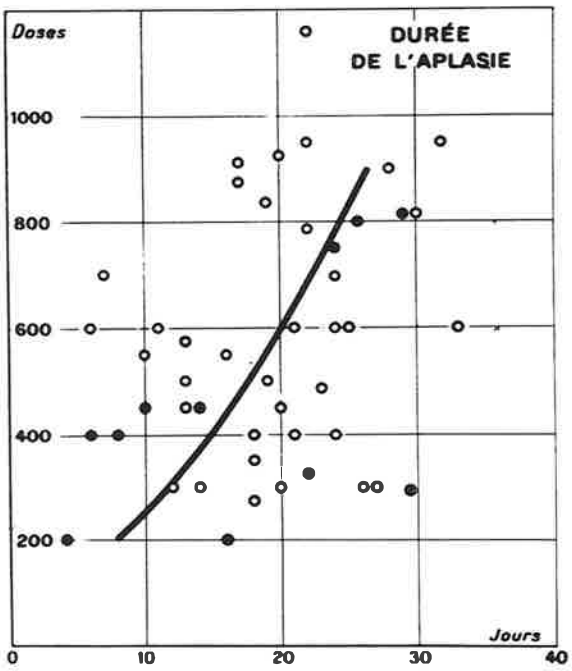
VAN BEKKUM This is, I think, essentially correct, and the only way to study this problem I believe, is in monkeys, because in mice one has very little time before the death of the animals. In addition, in the monkey, one would have to use an LD_{50} and not an LD_{100}. I would also suggest to give supportive treatment including thrombocyte-transfusions to these monkeys and only then can one study the effect of delayed bone marrow transplantation up to a period of maybe a week to 14 days.

SCHOFIELD If you give supportive therapy in the form of thrombocyte-rich plasma, then is not this rather obscuring the results you expect to get by giving bone marrow transplants?

VAN BEKKUM Of course one should perform this type of experiment with a large number of controls which are treated symptomatically and do not receive bone marrow and under such conditions, that a certain number of these controls will die. Only then can one assess the effect of the bone marrow.

MATHÉ I should like to show a slide demonstrating that dosimetry does not allow to predict what will happen. We have written to many people who

have irradiated patients and here (slide no. 1) we see the relationship between



Slide 1. Duration of aplasia

the duration of aplasia and the doses received. The black points show the patients who have a spontaneous recovery. You can see that for the same doses, or almost the same doses there are big differences. I think the physical dosimetry, even if it is well known, does not allow the clinician to guess about the prognosis. Here, the clinical sixth sense is obviously more important than dosimetry.

KURNICK I agree, but I think that until the experiment that Dr. van Bekkum described has been done in the monkey, it is my impression that one probably does the human patient a better service by treating only supportively with

platelet transfusions and whole blood and not with marrow. For the moment the evidence to me would appear to be, that if the dose is sublethal and you can keep the patient going with donations of non-reduplicating cells he will repopulate and if he is in the lethal range he will probably get secondary disease if you get a take, so there is a greater chance of doing him harm than doing him good by giving him marrow.

VAN PUTTEN I agree with Dr. Kurnick as far as it concerns whole-body irradiated patients but in accident cases it is very improbable that we shall ever see a homogeneously-whole-body-irradiated patient. You will undoubtedly encounter a number of patients where part of the bone marrow or the lymphatic tissue has been spared. And I think this will, to a very large extent, reduce the probability of their getting a permanent take and secondary disease.

KURNICK Well, then the only question is whether the temporary take is of any real benefit and whether replacement therapy with peripheral blood elements won't do just as well until he recovers in a similar way as he does when a temporary graft is rejected.

VAN PUTTEN Yes I agree, but I am not sure whether there is some evidence for this in mice. There is - from the Yugoslaves - some evidence for it in man, but maybe it would be a good thing to obtain the evidence in monkeys as well.

F. E. NEWSOME Billingham has found that in secondary disease the body temperature is lowered by 2^{o} C and I was wondering whether anyone has ever found similar results?

YOUNG I think we mentioned there was at times a slight temperature elevation. Actually this temperature elevation is related to what we have always considered as an infectious syndrome. We do see a temperature decrease just prior to death. Whether this decrease is exactly 2^{o} C I don't know.

WHITCOMB I'd like to say that in the six animals that received homologous bone marrow only, temperature elevation occurred in every animal of the six and appeared very definite to us. When we first observed this, we felt that it parallelled the temperature rise that had been observed in the human casualty cases at approximately 6 days following the accidents. We failed to see it in the autologously treated animals or in the control animals and in those animals which were treated with antibiotics alone. However, in subsequent experiments in which we preceeded the homologous marrow transfusion with chemical protection, (Dr. Melville's pattern of treatment) we also saw this temperature elevation again, only not quite to the same degree as before. We have no explanation for this.

VAN PUTTEN We too found a temperature elevation as long as the animals survive, though we did not work this out with elaborate base line values etc. When the animals go into shock terminally we observe a drop of body temperature to below normal values but this lower temperature seems not specific for the presence of secondary disease.

TECHNIQUES AND RESULTS AFTER HOMOLOGOUS BONE MARROW TRANSPLANTATION IN MONKEYS AS REPORTED FROM DIFFERENT INSTITUTES

	Brooks AFB	Buffalo		Memphis 1955 - 1957		Memphis 1958 - 1962	Rijswijk	
Weight of animals	5 - 7 lbs	2.5 - 3.5 kg		3.5 - 5 kg		2 - 3.5 kg	2.5 - 4 kg	
X-ray potential	250 kV	2 MeV		240 kVp		240 kVp	250 kVp	
HVL	2 mm Cu			2 mm Cu		2 mm Cu	2 mm Cu	
Dose measurement	skin dose in air	skin dose in air		midline dose in air		midline dose in air	midline tissue dose	
Interval radiation-BM infusion	48 hrs	24 hrs		30 - 40 hrs		24 hrs	24 hrs	
Radiation dose (in r)	900	700	800	650 - 700	800	700 - 1000	650 - 850	
Cell dose/kg (x 10^8)	10	4 - 11	0.7 - 1	5	5	3 - 4	0.2 - 2	2.5 - 12
Long term survivors (> 100 days)	none	10/18	0/4	3/16	none	none	none	none
Evidence of "take" of donor cells	+[1]	temporarily in 2[1,2]	?	?	?	-	-	+[3]
Evidence of secondary disease	+	-	-	?	?	?	-	+
BM regeneration in non-survivors[4]	+	-	-	±	±	± to +	-	+

1) By red cell groups, quantitative, does not prove proliferation of donor cells at time of sampling, unless percentage donor cells increases (technique as developed by Owen, see text).

2) By anamnestic immune response, measures immune system, specificity limited? (technique see text).

3) By sex chromatin in granulocytes, not quantitative, measures nearly instantaneous proliferation of donor cell due to short life span of neutrophils (technique: Magliulo et al., to be published).

4) + = regenerating to normal or hyperplastic.
$\pm$ = hypoplastic.
- = aplastic.

IMMUNOLOGICAL ACTIVITY OF THE FOETUS

EVIDENCE RELATING TO THE IMMUNOLOGICAL CAPACITY OF HUMAN FOETAL TISSUES.

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Introduction

Suspensions of foetal haemopoietic cells have been used in radiation protection experiments in mice and have in some circumstances been successful, particularly in that the incidence and severity of secondary disease have been significantly reduced (e.g. Uphoff,1958). In other experiments (e.g. Crouch,1959) this has not been the case but the occurrence of secondary disease in primates (Van Bekkum, Crouch,Van Putten and De Vries,1961) including man (Mathe, Bernard,Schwarzenberg,Larrieu,Lalanne,Dutreix,Denoix,Surmont, Schwarzmann and Ceoara,1959) suggests naturally the possibility of using human foetal cell grafts.

From the classical experiments of Billingham,Brent and Medawar(1956) there has arisen a tendency to equate the period of immunological immaturity with that of intra-uterine life (plus a few days) but, in fact, there was prior evidence in sheep (Schinkel and Ferguson,1953), that the foetus could reject skin grafts as early as 90 days (normal gestation 150 days). It is perhaps surprising that the foetus, immured and immersed within the remote confines of the uterus should be concerned with immune reactions before these became vitally necessary i.e. when exposed to microbial hazards in post-natal life. However, as we

shall see, it would appear that in primates also some sort of immunological potentiality exists from quite an early date in foetal life.

When speaking of immunity and tolerance it is important to introduce at once a number of definitions and qualifications. Firstly we must distinguish between the type of immunity dependent on circulating antibody and that imparted by immune cells. Although often concomitant and probably synergistic these types may be dissociated or apparently antagonistic. Certainly there seems to be some dissociation in the timing of their development and in the chick there may be an anatomical distinction between the antibody-forming capacity, dependent it would seem on development of the Bursa of Fabricius, and cellular reactions for which the thymus is essential (Burnet,1962).

In the second place it is agreed that the development of tolerance or immunity although dependent most closely upon the state of the lymphoid system can be modified by the mode of administration and dose of antigen. Both types of response are possible in both mature and immature individuals, and the classical Felton type of immunological paralysis may not differ essentially from the tolerance induced in young animals with small doses of antigen. It is the dose of antigen in relation to either the size of some cell compartment or the pace of some metabolic function which appears to be critical.

Thirdly we must qualify any statement relating to one antigen with the proviso that another antigen may be neutral or invoke an entirely different response.

Fourthly we must allow for individual variation. In mice there is some evidence that different strains show different rates of maturation of immune potency. Extrapolation from these differences of a few days only in the compressed span of murine development might indicate quite considerable differences in the more prolonged development of primates.

Finally there remains the distinction between the normal rate of development under normal circumstances and the potential rate of development under abnormal conditions, such as continual antigenic stimulation, or removal of cells to a new, perhaps adult, environment. It may be that antigens, or more precisely substances having 'adjuvant' properties, can accelerate the maturation of lymphoid tissue; also possibly that conditions in a foreign environment - for example the presence of γ-globulin or the absence of α-globulins (Kamrin,1958) - may potentiate or switch the reactions of those cells responsible for immunity.

Now having qualified, as it may seem, any general statements into insignificance we must examine the available evidence which for pre-natal man is of three main kinds. Thus we can study the morphogenesis of the Reticulo-Endothelial System from a purely anatomical or histological viewpoint and compare these observations with other species where direct evidence is available. We can examine pathological conditions where the foetus has been subjected to antigenic stimulation in utero or, most directly, we can review those cases where foetal cells have been used in therapeutic transplantations either into foetal or, almost equally unsuccessfuly, into adult recipients.

The Neonatal Period

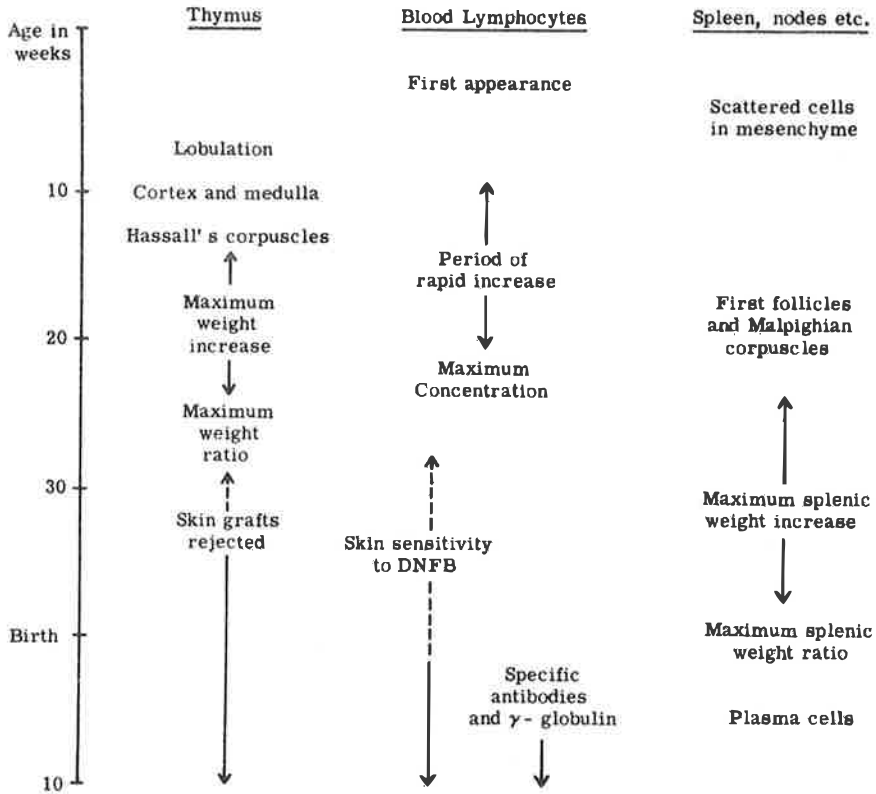
But first we must mention the state of the neonatal infant. Several strands of evidence indicate that it is born with a stock of maternal γ-globulin but has none of its own. Perhaps the best evidence for this statement is Zak and Good's case (1959) of an agammaglobulinaemic mother whose normal infant, born without gamma-globulin, took 40 days to reach a level of 115 mgm% γ-globulin despite repeated stimulation from birth by a variety of antigens. This may represent an exceptionally slow rate of maturation since many infants can make detectable antibodies - given the appropriate antigen - as soon as three weeks after birth (N.R. Butler - personal communication). The absence of plasma cells in young infants born to normal mothers is good confirmatory evidence that all their γ-globulin is initially of maternal origin.

Skin sensitivity on the other hand can often be stimulated from birth even in premature infants. Thus in 3/10 full-term babies and in 2/5 prematures, Uhr,Dancis and Neumann(1960) induced sensitivity to dinitrofluorobenzene given by percutaneous injection immediately after birth. The positive reactors included a foetus of only 30 weeks gestation. Although the numbers are small the individual variation is of considerable interest. Of other antigens that have been used, poison ivy seems to produce rapid sensitivity (Chase 1960) but tuberculin is ineffective up to 3 weeks of age at least (Warwick, Good and Smith 1960).

Fowler, Schubert and West (1960) applied skin homografts to new-born infants and found a normal adult type of rejection

Table 1

DEVELOPMENT OF LYMPHOID SYSTEM AND IMMUNITY IN MAN



at 12-20 days in 5 out of 6 including a 34-week premature baby. Their numbers are also small but it is pertinent that where the infants had received exchange-transfusions with fresh donor blood there was a considerable delay (68-148 days) in skin rejection accompanied in two cases by serious infections which raised the possibility that these might be the result of impairment of their immune system by foreign lymphocytes.

Lymphoid Morphogenesis

Information about neonatal infants, whether premature or full-term, is only partly relevant to our theme: it tells us nothing about the state of affairs in utero.

The early development of the lymphoid system is fairly well plotted out by classical embryologists. The difficulty is to define landmarks. The thymus is divided into a distinguishable cortex and medulla by about 11 weeks and Hassall's corpuscles appear at 13 weeks or so. The spleen acquires the first traces of Malpighian corpuscles at 18-20 weeks and lymphoid tissue in the intestine begins to be recognisable as such about the same time, although these seem to be rather variable milestones. Mature germinal follicles and plasma cells are never normally seen in pre-natal life. In the blood small lymphocytes appear at a very early stage, - they can be found as early as 1.8 cm.(C-R.) i.e. about 7 weeks - and the concentration increases to reach peak levels of about 7,000/cu.mm. at 24 weeks or so (Playfair,Wolfendale and Kay, 1962). Even in post-natal life this peak is not normally surpassed. We have found a rather similar increase in mid-

gestation in the foetal sheep but in the mouse and rat the main increase appears to be after birth. There is also a post-natal lymphocytosis in man - the peak is about 2 months (Kato,1935) with mean levels of 5-6,000/cu.mm. - but the pre-natal human levels almost certainly have no parallel in the rodents. Counts at day 18 in mice and rats for example are low, and the plain fact is that adequate lymphopoietic tissue does not come into existence until after birth.

The small lymphocyte is now known to be a very important cell immunologically so the question that naturally arises is this: are these small round cells in the foetal blood the same as those in post-natal blood? Morphologically they are, of course, rather anonymous cells but some preliminary studies we have done seem to show that their properties are identical (Wolfendale,Kay and Playfair,1962). Thus they have a similar motility, are non-phagocytic and do not take up tritiated thymidine but will, under the influence of phytohaemagglutinin, metamorphose to a blast-like cell and undergo mitosis. All these properties are shared by pre-natal and post-natal small lymphocytes.

It is possible to study and describe the evolution of the lymphoid tissues in the foetus especially, for example, the centres in the intestine and spleen and to point out that in comparison the earliest stage in follicle formation is not reached in the mouse until 5-6 days after birth. Quantitatively one can weigh either the spleen or thymus and express these as a proportion of the body weight. Of particular significance is the relative weight of the thymus

with its characteristic peak and subsequent decline even though the organ is still increasing in size for a considerable period of the whole life-span (Kay,Playfair,Wolfendale and Hopper,1962).

Antigenic Stimulation in Foetal Life

We thus have plenty of evidence that in man and the sheep the lymphoid system achieves a major part of its development during intra-uterine life but that immunological activity is not usually manifest until later. According to Silverstein, Kraner,Lukes and Brown(1962) this is due to lack of antigenic stimulation rather than incapacity. In their experiments with foetal sheep of about 100 days gestation precocious gamma-globulin synthesis was stimulated by injections of antigens combined with Freund's adjuvant and plasma cells appeared in the spleen. In one instance specific antibody was demonstrable. Presumably the only reason this does not occur naturally in sheep and man is that no antigens normally pass the placental barrier to initiate these responses.

Pathologically antigenic stimulation may occur and in man we can turn to certain pre-natal infections, for example syphilis, toxoplasmosis and tuberculosis. These are all rare nowadays, and the occasions when a late but non-macerated foetus can be examined are rarer still. Silverstein(1962) has examined a number of congenital syphilitics and on the basis of a plasma-cell reaction in the spleen has claimed that immunity can be initiated as early as the sixth month. The validity of this evidence depends on the positive identification of plasma-cells which in post-mortem material and in the presence of many

erythroid precursors may be difficult.

Foetal Haemopoietic Cell Grafts

Lastly I must mention such evidence as has been collected from the use of foetal cell grafts in man, the recipient being either another foetus or a marrow-depleted adult. In all cases the grafts have been composed of foetal liver cells in suspensions, injected either intravenously or intra-peritoneally. There is great difficulty in providing a sufficient quantity of cells for an adult recipient. The maximum we have ever achieved was 34 billion cells from seven different foetuses. In this and in one or two other cases there was dubious evidence of a temporary take of cells but nothing very convincing. Neither have symptoms attributable to secondary disease been encountered, but I suppose it is possible that in the fatal cases foetal, as well as adult, cells could have accelerated death by a graft-against-host reaction.

In the foetus-to-foetus transplants we are almost equally in the dark - literally. Of ten cases performed in Boston, London, Bristol and Dundee, it is worth considering four of the most recent and here I must acknowledge the major contribution of Dr. Diamond and his colleagues in Boston, Professor Browne at the Post-Graduate Hospital, London and Professor Walker at Dundee, as well as Dr. Goldsmith and Miss Giles at the Lister Institute.

Table 2

PRE-NATAL FOETAL LIVER TRANSPLANTATION IN ERYTHROBLASTOSIS FOETALIS

Case	Gestation (weeks)	Donor age (weeks)	Graft	Fate	Antigenic differences
6	13	?14(??18)	Fresh IP	Death in utero after ?3 weeks. Macerated foetus.	Donor O Recipient ?A and/or B
7	19½	19½	Fresh IP	Born at 34 weeks. Non-chimaeric, hydrops.	Donor MNS Recipient Ns
9	19	19	Fresh IV	Death in utero after 1 - 2 weeks. Macerated foetus.	
10	19½	15	Fresh IP	Born at 34½ weeks. Non-chimaeric, Hydrops.	Donor MS Recipient Ns

The four cases are summarised in Table 2. Previous cases had failed either for technical surgical reasons, because operation was too late, because preserved cells were used or for a combination of these reasons.

In these four the operation was technically a success. A suspension of cells from the whole liver of a healthy rhesus-negative foetus obtained at hysterotomy was injected after a delay of 6 to 36 hours into the recipient foetus either by the umbilical vein or into the abdomen (presumed to be into the peritoneal cavity).

In all cases there was evidence of continued vitality of the recipient foetus for 1 week or more but two evidently died between one and three weeks after the operation. Delay in delivery resulted in a hopelessly macerated foetus without the possibility of detecting chimaerism or of establishing the exact cause of death.

The other two continued to 34 weeks when labour was induced and two typical erythroblastotic infants were born. Neither was a blood-group chimaera and both were given immediate exchange transfusions. The more severely affected died but the other still lives - surprisingly perhaps as its three elder sibs were all premature stillbirths due to rhesus incompatibility.

There are a number of doubts about these cases. For example does the IP route enable enough stem-cells to reach the centres of haemopoiesis? How critical are the antigenic differences between donor and host? and, above all, of course, are these operations sufficiently early to effect mutual tolerance?

If, as now seems probable, both recipient and donor cells were immunologically potent then there may have been some sort of immune conflict with two donor and two recipient victories. If this is so then the advantages of foetal tissues are beginning to look very slender, although the lack of secondary immune responses might prove to be critical in some circumstances. Meanwhile we are in need of more direct evidence in primates to support our premises.

Acknowledgements

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DISCUSSION

VAN BEKKUM Could Dr. Kay state the numbers of cells that were employed in the foetus to foetus transplants?

KAY The cells derived from one whole liver were administered. From the liver of a 19 week old foetus about 1. 5 - 2 times 10^9 cells were obtained. There were two other transplants from younger donors which yielded under 10^9 cells, one about 8 times 10^8 and the other slightly less.

KROHN The results presented by Dr. Kay seem to suggest that the possibilities of inducing immunological tolerance in primates are not very good. However, since recent work with mice has shown that in this species certain degrees of tolerance can even be achieved in the adult animal, the difference might be still due to differences in cell dosage.

KAY Provided methods could be developed to specifically eliminate or suppress the immunologically competent cells from the donor suspensions - e. g. by incubation procedures as reported by Mathé and by van Bekkum - it would then become possible to increase the number of cells transplanted without increasing the risk of secondary disease for the recipient.

HUMBLE Were there any follow-up studies on Rh babies that had received exchange transfusions. Among them there had been a considerable number of prematures and donor skin transplants on them might have yielded information on the immunological status in these cases.

KAY AND KROHN referred to the investigations of Fowler et al. (Ann. N. Y. Acad. Sci., 87, 403, 1960) and those of Albert et al. (Biological Problems of Grafting, Blackwell, Oxford, 1959, pp 369). Both groups observed a prolonged survival of donor skin grafts in a proportion of their cases but there was no evidence of a chimeric condition in these patients.

VAN BEKKUM What is the yield of liver cells in relation to the age of the

donor and what is the number of foetal liver cells Dr. Kay would consider optimal in the treatment of bone marrow aplasia?

KAY The aim is to transplant as many cells as possible in cases of complete aplasia. This necessitates storage - which we do at present in dimethylsulfoxide - and although storage involves a certain loss of activity, this procedure should provide for theoretically unlimited numbers of cells to be administered. Usually 20-30 x 10^9 cells are given. When fresh cells are used the amount is entirely dependent of the foetal material that comes in during that particular period of a few days.

With regard to variation in cell type and yield per liver there seems to be a large variation in the number of hemopoietic cells at any one age. Roughly, at about 12 weeks the liver is 2/3 hemopoietic and by about 24 weeks this proportion is 1/3. There are furthermore bound to be variations in the proportion of stem cells and more mature cells and of these we have very little exact information. Also the method of preparation of the liver cell suspensions should be taken into account. When the liver tissue is chopped up and the cells are eluted from this mass a good yield of cells without clumping is obtained, but unfortunately Dr. Koller and collaborators found this type of suspension to be devoid of any protective effect in irradiated mice. Probably this means that the stem cells are not eluted by this procedure and therefore it seems necessary to grind up the liver particles which unavoidably leads to destruction of considerable numbers of cells.

VAN BEKKUM There are two aspects of the therapy with liver cell suspensions which should be considered separately. One is the restorative capacity of these cells in cases of bone marrow aplasia and the second is the severity of the graft versus host reaction once the transplant is established. With regard to the first property, from limited experience at Rijswijk with foetal liver suspensions in monkeys it seemed that their restorative properties were less than those of bone marrow from older animals. Did

Dr. Kay have evidence from the human material whether one or more cell types (e. g. thrombopoietic cells) were absent or deficient in the foetal livers?

KAY found that the foetal livers contained both erythropoietic cells and megakaryocytes and that myelopoiesis was not present to any significant extent. It is probable that foetal liver provides sufficient thrombopoietic stem cells because in one human case a very significant rise in platelets was observed following its administration. This was a case of lymphosarcoma which had developed aplasia as a result of multiple treatments with ^{32}P (Kay and Constantoulakis, Proc. 7th Congr. Europ. Soc. Haemat. 1959; part II, 1960, pp 964). The patient was suffering from hemorrhages and had a low platelet count when a suspension prepared from 2 livers of 20-24 week old embryos was injected. A dramatic increase of the platelet count occurred about 14 days later and the hemorrhagic symptoms disappeared. At the same time some reticulocytes appeared in the peripheral blood and some serological evidence was obtained that these reticulocytes were of the donor type.

DE VRIES An alternative explanation for the lower protective efficiency of foetal liver cells (in mice) could be that this tissue contains a much higher proportion of immature cells as compared to bone marrow and that comparatively more time is needed for these cells to proliferate and to maturate.

KAY Probably a more important difference between foetal liver suspensions and bone marrow suspensions is that a larger proportion of the former cells become trapped in the pulmonary capillaries and never reach the bone marrow, because it is very difficult to prepare monocellulair suspensions from liver tissues.

KURNICK asked Dr. Kay to comment on the use of foetal tissue for the treatment of agammaglobulinaemia. Kurnick had made two attempts at foetal spleen transplantations into adults with complete agammaglobulinaemia,

one patient was treated by intraperitoneal injection and the other two intramuscularly (although in the latter two cases intravascular transplantation was intended). In no case did gammaglobulins appear in the serum, the only evidence of a reaction was observed in one case which showed a beta globulin anomaly for a period of about two weeks.

KAY had treated two cases with foetal spleen and thymus cells. In one case the cells were injected intravenously, in the other case intraperitoneally. No evidence of a take was observed in either of the patients and the reason for this may be that agammaglobulinemic patients do show transplantation immunity reactions as evidenced by the rejection of skin transplants.

VAN LANCKER As reported by others the supernatant of liver suspensions can block the incorporation of thymidine C^{14} in bone marrow cells which may be a reflection of inhibition of mitosis. Could this not explain the lower efficiency of liver cell suspensions as compared to bone marrow?

VAN PUTTEN pointed out that the absence of takes in Kay's material did not necessarily mean that foetal human liver cells are much less effective than adult bone marrow, because with the latter material the number of takes has also been very limited.
It might be well to consider as well factors pertinent to the recipients which might have prevented the foetal cells to take. Among these factors are the absence of aplasia of the lymphatic tissues and possible sensitization due to previous blood transfusions.

KAY confirmed that nearly all his patients had had blood transfusions at some stage. Many had also been treated with slightly toxic amounts of alkylating drugs, probably not in sufficient dosage to depress the lymphatic system. In most cases this treatment was given elsewhere and foetal cells were requested as a last resort.

AN ATTEMPT TO DETERMINE THE AGE AT WHICH "CELLULAR IMMUNOLOGICAL MATURITY" DEVELOPS IN THE FOETAL RHESUS MONKEY

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The development of the capacity to recognise and reject immunologically foreign cells and soluble antigens has been intensively studied in the smaller laboratory animals. The age at which the immunological passivity of the foetus changes to immunological maturity varies according to the species and to the quantity and nature of the antigen. In mice, rats and rabbits this immunological maturity develops within a few days of birth, or somewhere around 20-30 days after conception. Little information is available for larger animals with longer gestation periods. The data on the human foetus have been summarised, but they are limited.

The placentation and general embryology of the rhesus monkey (gestation period 140-170 days) closely resembles that of the human conceptuus. Using an operative method developed two years ago giving direct access to the foetus, we have injected adult homologous marrow into 17 foetuses of various ages in an attempt to determine the age at which cellular immunological maturity develops.

We have a small breeding colony at the Institute and although we started with some pregnant monkeys imported from India, our study is not yet complete and this paper is thus an interim report.

Preparation of marrow cell suspension

All marrow donors were unrelated healthy females (except for B24 and B94 which were males). Marrow was obtained from the length of

the tibia by washing Earle's medium from syringes inserted in holes drilled into the marrow cavity. The marrow clumps were gently broken up and after concentration to 2 ml were injected into the recipient within 1-2 hrs. Numbers of nucleated marrow cells injected, see Fig.1, are conservative estimates.

Injection of the foetus

The length of gestation of the six rhesus monkeys imported from India was based on best estimates from size of uterus and foetus, and limb measurements at operation. The ages of the 11 other foetuses were calculated from the time of mating.

Marrow suspensions were injected intraperitoneally into the foetus under direct vision at open operation. At weekly intervals thereafter signs of foetal life were sought for, until birth.

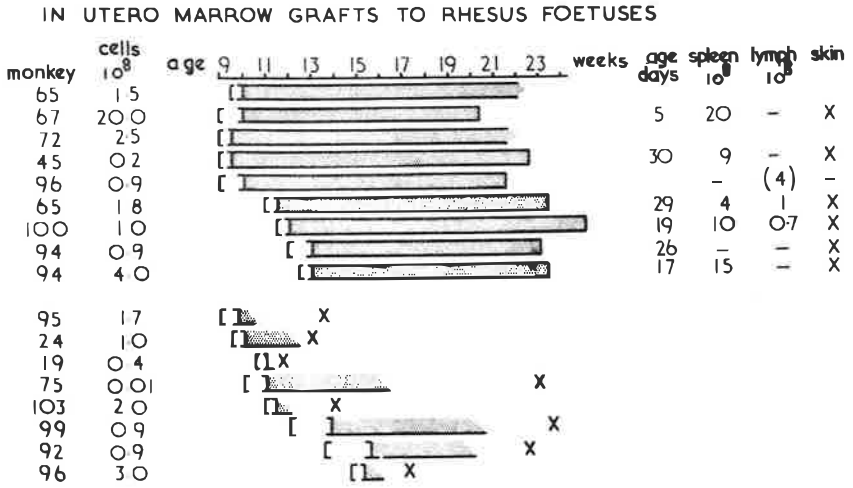

Fig.1 shows the 17 foetuses which were injected intraperitoneally, and the number x 10^8 of nucleated marrow cells used for each dose.

The estimated age of the foetus at operation is shown by the square brackets, and its survival is indicated by stippling. The figures to the right of the first 9 monkeys (those that were born alive) show the age in days when they were challenged with cells from the original donor; the number of nucleated spleen cells x 10^8, and thoracic duct lymphocytes x 10^8, given i.v.; and skin grafts (X).

The last 8 monkeys are those in which the foetus died in utero, and the time (X) of abortion or excision of the macerated foetus.

Five foetuses died in utero, probably for technical reasons at operation. Three more lived for several weeks but then died or did not survive birth. No material for histological study was obtained because they were either macerated when excised, or were destroyed by the mother. No evidence of 'secondary disease' was detectable in any specimen. All the babies born alive were quite normal, continued to thrive and gain weight and showed no haematological abnormality.

Challenge grafts

The time chosen to challenge those babies which survived was affected by several factors. Due to the difficulty of handling the mothers we were reluctant to disturb them for 2 or 3 weeks. Recently, however, we have challenged the baby within a week of birth. Babies were given a suspension of spleen cells intravenously from each respective marrow donor (for doses see Fig.1). When possible the donor's thoracic duct was also cannulated and a suspension of lymphocytes also transfused intravenously to the baby.

All the babies challenged in this way were examined every 2-3 days. They all developed a transient enlargement of the spleen but

otherwise showed no other sign of secondary disease whatsoever. They put on weight normally and there was no significant change in haemoglobin or blood cell count.

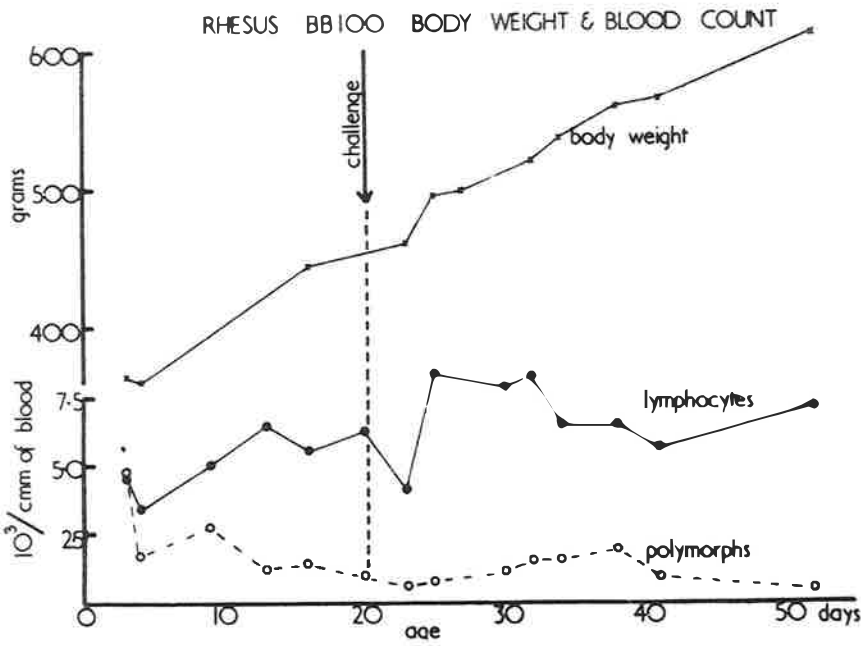

Fig.2 shows the effect of a challenge of 10^9 nucleated spleen cells and 0.7×10^8 thoracic duct lymphocytes given i.v. to BB100 at 20 days of age. There is no significant change in body weight gain or white blood cell levels. The graph is typical of the other babies challenged.

In most instances a full thickness skin graft from the abdomen of the marrow donor was sutured on the back or abdomen of the baby on the day when the spleen cells were given, together with control homologous or autologous grafts. It was impossible to cover the graft with any

dressing which would not be scratched off while the baby stayed with its mother. Among those which survived there was a fairly consistent sequence of events; the challenge skin was pale and oedematous after 3 days and became cyanosed and blotched by 5 days; thereafter it blackened and dried up but did not actually fall off until after 12-17 days. The grafts may have stayed on longer in these monkeys because of the large dose of antigen (spleen cells) injected concurrently. The initial reaction looked like an accelerated rejection characteristic of an immunised host, but we have not yet achieved adequate comparison with homologous control grafts to be sure of this.

DISCUSSION

The number of nucleated marrow cells injected into the foetuses was of the same order as that which induced secondary disease in irradiated adult rhesus monkeys, as described by Drs Crouch, van Putten, van Bekkum and de Vries. The irradiated monkeys, however, weighed 2.8 - 4.8 kg, while the 9-week foetus weighed about 25 gms. With some reservations we may say that the 9-week foetus is able to reject an immunologically competent graft. It is possible however that the foetal environment may be biochemically unsuitable to support the growth of adult cells or, second, the intraperitoneal route may not allow adequate dissemination of cells to occur in the young foetus.

With these two possibilities in mind the results such as they are so far suggest that the foetus of the rhesus monkey is already itself immunologically competent towards marrow cells by 9 wks. We have found lymphocytes present in the foetal blood at this age. This would correspond to about 16 wks in the human when lymphocytes are also present. Unless one postulates that much of this capacity resides in the placenta, we believe that human foetal haemopoietic tissue of this age should,

until directly investigated, be regarded as immunologically competent.

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DISCUSSION

OVERMAN In view of the gross differences in chemical anatomy between adult and foetal or early neonatal cells it should be kept in mind that other factors beside immunological reactions could play a role in the failure of adult transplants to survive in a foetus. As an example something as common as the sodium and calcium chloride distribution in erythrocytes does not approach normal adult values in the human until the age of 3 or 4 years. Secondly Dr. Overman liked to know whether attempts had been made to graft foetal skin into foetuses.

BANGHAM was very much aware of the discrepancies in the age of some of the grafts and that this may account for some of the bizarre effects that were observed. Foetus to foetus transplants had not been performed so far.

VAN BEKKUM referred to experiments by Dixon (J. Exptl. Med., 105, 75, 1957) on the lymph node transfer of adult cells into neonatal rabbits in which he observed a deficient antibody response of the transferred cells in the neonatal environment. Possibly a distinction between immunological and non-immunological factors determining the fate of the graft could be made in Dr. Bangham's experimental situation by subjecting the foetuses to sublethal irradiation before the transplantation.

KROHN suggested the transplantation of control homologous skin simultaneously with the skin of the homologous spleen donor and the use of control newborn recipients as well to facilitate interpretation of Bangham's results. He did not think however that the differences in age between the donor and the host would influence the results very much. In Dr. Krohn's laboratory newborn rhesus monkeys had been injected with massive doses of adult spleen cells from 1 or 2 homologous donors. Part of suspension containing mostly free cells ($5\text{-}9 \times 10^8$) was injected intravenously and the rest of the

suspension which was much more particulate, was administered intraperitoneally. These injections had no effect whatsoever on the growth and the development of the monkeys over a period of 16-18 months. They have grown up entirely normally. These monkeys were tested with skin grafts of the original spleen donors and with control homologous grafts at the weaning age of about 6 months. Rather surprisingly it appeared that both grafts were destroyed at the same time which would indicate that the spleen injections had neither sensitized the monkeys nor rendered them tolerant. However, the dressings were not removed until the 10th or 11th day after grafting so that differences in the rejection pattern within that period cannot be excluded.

KROHN could not follow Bangham's suggestion that this monkey material should be more genetically heterogenous than the human material. It could possibly be even the reverse because the area covered by the collectors of monkeys is probably less than the area covered by the collector of human foetuses (Dr. Kay).

BANGHAM answered that his remarks on the possibility of larger antigenic differences between the monkeys was based on the fact that no attempts had been made in his experiments to select the donors and the recipients whereas Dr. Kay very carefully matched at least blood groups.

BANGHAM AND KROHN both answered to relevant questions that they did not in their experiments employ mother to child nor the reverse transplantations.

CORRELATION OF SKELETAL GROWTH AND EPIPHYSEAL OSSIFICATION WITH AGE OF MONKEYS
II - MATURATION EXTENDED THROUGH 75 MONTHS

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INTRODUCTION

The level of sophistication in biomedical research has increased through the use of precise laboratory techniques and devices, electronic parameter measurements, quality data recorders and processing, and high quality biological specimens maintained in a scientific manner. In those laboratories measuring the physiological parameters of monkeys, the first 2-4 years of history are frequently unknown except that they are imported from their natural environment.

For many years a variety of procedures for estimating the age of laboratory monkeys has been used. Dentition dates of deciduous and permanent teeth are documented in great detail (Schultz, 1933a). Weight charts are relatively constant (Schultz, 1933b). Organ weights and body measurements are well documented; however, using these end points is time-consuming when dealing with large numbers of animals, and often requires one or more highly trained technicians for interpretation (Fremming, Benson & Young, 1958).

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The aim of this study has been to develop a more accurate method of estimating the age of imported monkeys in order to provide a valuable portion of an acceptable medical history for the biologist. Throughout the last few years, age estimation by maturation of skeletal growth has been used in the monkey and to date it appears to be relatively easy, inexpensive, and accurate.

***

A few years ago this laboratory became interested in a better approach to the problem of estimating monkey age. A pilot study on bone development and maturation appeared promising. The study started in January 1958. Initially, 50 animals ranging from birth to 8 years of age were used. Subsequently, all animals born in the colony were integrated into the study at birth. As the investigation developed, aging criteria were ascertained only in those animals whose exact age was known. To date, there are aging criteria through 80 months.

This study outlines a method of age estimation that has been developed for this monkey colony. Gertrude van Wagenen and C. W. Asling (1958) have reported a similar study. Although objectives are not identical, criteria and conclusions are complementary.

METHOD

The animals are weighed, anesthetized with a short-acting barbiturate, positioned, and taped to the x-ray table face downward over a 14-inch

by 17-inch cassette. The appendages are so positioned that the left hand and left foot are taped with a volar and plantar surface flat on the table. The right appendages are positioned so that as much of them as possible reflects the lateral position on the film. Technique varies according to thickness, and includes use of Potter-Bucky screen. A more elaborate series of radiographic exposures would give the added advantage of more extensive coverage of views; however, this arrangement has been used in order to keep the procedure simple and inexpensive.

The bone-maturation criteria used for interpreting the age from the radiographs have been selected from this positioning of the animal. As the number of serial radiograms increased, possible aging factors were added, changed, and deleted. Radiographs at quarterly intervals were found sufficient for development of growth criteria (figure 1).

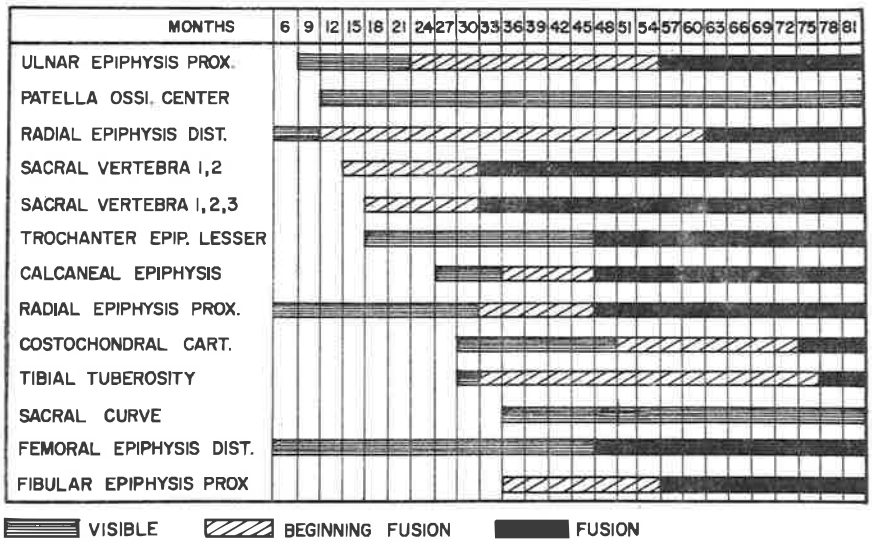

Figure 1 AGE ESTIMATION GRAPH (MALES)

RESULTS

Presently, no attempt is being made to catalogue bone change in animals less than 6 months old. A monkey of this age either has been born in this colony or has been purchased from a colony where the age is known.

By the sixth month the proximal ulnar epiphysis is visible. The patellar ossification center is usually visible, especially in females.

The ninth month reveals a constant patellar ossification center. The distal radial epiphysis has developed to the point of visual continuity with the shaft.

By the twelfth month the fusion of the first and second sacral vertebrae has begun. The ossification centers of the humeral head have united.

At fifteen months the second and third sacral vertebrae have coalesced. The femoral lesser trochanter epiphysis is present.

By the eighteenth month the second and third sacral vertebrae have fused. The calcaneal epiphysis is occasionally visible.

At twenty-one months the proximal ulnar epiphysis begins to fuse. In the female, the first metatarsophalangeal joint sesamoid becomes visible, and the head of the radius begins to fuse.

By the twenty-fourth month the distal humeral epiphysis shows fusion. In the female the calcaneal epiphysis is visible, and the proximal ulnar epiphysis shows evidence of fusion.

The twenty-seventh month shows the costochondral cartilages definitely visible. The proximal ulnar epiphysis appears about 30 percent united. The tibial tuberosity may become visible in females.

By the thirtieth month three sacral vertebrae have fused. Beginning fusion of distal ulnar epiphysis is visible and the proximal radius is fusing in the female.

By the thirty-third month there is fusing of the distal radial epiphysis and the calcaneal epiphysis.

The thirty-sixth month shows beginning fusion of the proximal fibular epiphysis, and the sacral lordotic curve is beginning to form.

By thirty-nine months the ossification center of the tibial tuberosity is generally fused to the proximal epiphysis. Fusion of the proximal radial epiphysis in the female is practically complete.

At forty-two months the distal radial epiphysis is uniting to the radius. Fusion of the proximal radius in the female is complete. A constant angulation between the second and third caudal vertebrae is seen. The distal femoral epiphysis is almost completely fused.

By the forty-fifth month fusion of the tuber calcanea, proximal radius, and lesser femoral trochanter is complete.

There is practically complete fusion of the distal femoral epiphysis and most long-bone epiphysis at forty-eight months.

At fifty-one months the proximal ulnar epiphysis is fused and fusion is complete in the metacarpal and phalangeal epiphysis.

At fifty-four months the proximal fibular epiphysis has fused completely.

By sixty months the distal radial epiphysis has completely fused.

From sixty-three to sixty-nine months no measurable skeletal changes have been shown.

By sixty-nine months the distal ulnar epiphysis is completely fused.

At seventy-two months the proximal humeral epiphysis is completely fused and the costochondral cartilage is mature.

By the seventy-fifth month the tibial tuberosity is completely fused.

DISCUSSION

Observation of skeletal growth has been the most satisfactory method of estimating the age of imported monkeys.

Females generally meet the maturation criteria 3-5 months sooner than males before puberty. After the onset of female puberty the majority of epiphysis in the female fuse six to ten months ahead of the corresponding fusion in the males (Van Wagenen & Asling, 1958).

The age estimation graph depicts over a dozen of the more dependable and constant maturation factors found in the male. Complete ossification of the extremities would be complete in females at 5 1/4 years and in males at 6 to 6 1/5 years (Van Wagener & Asling, 1958).

Although only the rhesus (Macaca mulatta) was used in this study M. irus (Cynomolgus) have exhibited a similar maturation pattern (as indicated by Dr. Wm Greer, March 1961).

SUMMARY

A method of estimating age in the rhesus monkey has been presented. Some thirty-five ossification changes have been used as age-estimating criteria. Females generally meet the maturation criteria 3-5 months sooner before puberty and 6-10 months sooner than males as the age increases. After 75 months all observed centers of ossification appeared complete in the male.

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DISCUSSION

VAN PUTTEN Did you find any retardation in growth or epiphyseal ossification due to infectious disease in monkeys?

GISLER To our surprise we have found little or nothing to date on this point.

BENSON Do you have any information on the effect of diet on skeletal growth?

GISLER We could detect no differences between animals brought from widely different areas and those in our own colony which were fed a known diet and it therefore appears that dietary differences, within reason, do not materially affect skeletal growth and ossification.

BENSON Do you believe that this technique can be carried out by technical personnel?

GISLER Certainly the positioning of the animals and the production of adequate X-ray plates can be done by technicians.

OVERMAN Can you give me some idea of the variability encountered in the time of occurrence of particular epiphyseal ossifications?

GISLER Yes. Although there is some variability depending upon what particular age group you are dealing with, you can generally be accurate to at least 3 to 6 months. I should like to ask the members of the symposium what they believe to be significance of knowing the age of animals?

AMBRUS Perhaps one of the important areas for developing age criteria would be in embryos.

GISLER We have not done any work in animals under 6 months of age.

BENSON Do you feel that this X-ray method has promise of being applied to embryos?

GISLER Yes, due to the fact that at birth a number of radiographic signs are already present, it should be possible to go back into embryonic life.

CROUCH I think there are some very well established lines in human embryology for aging the foetus by the time of appearance of various ossification centers or even centers of cartilage formation which might well be adapted to the foetal monkey. Once all the centers are ossified, Dr. Gisler, do you know of any way of determining animal age?

GISLER None are known to me except that based on examining the teeth.

WHITCOMB Because of its possible importance in bone marrow transplantation studies between pre- and post-pubertal animals, I wondered how bone age correlates with other secondary characteristics of puberty and with animal weight?

GISLER We cannot pinpoint the onset of puberty by this method but we can come within three months of the real age and can correlate age with the onset of puberty determined by other methods.

BENSON Dr. Gisler has indicated that skeletal growth does not seem to be greatly affected by diet or by infectious disease. I wonder if the technique is sufficiently sensitive to be used in radiation studies?

GISLER Young animals surviving acute radiation exposure are noticeably retarded in weight gain; however, insufficient data is at hand to know how carefully this retardation can be followed by the X-ray method described here.

RIOPELLE Do the data reported here apply to mother-reared monkeys or to animals that were separated from the mother soon after birth?

GISLER We have studied animals reared in both ways and have seen no marked or consistent differences between them.

CARE OF CHIMPANZEES FOR RADIATION STUDIES

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Interest in the use of primates for research is growing rapidly around the world. This growth is due to many factors, at least one of which is the greater recognition of the importance of biological research as evidenced by greater financial support to investigators. Investigators for the first time are able to use what are often thought to be expensive animals. In many cases, however, the expense is illusory and the greater familiarity with primates in research has resulted in the further awareness of their special advantages and has led to more sophisticated research than was possible with lower species.

In selecting a primate as an experimental subject, the primary determinant should, of course, be the use to which the animal will be put. Typically, three research functions for primates can be discerned. First is the substitute for the rat. Investigators who are looking for a replacement for the white rat, which phylogenetically is nearer to man, usually require very large numbers of subjects and they wish to have the smallest animal possible that will serve their needs. This use implies the selection of a rather primitive primate, perhaps a

tree shrew or a marmoset, for these are the smallest of the order.

Other investigators want a general-purpose animal which is hardy and relatively inexpensive, yet not too big. The rhesus monkey has served this function admirably. Being neither the most complex of the primates nor the most primitive it can serve at least as a nearly suitable animal if not the ideal one for a wide variety of research problems. Furthermore, because it is so widely used in research, a wealth of background information is available on this species which can be drawn upon for the establishment of physiological standards.

The third use is as a substitute for a human. For this purpose the scientists are looking for an animal that will display with highest fidelity the kinds of reactions to disease and environmental stress that humans show. They may, for example, want the species to contract infections that are common in man or they may wish the species to show the same kinds of degenerative changes with age that are shown by man. The most practical primate for such studies is the chimpanzee (Pan troglodytes).

The chimpanzee is surely an expensive experimental animal; it is expensive to procure, to raise, and to maintain. Despite the apparent extravagance in using chimpanzees for research, they can, with proper colony management and selection of research

problems, be used in such a way that the cost of using them may actually be cheaper than that of using other species, especially if the others are not perfectly suited for the task. To illustrate, in a review of the autopsies performed within the past few years at the Yerkes Laboratories it was noted that most, if not all of the adult chimpanzees showed a significant amount of sudanophilic staining of the aorta and, in some instances, of the basilar arteries. The relative frequency of this spontaneously occurring pathology was more than 10 times the frequency found in other, "cheaper," primates studied thus far. If this continues to be true, the economic advantage of the cheaper primate for the study of atherosclerosis may well disappear.

Other observations in our laboratory strongly suggest that the chimpanzee may be capable of contracting viral hepatitis and of transmitting it to man. The chimpanzee might well be the only suitable experimental animal and regardless of its apparent expense its use will be required if progress is to be made. It's cost will be negligible compared to the annual economic toll due to hepatitis.

Because the chimpanzee can be used simultaneously for many different kinds of research, costs can be shared by the different projects. Since we also know the techniques for maintaining a virtually self-sustaining colony, there need be only a small

drain on the natural resources of Africa, hence no international problems are created.

Very few chimpanzees are now used in research. We estimate that well under 200 chimpanzees are used annually in the United States, the only country with which I am familiar. This should be contrasted with the annual use of nearly 20,000 rhesus monkeys not counting those used in polio vaccine production. Even if the number of chimpanzees were increased to 1,000 per year it would still represent only 5% of the number of rhesus used in direct research.

The number of chimpanzees used in research is gradually increasing and it will continue to rise. The opportunities for use of chimpanzees for radiation studies will also increase because more will be available. Even if the chimpanzee does not become the primary animal for such studies, it can serve a useful function in checking the results of experiments of the smaller animals. In view of the anticipated increase in the requirements for chimpanzees, we should like to note some of the experiences gained and procedures developed during the past 30 years at the Yerkes Laboratories in the care and maintenance of chimpanzees.

Growth

The chimpanzee is small and helpless at birth, but at maturity is large and powerful. This fact alone is the most important single consideration in the design and operation of a

laboratory using chimpanzees. In few other species is the helplessness of the infant transformed, in adulthood, into the powerful monster that the chimpanzee is. Physical facilities must therefore not only take into account the chimpanzee's particular stage of maturity but also its rapidly increasing strength.

At birth the chimpanzee weighs approximately 1.9 kilograms. An animal of this size can be kept in cribs and incubators, much as one would keep a human infant. At about 6 months of age the chimpanzee becomes too active to be restrained in this small crib and must be moved to a larger cage to permit the development of climbing and other locomotor skills.

High concentrations of chimpanzees in small spaces is certainly inappropriate in handling adults, and if breeding is contemplated some large cages especially designed for this purpose must be constructed. Effective breeding cannot and will not take place in small cages. Because of the marked difference in strength of the infant and the adult, no single cage can be used for all purposes to which the chimpanzees may be used.

Birth

Almost every infant chimpanzee born at the Yerkes Laboratories within the past 10 years has been removed from its mother as soon as possible after it was born. Whenever a

chimpanzee infant is expected (the gestation period is approximately 7-$\frac{1}{2}$ months) the automatic watering device located in the cage is turned off each night and turned on again in the morning if there is no baby. The purpose of this is to make sure that the chimpanzee mother is thirsty in case it becomes necessary to give her an oral anesthetic to remove the baby.

The cage in which the mother is placed while expecting a baby is an individual cage with both indoor and outdoor facilities. The animal may be locked either in or out and it may be transferred from cage to cage by means of sliding doors between each of the adjacent cages.

On the morning the infant is found in the cage (births usually occur at night) the mother is lured from one cage to another and back again. Very frequently it occurs that the mother lays the infant aside in her travels from cage to cage to obtain food or drink. When this happens, the door between them is closed and the caretaker retrieves the infant, which is then taken indoors, weighed, cleaned and diapered.

Experience has shown that the chimpanzee raised under the optimal conditions of the laboratory is stronger, weighs more, and is healthier than one reared by its mother, even one reared by its mother at the Yerkes Laboratories. Nissen and Riesen (1949) separated infants from their mothers at various times

up to 1 year after birth. Those animals weighed less at time of separation than the laboratory reared animals and they tended to recover their weight deficit when placed on the laboratory regimen.

Growth curves of chimpanzees have been published (Gavan, 1953; Riopelle, in press) and they indicate that until about 9 years of age there are no sex differences in weight but that shortly thereafter the male is the heavier. Typical weights at different ages are shown in Table 1. By the time a

Table 1

ANNUAL WEIGHTS OF CHIMPANZEES
BORN AT YERKES LABORATORIES OF PRIMATE BIOLOGY, INC.

	MALES		FEMALES	
AGE	NO.	MEAN	NO.	MEAN
Birth	15	1.8 kgm.	10	1.9 kgm.
1 yr.	22	6.7	18	6.3
2 yrs.	22	10.2	17	10.4
3 yrs.	21	13.4	17	13.6
4 yrs.	21	16.7	16	17.3
5 yrs.	22	19.9	15	20.8
6 yrs.	22	23.3	15	25.5
7 yrs.	21	26.2	15	28.6
8 yrs.	21	30.6	15	32.2
9 yrs.	21	36.1	15	36.5
10 yrs.	21	40.2	17	38.6
11 yrs.	20	43.2	17	39.6
12 yrs.	18	46.2	16	41.3
13 yrs.	19	49.8	17	39.7
14 yrs.	16	50.0	13	42.1
15 yrs.	12	50.5	12	43.5

chimpanzee is 15 years old its weight has become fairly well stabilized and this suggests that maturity has been reached. Another index of maturity is the onset of ossification in the epiphyses and shorter bones of the extremities. Data based on a roentgenographic survey of 16 chimpanzees born at the Yerkes Laboratories have been published by Nissen and Riesen (1949) which include 70 centers of ossification on the left side of the body. From those data and the weight table above, one may estimate the age of an unknown chimpanzee.

Puberty is usually defined as the period immediately following menstruation in the female and the development of certain other sex signs in the male. Since these other sex signs do not appear abruptly, and are not so easily identifiable in the male, the female definition is the most practical one. Records gathered at the Yerkes Laboratories by Dr. Nissen show that the average age of the onset of menstruation is just short of 9 years. This can be compared with 12 to 15 years in a human.

Adulthood is that period when the long bones no longer increase in length. Roentgenographic records at the Yerkes Laboratories show that the proximal epiphyses of the tibia are closed at 10.8 years, the distal epiphyses of the tibia close at 11.1 years and the distal epiphyses of the ulna close at 12.2 years.

Another measure of maturity is the eruption of the final

permanent teeth. As yet unpublished studies by Nissen and Riesen show that the 3rd molar erupts 1 millimeter between the 10th and 11th year of age and the second bicuspids appear on the average of 7-1/3 years. These same teeth erupt approximately 50% later in the human.

Extrapolating from the human lifespan of 100 years to that of the chimpanzee by assuming that the ratio of the life-spans is the same as the ratio for these indices of maturity, we estimate the lifespan of the chimpanzee to be between 50 and 65 years.

It is of interest to know that the oldest chimpanzees at the Yerkes Laboratories are females and all continue to menstruate. The oldest chimpanzee, Pati, now 42 years of age became pregnant the last time she was bred, which occurred only 2 years ago.

In this brief survey we wish now to turn away from the growth characteristics of the animal and to describe some of the procedures and facilities developed for maintaining the animals.

Caging

It has been noted already that infants taken from their mother at birth are placed in incubators similar to the isolettes used in hospitals. The temperature of the chamber is maintained between 75 and 90 degrees fahrenheit. The

flooring of the cage is made of nylon screening which is firm yet will stretch, is nontoxic and does not rust. Animals over 6 months old may be placed in cages that should be at least 3 feet long in every dimension. This cage will suffice to hold the animals until they are about 3 years of age.

Chimpanzees weighing over 30 kilograms can be easily kept in indoor-outdoor cages. The cages at the Yerkes Laboratories hold 2 juveniles or 1 adult. The buildings for housing of breeding adults have cages large enough to hold 2 or 3 animals.

Juvenile and adult animals require a sleeping perch of some impervious material located off the floor. Sliding doors rather than swinging doors are used wherever animals will be transported, in order to provide no opportunity for an escape of an animal when going from cage to cage. Cages are equipped with indoor and outdoor locks to permit daily cleaning and inspection of the quarters. The purpose of the double locks is to prevent the inadvertent release of an adult animal into a cage while it is being cleaned.

Cage cleaning is one of the most important labor costs in maintenance of a laboratory colony. Every investigator using primates recognizes that there is no substitute for sanitation. Ordinary household cleanliness is adequate for

the incubators and cribs since the infants are relatively immotile and do not soil the cribs. Diapers may be placed on the younger juveniles until they are 3 years old to contain the feces, but it becomes increasingly difficult to keep them on the active youngsters as they grow more active. When diapers are changed frequently the cage will not get very dirty. If only a few infants or juveniles are to be housed in a laboratory, individual care in feeding and cleaning is easily given. If many animals are to be maintained, however, thought should be given to ways of reducing the labor by dispensing with diapers and using throw-away cage-floor inserts.

Older juveniles and adults living outdoors wear no diapers. Cleaning of those cages consists of 3 steps: first, the removal of all solid matter, second, spraying the cage with a commercial quaternary of ammonia or activated iodine solution, and finally careful steam cleaning of the floor and all cracks and crevices. A steam generator is essential, since it kills the ova or intestinal parasites that may lurk in the cracks. It is important to keep this number at an absolute minimum because the chimpanzee is not fastidious about his diet and frequently practices coprophagy.

Nutrition

Dietary requirements for a chimpanzee are very close to

those of an active vigorous human. Adult chimpanzees will eat between 2500 and 3000 calories per day, 15% or more of which should be of high quality protein. Few areas of animal care are so replete with pet recipes and impressions. The chimpanzee is a versatile animal and will eat a varied diet. It can also accomodate itself to many different diets. It is our view that the general principles of human nutrition apply in feeding the chimpanzee.

Chimpanzees in the wild do not eat meat except on the rarest of occasions, and then they are motivated perhaps by curiosity rather than by nutritional requirements. They instead eat large amounts of vegetable matter which is high in cellulose. Recent studies in collaboration with Dr. Oscar Portman of Harvard University show that cellulose is not essential. Animals placed on a cellulose-free synthetic diet were maintained over a year with adequate weight retention and vigor of action. This diet is also adequate for raising infants from the day of their birth although our experience with this is less extensive. None of the animals at Yerkes Laboratories receive meat. They do however get high quality protein in the form of milk and soybeans.

Transport of animals

Infants can be carried about the laboratory in one's

arms, and juveniles up to the age of 3 or 4 years, depending on the intimacy of contact with humans, can be walked around the laboratory on a leash. Older animals must be moved in a transport cage. The device in use at the Yerkes Laboratories is about 18 inches wide, 3 feet long and 2 feet high. No great problem exists in training the animals to enter the transport cage and the device is useful for the monthly weighing as well. Since animals are moved frequently, adaptation to the transport cage is readily maintained. For long distances the transport cage is carried on a truck, but for short distances it is carried by hand.

Anesthetization

Physical examinations can be conducted on infants with no restraint or manipulation. Juveniles on the other hand must be trained to cooperate in the investigation. The risks are too great for any but the most intimately familiar persons to depend on cooperation with older juveniles and adults, therefore an efficient anesthetization system must be worked out. There is invariably a danger in anesthetizing an animal. This is especially true in dealing with old animals or animals that are sick or injured. Injury is an additional hazard.

Recently there has been introduced a new anesthetic which seems peculiarly well suited for primates and the usual

procedure with this drug will be described. The first step in anesthetizing an adult animal is to transfer it from the transport cage to an anesthetization chamber. Then ether or a combination of nitrous oxide and oxygen is insufflated into the cage as rapidly as possible. The sole purpose of these gasses is to inactivate the animal just long enough to permit injection of an intramuscular solution of 1-(1-phenylcyclohexyl) piperidine hydrochloride administered in the amount of 1 milligram per kilogram. (Chen and Weston, 1960). As soon as this intramuscular injection is given, the ether chamber is ventilated and within one or two minutes the animal arises and is fully alert. Approximately 10 minutes later it becomes immotile without passing through any excitatory phase. Anesthesia lasts 20 to 40 minutes at this dosage and supplements, usually at doses of .5 milligrams per kilogram, are given as needed. Deep abdominal and brain surgery has been performed with this drug at a dosage of 3 milligrams per kilogram. The sole practical effect of the increased dose is that the animal is anesthetized for a longer period of time. In over 600 experiences with this drug not a single fatality or fright can be attributed to it despite the fact that on some occasions doses of 5 to 8 milligrams per kilogram have been administered. In view of the high safety of the drug and its ease of use, barbiturates

are no longer used at our laboratories. This anesthetic has also proven effective for gibbons, gorillas and monkeys without loss of life.

The drug can also be administered orally if it is mixed with grape juice or a carbonated sweet beverage. Onset of immobilization is delayed for 30 minutes or more and heavier doses must be given. Since absorption of the drug may be retarded, the animal may be immotile for up to 24 hours. There is no great cause for alarm should this occur for respiration and cardiac output are maintained.

Manipulation of adult animals, such as is required for injections, radiation, or recordings of the nonanesthetized animal can be done only with the aid of a strong restraining board. The board we have used has steel pipes for the arms and parachute webbing for the chest, abdomen and legs. Padding is necessary to prevent back sores.

Breeding

The various phases of the menstrual cycle of females are accompanied by differential visible signs. At time of ovulation, which occurs about midway in the 35-day cycle, the perineal region reaches its maximum swelling due to the engorgement of spongy tissue with fluid. This swelling subsides after ovulation has occurred and disappears at time of menstruation.

The male is placed with the receptive female for breeding only after being located in adjacent cages for at least a month. The gate between the two adjacent cages is opened when the female is at maximal swelling and both she and the male show signs of excitement. The swelling is an important stimulant to the male. Occasionally a violent fight ensues, but in most occasions the encounter is peaceful. Fights, when they occur are terminated with streams of water from hoses. If the couple appears compatible they will be left in the same cage so that copulation can take place often while the female is in swelling.

A word should be said about psychological problems in breeding. Nissen has observed that many of the chimpanzees born in the laboratories do not breed as adults. This is something of a surprise to researchers with rodents or rhesus monkeys who frequently must house their animals in individual cages to prevent pregnancy. The reasons for this lack of sexual drive in the laboratory reared chimpanzee are these: Chimpanzees raised in the laboratory do not have opportunity to associate with as many chimpanzees during youth and adolescence as does the forest reared animal hence does not learn as many social skills; Secondly, sex behavior in the chimpanzee is controlled more by cortical than by hormonal mechanisms and learning is very important. Observational

learning at time of puberty or of maturity is inadequate to assure competent sex behavior.

Health

In the maintenance of any colony it is as important to obtain standards of health as it is to have records of disease. This requires information about the normal values of the usual measures obtained in physical examinations. Since the animal cannot provide us with a list of its complaints it is important to obtain a health profile on each animal. Comparisons with the normal profile are essential for the early detection of disease. Indeed, to become alerted to the possibility of illness is itself important. Two of the first signs of illness are lack of appetite and reduction in gross, outwardly directed activity. Therefore observations of these aspects of behavior should be made routinely, and any reduction in either should be taken as an indication of illness.

The normal values and ranges for the usual clinical signs of health have been published by workers at the Aeromedical Research Laboratory, Holloman Air Force Base. (Cook, Fineg, and Miksch, 1960; Staten, Cook, Edwards, Fahlstrom, Goins, Cooper, and Schwandt, 1961; Weisler, Fineg and Warren, 1961.) In general these values are more similar to those found in humans than in simians. Studies at the Yerkes Laboratories point to the same

conclusions. Because of this fact, the literature on human health may be consulted freely. Because the norms change with age and sex of the animal, no complete listing of the norms is available for a broad range of animals known to be without parasites or illness.

A few additional observations may be made. Body temperature taken rectally shows fluctuations with the seasons of the year and is consistently below, and occasionally is 10 degrees F. below that for humans. The average is 1 degree F. below the human standard. Blood pressure shows a slow decline with depth and duration of anesthesia. The sedimentation rate is extremely variable in chimpanzees. It contrasts sharply with the stability of values for monkeys and gibbons. Whereas rhesus monkeys generally have sedimentation rates under 2 or 3 millimeters per hour and gibbons show virtually no sedimentation in this same period of time, that for chimpanzees may reach 40 mm. per hour in apparently healthy animals, especially if intestinal parasites are present. Thus it is a sensitive measure of health in chimpanzees, as it is in man, but is a crude measure in these other species.

Diseases

Chimpanzees are often used in research because they are susceptible to diseases contractable by man. The very fact

that these animals do contract these diseases imposes a risk both on the caretakers and the animals.

Chimpanzees are known to be susceptible to tuberculosis. Recognizing this fact, many zoos separate the animals from their human observers by glass walls to prevent airborne contamination. When given adequate amounts of fresh air and separation from contaminated humans, there is little or no risk of contamination of a healthy colony. Indeed, the Yerkes Laboratories has not had a proven or suspected occurrence of tuberculosis in over 32 years. The only case occurred 32 years ago in an animal that died within 3 months after it was brought into the colony. The illness doubtless was acquired before arrival. The great success of the Yerkes Laboratories in keeping a colony free from tuberculosis for more than 30 years testifies to the importance of proper sanitation, cage design, isolation, and a climate which permits the animals to have free access to the out-of-doors.

Animals born into the colony if raised indoors may be kept free from many parasitic diseases for a very long time. They are likely to acquire them, however, when placed in outdoor cages.

The usual intestinal parasites are a persistent problem in primate husbandry. We are familiar with Necator, Enterobius,

Trichuris and Oesophogostomum. Strongyloides and Ascaris are rarely seen in our permanent colony; they present an important and difficult problem, however, in newly imported animals. We have also seen Capyilaria hepatica and Tenia in imported animals.

This past year vigorous attempts were made to eradicate helminths from the entire colony. In addition to the water and cleaning described above, all infected animals were placed on a 10-day regimen of dithiazanine iodide. This regimen was repeated a second and third time where necessary and intestinal helminths have virtually disappeared from the colony.

Balantidium coli is a persistent inhabitant of the gastrointestinal tracts of many of our animals and has resisted all attempts to eradicate it.

Chimpanzees are very prone to upper respiratory infections which sweep through the entire colony in a few weeks. These infections regress spontaneously after a week or ten days.

Animals purchased directly from Africa are not, in our experience, suitable as experimental subjects until after several months of conditioning. All are products of malnutrition and infection by bacterial, viral and parasitic agents. Liver biopsies of such animals characteristically show fatty degeneration, cell destruction, leukocytic infiltration and cell division. The use of such animals for radiation and other studies

would lead to erroneous results. It is difficult enough to maintain irradiated primates through the period of acute radiation sickness without having to cope with the extra burden of disease. And the diseases themselves have an effect on the physiological functions one wishes to measure in experiments.

Since most of these infections can be contracted by man, it is essential to maintain extraordinary vigilance to prevent contaminating the staff personnel. Several persons at the Yerkes Laboratories contracted infectious hepatitis shortly after the importation of six chimpanzees. This is not without precedent; indeed, over 50 persons have had the disease in association with newly imported chimpanzees. Obviously, strict quarantine is required despite the universal appeal of the affectionate young chimpanzee.

Experience with irradiated chimpanzees has been limited to the behavioral observations on 13 animals given 375 r of gamma radiation in a single 12-hour period (Riopelle, 1962). The surviving animals have been given numerous behavioral tests, few of which indicate any loss in intellectual functioning. Motivational decrements have been observed, however, during the period of radiation sickness when the animals refused to eat. Some studies required depriving the animals

of food for extended periods. This additional stress can be tolerated in only those irradiated chimpanzees which have no parasitic burden. Although no new principles of animal care are necessary for irradiated chimpanzees, the existing problems are intensified.

Summary

This paper has described the general characteristics of the chimpanzee as a laboratory animal and the procedures and precautions in its maintenance and handling.

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DISCUSSION

CROUCH Did I understand you to say Dr. Riopelle that you have been able to get rid of all helminth infestations (including specifically oesophagostomum) using multiple treatments with Delvex?

RIOPELLE We do not see oesophagostomum in the animals that have been in our colony a long time but rather in young animals we have imported, so I do not have sufficient data to answer this question positively.

GISLER I was interested in the diet you feed the chimpanzees and wonder if the Purina diet often used for monkeys should be adequate?

RIOPELLE We give Purina diet, but not exclusively. Many years ago we developed a basic diet for chimpanzees at the Yerkes Laboratories which is known as "Chimp Crackers" and we use this supplemented with natural foods. The basic diet consists of 65 per cent flour, 25 per cent casein and the balance is the usual additions, salts, protein and carbohydrate and the animals do very well on this.

VAN LANCKER You mentioned that at autopsy some of your animals had obvious atherosclerotic plac ques. Do you know the age at which these appear and whether they ever show the associated classical lesions (as myocardial infarcts) of human atherosclerosis?

RIOPELLE I would prefer to refer you to Dr. Stephen Andrews at Harvard who has been doing the pathological work-up on our autopsy material. In general I believe the findings in regard to atherosclerosis depend upon the age at which the animal dies or is sacrificed. Our animals have been much older than the usual rhesus monkey that we autopsy.

AMBRUS The people in the East African Baboon Research Institute have reported similar pathologic lesions both in captive and wild baboons and they reported traces of myocardial infarcts as well. I should like to ask

Dr. Riopelle whether he believes that the nutrition of chimpanzees can be improved by adding liver to the diet?

RIOPELLE I have no information on that point and the only adequate defense I have of the diet we use is that in the past 30 years we have had 140 live births and that several animals in the colony are over 35 years old and are apparently quite healthy.

KROHN We have had rhesus monkeys alive for 25 years in our colony but we have recently discovered that they are grossly deficient in vitamin B_{12}, the blood levels ranging from 0 to about 40 micro-micrograms per ml whereas monkeys newly arrived from the wild show blood B_{12} levels virtually equal to those of human subjects (300-400 micro-micrograms per ml). We believe our diet is responsible for these low levels which resemble those seen in human subjects living on a strict vegetarian regime. Such subjects show very low B_{12} blood levels but, like our monkeys, have no hematological signs and only occasionally show neurological symptoms.

YOUNG I would like to ask Dr. Krohn if the animals having a B_{12} deficiency showed "cage paralysis"?

KROHN Yes. This is what started our whole investigation on B_{12}. One animal began to show paralysis of the lower limbs which was improved but not completely abolished by B_{12} treatment.

WHITCOMB I'd like to ask Dr. Krohn whether he has extended his studies to pyridoxine deficiences? I am thinking of the possible problem of prophylactic treatment for tuberculosis with isonicotinic acid hydrazide which might induce a pyridoxine deficiency.

KROHN We have not studied pyridoxine levels since we have been fortunately free of tuberculosis in our colony for a long time and use no prophylactic treatment for this disease.

BENSON I'd like to ask Dr. Riopelle whether the presence of hepatitis has been confirmed in any of the chimpanzees in your colony and if so (1) what has been its incidence and (2) what methods of prophylaxis or diagnostic techniques are used in relation to this disease?

RIOPELLE We have been quite excited about the possibility that the chimpanzee might prove to be a useful experimental animal in hepatitis research and so have begun a wide-spread examination of these animals both in our own colony and in those held by zoos and importers. We have found some elevated liver function tests and have found suggestive morphological changes in the liver both from biopsies and autopsies. While these findings have been only suggestive, we must remember that most of the animals studied have probably been in the recovery phase of the disease. Our attempts to innoculate chimpanzees with material thought or known to contain virus have been completely negative.

BENSON Do you practice routine tuberculin testing and if so, what tests do you use?

RIOPELLE Yes, we take routine chest X-rays and employ the old tuberculin test once or twice a year in our animals. Apparently the chimpanzee does not contract the fulminating type of TB which is often seen in the rhesus monkey.

PROBLEMS IN THE SANITATION OF MONKEYS FOR WHOLE-BODY IRRADIATION EXPERIMENTS

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In previous papers of this group on bone marrow treatment of irradiated monkeys, data were reported on the incidence of various infections with parasites and micro-organisms and their possible significance for the outcome of the experiments. The enhanced susceptibility to infections in irradiated animals and especially radiation chimeras require that every effort should be made to obtain a thorough sanitation of the monkeys before they are used for irradiation experiments.

In this report current procedures for detection and elimination of pathogens will be described as well as their influence on the post-irradiation condition of the animals. Except for a few pig-tail monkeys (Macaca nemestrina) and cynomolgus (Macaca irus) monkeys, our colony consists of rhesus monkeys (Macaca mulatta). Nearly all our information about sanitation problems concerns the latter group. Our last three shipments of rhesus monkeys were supplied according to Tree-to-Laboratory arrangement, which means in practice, that the animals are stocked in India for a few days only before they are flown to Europe. During shipment the animals are kept in individual cages. By reducing the period of exposure to their caretakers in India to an absolute minimum, it is hoped to limit ex-

* This work was performed under contract with Euratom (European Atomic Energy Community) 51-53 rue Belliard, Brussels, Belgium.

posure to human sources of infection. Shortly after arrival the monkeys are put into individual laboratory cages and during the following few days handling is avoided and the animals are disturbed as little as possible. During that initial times feces is collected from the cage pans for bacteriological and parasitological examination. The results of these investigations and data relating to the effectiveness of the current sanitation procedures in preparing the monkeys for radiation experiments will be discussed.

RESULTS

Salmonella and Shigella

As Salmonella and Shigella are the most common causes of enteric bacterial disease in newly imported monkeys, special attention is paid to their detection. This is done by a slightly modified version of the procedure outlined by Galton et al. (1948). The infection usually exists already at the time of arrival of the monkeys, when some of the animals have watery diarrhea. We never saw monkeys die shortly after arrival with hemorrhagic diarrhea, as Fegly and Sauer (1960) and Schneider et al. (1960). From the feces of 198 newly arrived monkeys, Shigella was isolated 55 times and Salmonella 28 times (table I). Because the shipments never con-

NUMBER OF MONKEYS WITH PATHOGENIC INTESTINAL BACTERIA OUT OF 198 ANIMALS

TIME OF INVESTIGATION		SHIGELLA					SALMONELLA				
		Flexneri II	Flexneri III	Flexneri Y	Newcastle	Sonnei	Paratyphi A	Paratyphi B	Bareily	Newport	Typhimurium
At arrival		9	36	5	4	(1)	1	18	1	5	3
Recurrence	1x	1	1	-	1	-	-	1	-	-	-
Recurrence	2x	1	4	-	-	-	-	2	-	-	-
Recurrence	3x	-	2	-	-	-	-	-	-	-	-

Table I

sisted of more than approximately 30 monkeys, individual treatment of the animals was practicable. Antibiotics were preferred to chemotherapy because of their greater effectiveness when administered intramuscularly.

When the feces were positive for Salmonella, the monkeys were treated with chloromycitin for 5 successive days. In case of Shigella infection, treatment with terramycin* was instituted during a similar period. The antibiotics were both administered intramuscularly. In table I the results of the treatment may be evaluated with respect to the number of recurrences. Recently we attempted to decrease this very low incidence of recurrence even further, by testing the sensitivity to a number of antibiotics with test tablets, immediately after isolation of either of these two organisms. If necessary, treatment was then continued with a more effective antibiotic. There are still not enough data on the results of this last procedure to warrant any conclusions. During the period of administration of antibacterial drugs, even when only one or two animals turned out to be infected, the entire colony was kept in quarantaine until negative cultures were obtained from all members of the colony. This measure probably accounts for the fact that spreading of the infection through the colony occurred only rarely. From the feces of the irradiated monkeys that died with hemorrhagic diarrhea, a Salmonella had been isolated in one case only. In two irradiated monkeys Shigella was cultured from one of the daily feces samples.

Intestinal parasites

Among the intestinal parasites, the nematodes are most frequently found in monkeys. Chiefly, three genera of worms may be present, Oesophagostomum, Strongyloides and Trichuris. The members of the second

* A generous gift of terramycin from Pfizer N. V. Nederland is gratefully acknowledged.

genus, represented by a number of species, are more frequently encountered than the others. Only Oesophagostomum however, seems to be of importance to the post-irradiation condition of the host. This is explained by the habit of this worm to burry itself deeply in the intestinal wall. Secondary bacterial infections of the worm lesion may be a fatal complication. The diagnosis of helminth infection was established by the Faust-method of concentration of worm eggs as described by Mackie et al. (1955), while concentration of cysts was carried out by Mansoer's method (1959). Data obtained by these methods are given in table II.

PERCENTAGE OF MONKEYS WITH WORM-EGGS AND/OR CYSTS
(30 MONKEYS)

WORM-EGGS		CYSTS	
Oesophagostomum	88	Entamoeba histolyt.	64
Strongyloides	91	Entamoeba Polecki	8
Trichuris	75	Entamoeba Hartm.	12
Unidentified	3	Iodamoeba Buetschlii	7
		Endolimax nana	44
		Lamblia intest.	3
		Blastocystis	51

Table II

Nearly 10% of the irradiated monkeys showed multiple nodules, harbouring Oesophagostomum larvae, in the wall of the large intestine, the omemtum, and mesenteries and less frequently in the stomach, abdominal wall and liver. Secondary bacterial infection of these nodules apparently was the cause of the local or general peritonitis, which was observed in several cases. The lesions described are characteristic for this worm. The pre-adult larvae obtained from the nodules of two monkeys were classified as Oesophagostomum apiostomum by Prof. P. H. van Thiel.*

* From the Department of Parasitology, Institute of Tropical Medicine, Leyden, The Netherlands.

The Oesophagostomum eggs pass in the feces where, under suitable conditions the development into an infectious larval stage occurs in the course of about 5-7 days. It is assumed that the mode of entry of the larvae is by way of the mouth, although it is stated by Graham (1960) that the possibility of a percutaneous route of entry in primates should not be excluded in view of the findings of Mayhew (1939) in calves. The ensheated third-stage worm enters the wall of the cecum and colon, where nodule formation occurs (figure 1). Inside the nodule the worm passes through its third

Figure 1

Noduli in the wall of the colon of Macaca mulatta, caused by Oesophagostomum apiostomum.

ecdysis and loses its cuticle, to become a pre-adult worm. This fourth-stage worm usually re-enters the lumen of the intestine, but when the host is highly immune, it appears to be permanently trapped in the nodule. (Graham, 1960). As a rule the adult worms do not attach themselves to the wall of the colon to suck blood like the Ankylostomum species. The period

during which the larvae reside in the intestinal wall is still uncertain, but it is believed to be some seven days in non-immune animals (Graham, 1960 and Ruch, 1959). To establish a possible relation between the mean number of nematode eggs per gram of feces and the number of nodules in the intestinal wall, 5 separate portions of feces of each monkey that arrived with the last shipment, were investigated. As is shown in table III, no relation was demonstrable between the average number of Oesophagostomum eggs found per gram of feces and the number of nodules found at autopsy some weeks later. An additional purpose of the investigation was to get an impression of the severity and incidence of Oesophagostomum infection with nodule formation among newly arrived animals, in comparison to animals that have been

THE RELATION BETWEEN THE MEAN NUMBER OF OESOPHAGOSTOMUM-EGGS PER GRAM FECES OF FIVE SEPARATE PORTIONS IN NEWLY ARRIVED MONKEYS

Incidence of nodules in the wall of the colon	Mean number of eggs
++	10.7
++	8.5
++	4.3
+	4.0
++	3.9
+	2.5
++	2.5
++	2.3
++	2.0
-	1.9
++	1.8
+	1.8
-	1.8
+	1.2
+++	1.0
++	1.0
-	1.0

Table III

under laboratory conditions for some time. As compared to the frequency of nodules in animals that have been under laboratory conditions for 6 months or more, the incidence shown in table III is rather high. The possibility exists that the number of nodules has a tendency to decrease in the course of time when the monkeys are kept in strict isolation and when

measures are instituted to prevent re-infection. However, a more systematic investigation has to be performed in order to prove this hypothesis.

In our opinion the detection of Oesophagostomum eggs in the feces of newly arrived monkeys, probably is only of value if their mean number is compared with similar egg counts at a later date. An increase of eggs at that time, could indicate that a massive infection with many nodules had been present initially and that the larvae of the nodules have grown into adult worms between both observations.

An attempt has been started to destroy the adult worm with the new antihelmintic drug Alcopar (Bephenium Hydroxynaphtoate), in a group of 28 monkeys. After an initial egg count was performed in the concentrate of one gram of feces, the drug was given orally in amounts of 2 gm/kg body-weight following a fast period of 24 hours. The egg count was repeated every three weeks during the following 4 months. Twelve weeks after the first treatment with Alcopar, a second similar dose was given to those monkeys that still had Oesophagostomum eggs in their feces. In figure 2 the mean number of worm eggs in the feces of all 28 monkeys is plotted against the time of investigation. The effect of the drug on adult Oesophagostomum in the intestinal lumen seems to be rather favourable. The fact that Strongyloides usually embryonate very early (in some cases the larvae may even develop in the last part of the colon) may explain why Strongyloides eggs do return in great numbers rather soon after the administration of Alcopar. To eliminate Strongyloides infection the drug should perhaps be administered more frequently.

Protozoal intestinal infections

Infection with intestinal protozoa may have contributed to the death of two irradiated monkeys. In these animals Entamoeba hystolitica was demonstrable in colonic ulcers. The frequency of histolityca cysts in the feces

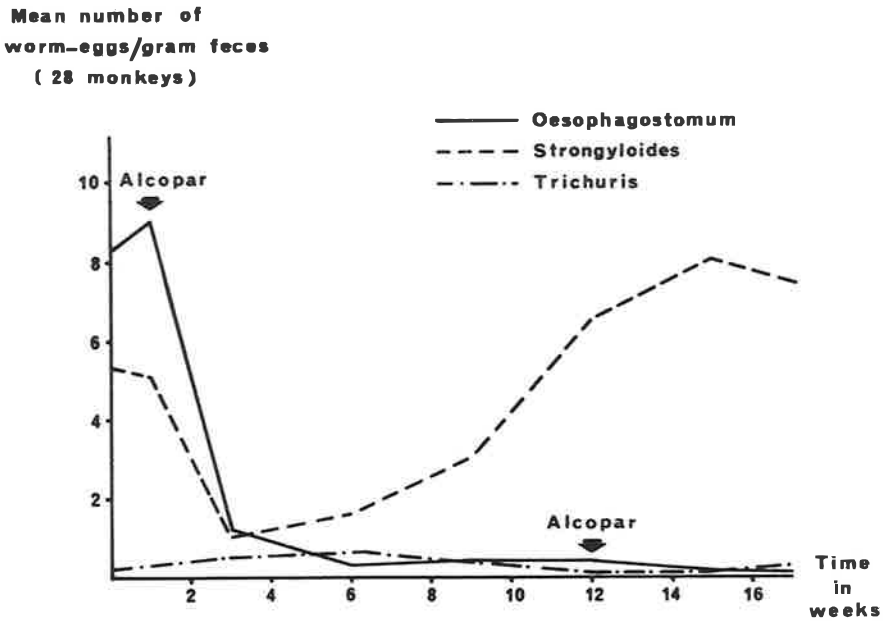

Figure 2

Mean number of worm-eggs per gram feces of 28 monkeys as influenced by treatment with Alcopar.

of our monkeys (table II) is rather high in comparison to data reported by Ruch (1959), but since invasiveness of the Entamoeba was so rarely found in the absence of specific treatment, it is not believed that this parasite is of great significance. It has been suggested by Ruch (1959) that Entamoeba histolytica is of minor importance, either because it is less pathogenic for monkeys or because the monkey has a stronger resistance against this infection than man.

Tuberculosis

Three monkeys died with widely dissiminated pulmonary tuberculosis, which unfortunately was not diagnosed until the monkey was autopsied. A few days after arrival, each animal was tested intracutaneously with a di-

lution of 1:10 of Koch's old tuberculin in the right eyelid. The dose administered is equivalent to 10 mg which dose is also being used by Young et al. (1957). This procedure was repeated monthly during the first three months after arrival and thereafter quarterly. The tuberculin solution used in these skin-tests was checked afterwards in two tuberculous guinea pigs, and this resulted in positive reactions in dilutions of 1:100 and 1:1000. When the first case of tuberculosis was discovered at autopsy, the Mantoux-test was repeated immediately on the monkeys in the same room and turned out to be negative in all cases. It should be noted that in the three tuberculous monkeys the Mantoux-tests had also been negative. The first two affected animals were rather ill during the last week before they died. The last one on the contrary, showed no loss of weight but was coughing, probably as a result of pressure on the trachea by an enlarged para-tracheal lymphatic gland. The second and the third case were members of the same shipment and for that reason the whole group was killed. At autopsy no suspicious foci were found in these animals. In smears of the spleen and lung stained by the Ziehl-Neelson method no acid-fast rods were demonstrable.

The results show that the Mantoux-test in our hands is a highly unreliable index of tuberculosis infection in rhesus monkeys. On the other hand the contagiousness of the disease is apparently very low; although the infected monkeys had been coughing for several days and even for several weeks in the last case, all neighbouring monkeys proved to be negative at necropsy. These findings differ from those published by Young et al. (1957) and Gisler et al. (1960) who obtained satisfactory results by using the Mantoux-test and also reported a rapid spread of the tuberculous infection in their monkey colony, if they had an infection at all.

ACKNOWLEDGEMENT

The technical assistance of Mrs. A M. de Bruin-van Diessen is gratefully acknowledged.

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DISCUSSION

CROUCH Dr. van der Waay, you mentioned doing sensitivity tests in making the choice of antibiotics for the treatment of Shigella. Which antibiotic did you have the most success with in preventing recurrence of Shigella infestations?

VAN DER WAAY We used terramycin in the monkeys reported on here and we treated only those from which we had obtained positive cultures.

PITCOCK Do you feel that you can reliably distinguish between Oesophagostomum and Strongyloides prior to the embryonated stage? Although there is a slight difference in size, we have felt that we could not reliably differentiate them.

VAN DER WAAY This is quite a difficult problem because so many Strongyloides species are found in the feces of rhesus monkeys that the embryonating time of some of them may overlap that of Oesophagostomum.

RIOPELLE I understand that Merck, Sharp and Dohme Co. of West Point, Pennsylvania have an experimental single-shot anti-Strongyloides drug known as Thiabendazole which is not yet commercially available. This compound has been used by Dr. Pickering at the Oregon Primate Research Center on infested rhesus monkeys with excellent results.

YOUNG Although we have a much lower incidence of Shigella than Dr. van der Waay reports, our primary problem is Strongyloides. Using Delvex (which was mentioned earlier) together with a product made by Lederle Laboratories called DNP we have been able to get about a 36% clearance in our infected animals.

On the tuberculosis problem: at one time we had an incidence of 20% tuberculosis in our colony and were forced to annihilate the animals and start over. All our animals now receive isonicotinic acid hydrazide (INH) at a

prophylactic level and are tuberculin tested at 4 month intervals. We have noted a much higher incidence of tuberculosis in larger (and older) animals imported into the colony. Out of 90 such animals, 11 gave a 1+ tuberculin reaction (slight erythema and edema). Of these, 3 came down with active tuberculosis while in quarantine whereas 3 more have been sacrificed and found free of TB. We are now using PPD for our first two tests and then going to old tuberculin. In addition we are X-raying our animals and any that look suspicious are getting three gastric lavages which are cultured before the animal is released.

BENSON I would like to comment on tuberculosis. I suspect that there is a high percentage of false negatives in situations where overwhelming, fulminating cases are occurring.

GISLER Yes. The 90-day quarantine idea is based on this suspicion. If they are quarantined and get a fulminating disease, even though the tests have been negative, they will not infect the rest of the colony.

WHITCOMB The large animals mentioned above by Dr. Young did not appear to be chronically ill and showed no evidence of weight loss at the time they were received. The skin tests were negative and when they were autopsied the lesions were not acute. From the gross findings it appeared that they had had the infection for some time. Thus, we perhaps cannot rely on the 90-day quarantine to protect us.

VAN PUTTEN Do we have the implication that if there are three negative skin tests and a normal sedimentation rate that it is safe to introduce an animal into an experimental colony?

BENSON Assuming that the animal is considered healthy in all other respects, I would venture to say yes.

CROUCH And yet many investigators who have followed such a procedure have introduced tuberculosis into their colony. It seems to me that the skin

test is often unreliable and I wonder whether we should use it at all?

BENSON In our experience, by retaining these animals in a 90-day quarantine under a heavy regime of parasitic and bacterial prophylaxis and with dietary supplements, we have had an excellent response.

SCHOFIELD I am somewhat worried about the use of tetracycline before irradiation on the ground that some bacterial resistance will develop in organisms that persist to cause trouble after radiation.

VAN PUTTEN We have employed tetracycline extensively and successfully to combat Shigella infection during the pre-irradiation sanitation procedure. In many of these monkeys tetracycline was again administered post-irradiation to prevent infections and this seemed to be quite effective. Therefore, we assume that just as in humans, the resistant bacteria disappear in many cases in the course of time.

DE VRIES To return to the tuberculosis problem: the conclusion seems to be that the tests to be performed are: (1) chest X-rays which may be difficult to interpret if lung mite (pneumonyssus) lesions are present, (2) cultures of gastric lavages and (3) liver biopsies. I should like to ask Dr. Riopelle whether he thinks doing liver biopsies is either difficult or hazardous to the animals?

RIOPELLE Although our experience with liver biopsies is limited to 40 or 50 animals, we have had absolutely no ill effects which I attribute, in part at least, to the use of the ICO needle, an Italian invention. This needle is inexpensive, fits a standard hyperdermic syringe, delivers an adequate amount of tissue for study and with it one can do a liver biopsy in exactly one second.

TAMARINUS NIGRICOLLIS AS A LABORATORY PRIMATE[1]

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The use of primates for biological studies in the laboratory has most often involved the rhesus (Macaca mulatta). Relatively few studies have been made with other primates, although the literature contains studies on a variety of species, each involving analysis of a particular physiologic trait that the investigator feels may be conveniently studied in the test animal he has chosen. However, the use of primates in basic research has gradually increased, primarily as an initial attempt to decrease the broad physiologic gap existing between man and the experimental animal. This problem is in evidence at this symposium, where the difficulties encountered in the protection of primates from radiation injury are being contrasted to the well-documented effectiveness of various procedures in experimental rodents. This paper presents a preliminary analysis of the feasibility and practicality of using a small South American primate, Tamarinus nigricollis, as a laboratory animal for radiation studies.

THE TAMARINS

In primate classification, tamarins are placed with the small marmosets in the Hapaloid family, lying between the more primitive Tarsioid family and the Pithecoids, in which is found the common squirrel-monkey of South America (Sanderson, 1957). The tamarin used in this study is from that group called Tamarinus, for which Sanderson (1957) reports 12 species and

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Monitoring Laboratory: School of Aerospace Medicine, Aerospace Medical Division (AFSC), Brooks Air Force Base, Texas.

21 subspecies. Tamarinus nigricollis, the white-lipped, black-necked tamarin is the particular species used in our laboratory. The tamarins originate in the upper Amazon basin, ranging through the equatorial forests of Colombia, Ecuador, Peru, and Bolivia.

The adult animals are about 19 to 21 cm from head to rump and have a nonprehensile tail measuring 23 to 30 cm. A unique characteristic of the tamarins is the presence of clawlike nails on all the fingers and toes except the opposable great toe, which has a flat nail. The hands, despite the lack of opposable thumbs, are prehensile and are frequently used in feeding while the animal sits on its haunches. The body weight of the young adult may range from 200 to 400 grams, thus approximating the weight of a laboratory rat.

The tamarins used in this study were not cage-bred animals. Many of them have been acclimated to captivity for two or three weeks and others were received directly from South America.

Caging and Handling.

Caging the tamarins in the laboratory is accomplished by using a box 20" x 18" x 18" made of pressed galvanized metal. As shown in Fig. 1, six of these cages can be placed conveniently on a portable rack, providing excellent mobility for any group of animals. Drop trays are located seven inches below the cages and contain an absorbent material, with most droppings passing through the small openings of the metal cage. Trays are changed daily and the cages are washed with disinfectant each week. Cage doors are sheets of metal, which slide down into grooves and require no lock because the weight itself makes it impossible for an animal to pry it open. Two wooden perches of varying height are placed in each cage. The simplicity and mobility of the cage and rack facilitates clean, open quarters and three such racks will permit the housing of 72 animals in a 12' x 15' room, provided each

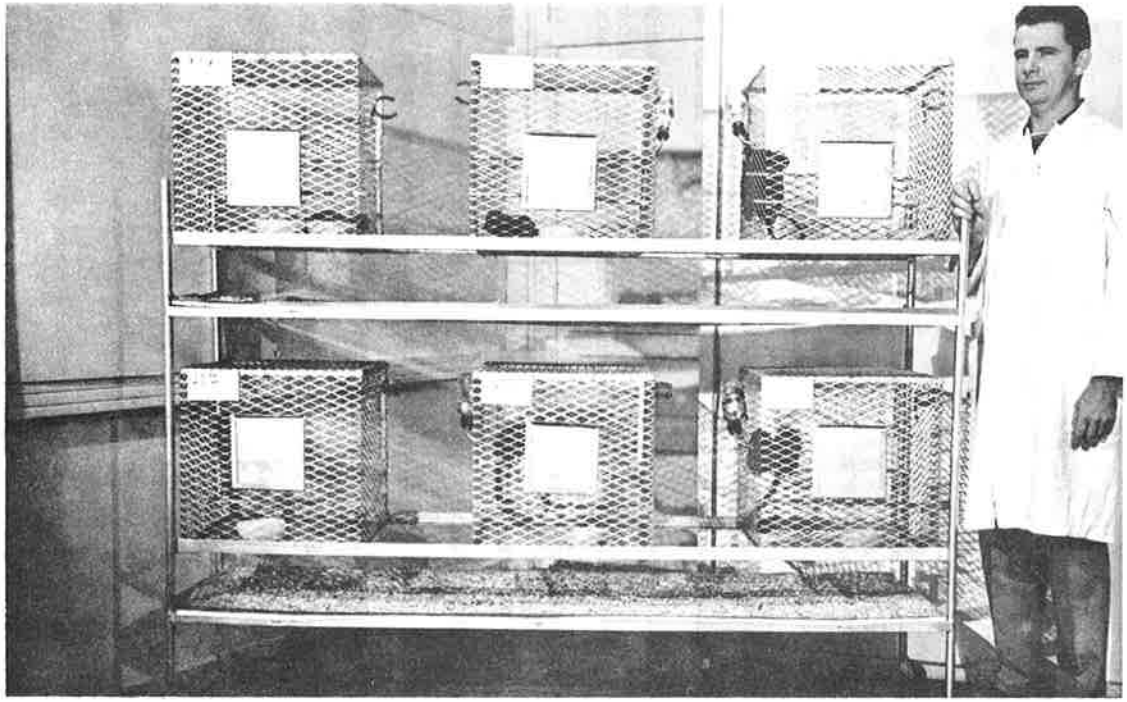

Figure 1. Six cages, containing up to four tamarins per cage, are maintained on a portable rack.

cage contains the maximum of four animals. At present, when space and cage quarters permit, we place only two animals per cage for general maintenance; and for radiation studies we house individual animals in similar cages. Room temperature is maintained at 78° to 84° F., and humidity at about 25%.

Removal of an animal from the cage is accomplished easily by using a small fish net. Our experience has shown that this method is least traumatic for both the caretaker and animal, for the tamarin is "fished out" in about 20 to 30 seconds. Freeing it from the netting requires leather gloves (lined with rubber) and a protective arm sleeve for the caretaker. Although the animals have not shown any tendency to attack a person while they are being fished out of the cage, they do not like to be handled and protective material is necessary to prevent bites from their sharp canine teeth. Figure 2 shows the size of a tamarin in relation to a man's hand.

For routine laboratory work, such as injections and bleedings, the tamarin's hind legs are immobilized by rubber bands attached to metal screws on a board improvised for this purpose (Fig. 3). Placing a flat-edged piece of material (here a lucite strip) against the base of the feet keeps them in a flexed position, preventing the animal from slipping his legs out from under the rubber bands. The caretaker holds the animal's upper body in place and the investigator may use two hands freely in doing any hematologic work on the animal. Anesthetics, thus, are not required. The femoral vein is used for venipuncture to obtain routine clinical hematologic data, and also for bleedings and injections, which are performed with a 1-ml tuberculin syringe and 25-gauge needle.

Feeding of the Tamarins.

Solving the problem of maintaining the tamarins on an adequate diet has been facilitated by the aid of Dr. L. Rane of the University of Miami who has successfully maintained these animals for about two years. Dr. Rane has made available to us a complete high-protein diet having a bread-like

Figure 2. Protective covering on hands and arms is necessary when handling the tamarin.

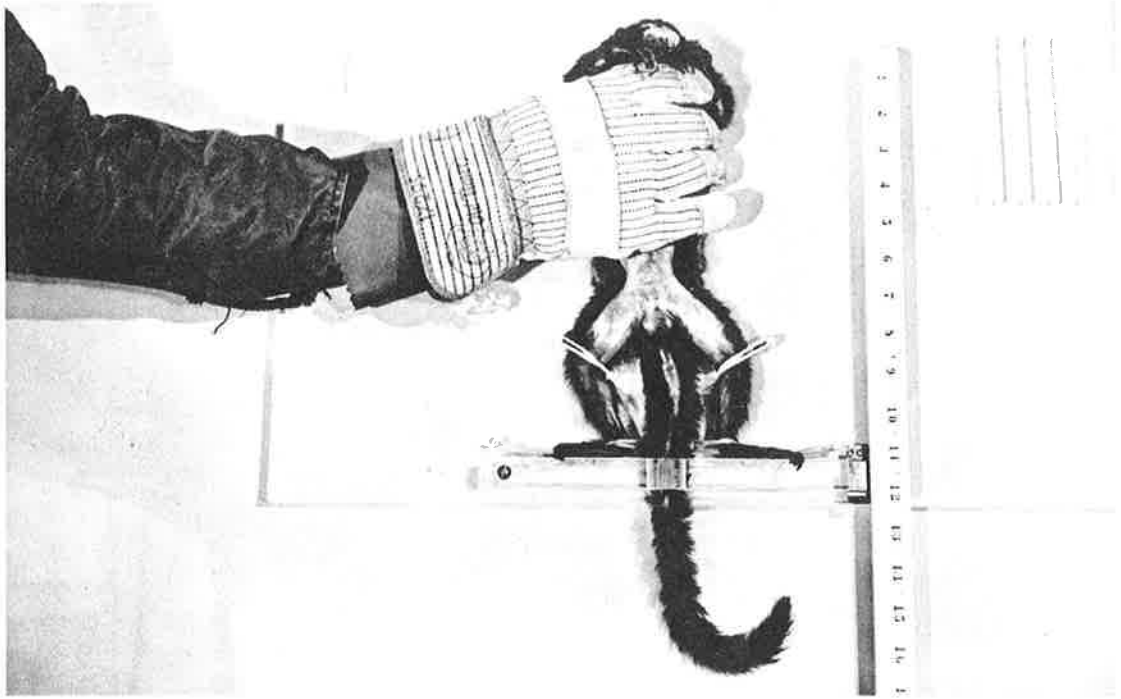

Figure 3. Procedure used to immobilize the tamarin for routine laboratory work.

consistency, which was formulated at the University of Miami expressly for his primate colony. In addition to this prepared diet, we have found it expedient to provide the animals with other foods such as Purina Monkey Chow pellets, eggs, fresh fruits and vegetables, and Pervinal, a multivitamin supplement. The animals are fed twice daily, receiving at noon the prepared diet sprinkled with Pervinal, and Purina pellets moistened with milk. In the evening they are given small portions of fresh fruits and vegetables, which are varied from day to day. The offering of a variety of foods appears to increase their appetite considerably and compensates for the day-to-day like or dislike for any particular food. A typical diet schedule and portion of food given for three consecutive days is shown in Table I.

Water is provided ad libitum by bottles attached to each side of a cage, the tamarin being readily adaptable to drinking from spouts similar to those used for rodents. The efficacy of the food in producing gain of body weight is shown in Fig. 4. On arrival at our laboratory this group of 31 animals showed a mean body weight of 274 grams and subsequently over a period of 98 days had a mean body weight of 314 grams, representing a 15% increase during this time. We have found that most animals will reach their maximum gain within two months after arrival, subsequently showing a leveling off or even a slight drop in weight thereafter.

Parasitism, Disease, and Mortality.

As with most primates, tamarins are afflicted with a variety of parasites. Most damaging has been a thorny-headed worm, an acanthocephalan, identified as Prosthenorchis elegans by Dr. Helen L. Ward of the University of Tennessee. Nearly every tamarin examined at autopsy is found to have these worms in the lumen of the ileum and attached to the mucosa, often resulting in chronic abscesses characterized as "pearly" nodules on the serosal surface of the ileum. Eradication of this parasite by oral treatment with piperazine citrate, dithiazanine iodide, or diethylcarbamazine has met with limited success as has been noted by others. Unfortunately, this

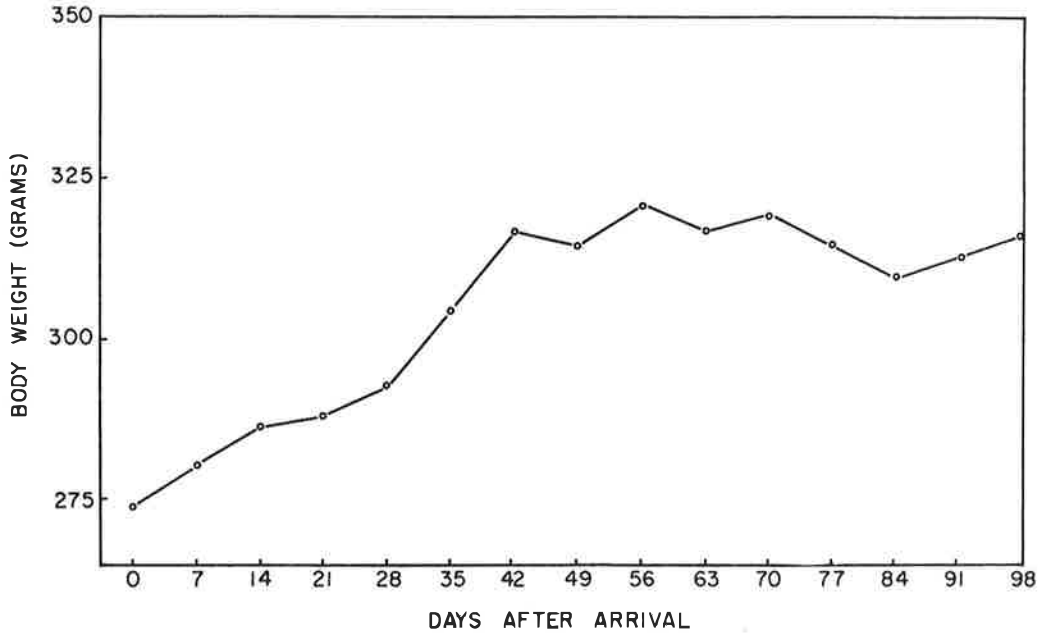

Figure 4. Mean body weight increase of 31 tamarins over a period of 98 days after arrival.

Table I

Food Schedule for Tamarins for Three Consecutive Days*

	Noon Meal (11:00 A.M.)	Evening Meal (4:00 P.M.)
Day 1	Diet**	Apples
	Lettuce	Sweet potatoes
	Monkey pellets	Monkey pellets
Day 2	Diet	Bananas
	Egg	Orange
	Monkey pellets	Monkey pellets
Day 3	Diet	Grapes
	Carrots	Apples
	Monkey pellets	Monkey pellets

* Proportions of food given per four animals is as follows:

Prepared Diet.....80 (g)	Apple.....1/2
Carrot (cooked).....1/4	Orange.....1/4
Egg (hard boiled).....1/2	Sweet potato (cooked).. 4 slices, 1" x 1/4"
Grape.....12	Lettuce.....1 leaf
Banana.....1/2	

Monkey pellets (moistened with milk).....4

** A prepared diet obtained from University of Miami, sprinkled lightly with Pervinal, a multivitamin supplement.

parasitism has occasionally contributed to the death of the animal here and elsewhere (Graham, 1960 and Ruch, 1959). A smaller, threadlike nematode, identified by Dr. Arthur Jones of the University of Tennessee as belonging to the family Spiruridae (possibly the genus Spirura) has been observed in the esophagus, often attached to the mucosa. This is found less frequently than the acanthocephalan and as yet does not appear to be a serious pathogen, although large masses of them sometimes almost occlude the esophagus. Filaria have been found in occasional tamarins in some shipments, with many adult forms in the peritoneal cavity and microfilaria in the blood. Sarcosystis parasitism in the skeletal muscles has been noted occasionally. Less frequently observed is the encysted form of a degenerate arthropod related perhaps to the "tongue-worm" of canines (genus Pentastomum), which is found in the lung, spleen, liver, mesentery, and abdominal lymph nodes, again without apparent ill effect.

A protozoan parasite discovered unexpectedly during routine examination of peripheral blood smears is a trypanosome, not further identified. Most, if not all, tamarins received to date have had the trypanosomes in their circulatory systems. The significance of this parasitism is not known, although one can speculate that the tamarin in its wild environment represents a natural reservoir in the life cycle of the trypanosome.

Tests for bacterial pathogens in the stools and blood have thus far been negative. Cultures of the feces have shown predominantly Proteus, and occasionally Aerobacter aerogenes, Pseudomonas aeruginosa, and Escherichia coli. Tests for Shigella and Salmonella have been negative. Cultures of the blood have occasionally shown Staphylococcus albus (coagulase negative) and Escherichia coli. The latter are detected frequently in postmortem blood cultures and are thought to represent a bacteremia often attributable to abscesses caused by the acanthocephalans.

Although it has been possible to successfully keep most of our primates healthy, a high mortality from a variety of ill-defined causes has occurred. Usually most deaths occur within the first two weeks after the animals are received, although this varies considerably from shipment to shipment. We feel these early deaths may be attributed to two interdependent factors; 1) the quality of animal initially shipped, and 2) handling and environmental conditions while in transit. In many deaths occurring beyond this critical period, autopsies have failed to reveal the exact cause. The most common and definite cause of death is peritonitis from the abscesses produced by the acanthocephalans in the ileum. One as yet unexplained observation is the extreme osteoporosis found at autopsy in some animals. The afflicted tamarins have bones that are readily broken or cut, while other tamarins have bones that are hard and strong. Detailed pathologic studies are now being performed on a number of the tamarins that died after several months in our laboratory and will be reported by Dr. Bill M. Nelson at a later date.

Breeding of the Tamarin.

One desirable feature of any laboratory animal, and primates in particular, is the ability to breed them successfully in captivity. Twinning is reported to be the rule in litters of this species, which sets them off uniquely from other laboratory primates (Sanderson, 1957). To ascertain the feasibility of breeding tamarins, four males and four females were caged in pairs and provided with small nesting areas. Two females became pregnant and each subsequently delivered twins. (The gestation time, although not known definitely, in one animal appeared to be about 130 days.) One female delivered prematurely by about two to three weeks and both twins died shortly after birth. In the other set of twins, one young was stillborn, with the recently notorious malformation known as phocomelia. (No sedatives were given to the mother.) The second twin apparently was born healthy but died three

months after birth. The mother of the baby tamarin died six weeks after delivery, and although the infant was apparently weaned at that time and subsequently cared for by the father, the absence of the mother may have contributed toward its death six weeks later. In any event, this success in breeding, considering that it was attempted on such a small scale, appears to offer excellent possibilities for future studies in hematopoietic transplantation and related immunologic problems. Dr. Rane has also indicated successful breeding of tamarins in his laboratory, substantiating further the feasibility of breeding these animals in numbers sufficient for experimental work.

Laboratory Studies on the Normal Tamarin.

It cannot be presumed that the tamarins in our colony presented a true "normal" picture of their health, but within themselves presented a set of biological values for primates conditioned to a laboratory. Evaluation of various biological parameters in the tamarin was performed in an attempt to define some base line for members of this species. This was done primarily in anticipation of future radiation sensitivity studies, and also for comparison of some of these values with the well-documented data existing on the rhesus.

Twenty-one randomly selected adult tamarins, 6 females and 15 males, the ages of which could not be determined, were bled by venipuncture at two-week intervals for 12 to 14 weeks. The animals were maintained under routine laboratory conditions and blood samples were always collected in the mornings before feeding. Samples were drawn for red blood cells (total count, hemoglobin, hematocrit) and white blood cells (total count, smears for differential analysis). A total of 162 to 163 observations was made for each parameter during this period. The mean values, range, and the standard deviation from the mean were determined. The values are given in Table II.

Table II. Analyses of Various Biological Parameters in *Tamarinus nigricollis**

Parameter	Number of Observations	Mean	Standard Deviation	Range
Red blood cells (x $10^6/mm^3$)	163	6.66	.910	4.47 - 9.35
Hemoglobin (g/100 cc)	162	16.0	1.4	11.4 - 19.1
Hematocrit	162	54.8	4.4	41 - 65
White blood cells (x $10^3/mm^3$)	162	15.0	5.9	6.8 - 45.9
differential, %:				
lymphocytes	163	55.2	15.1	20.5 - 91.0
segmented neutrophils	163	39.2	15.1	4.0 - 72.5
monocytes	163	2.7	2.0	0 - 11.0
basophils	163	1.5	1.3	0 - 7.5
eosinophils	163	1.2	1.2	0 - 11.5
Platelets (x $10^3/mm^3$)	31	430	36	232 - 713
Body temperature (°C, rectal)	34	39.3	0.5	38.1 - 40.3
Body weight (g)	41	314	40.2	227 - 436
Serum protein (mg N/ml)	19	12.5	1.3	9.9 - 15.1
Electrophoretic distribution %:				
gamma globulin	19	20	4.4	13 - 27
beta-2 globulin	19	6	1.4	4 - 9
beta-1 globulin	19	16	2.3	13 - 20
alpha-2 globulin	19	8	1.4	6 - 11
alpha-1 globulin	19	<2	0.7	1 - 2
albumin	19	49	5.1	39 - 58

* Values in the upper half of the table were obtained on 21 tamarins bled every two weeks over 12 to 14 weeks. The values in the lower portion of the table were obtained from single observations on individual tamarins, the numbers used shown in the left hand column.

Although there was an appreciable range for the total red blood cell count, hematocrit, and hemoglobin, the standard deviation for each of these parameters indicates that the majority of the values approximated the mean, thus showing a fair degree of homogeneity in these three parameters. Relative to the erythropoietic system, nucleated red blood cells were occasionally seen in the peripheral blood, the numbers ranging from 1 to 10 per 200 white blood cells in a differential analysis (data not tabulated). Diffusely basophilic red blood cells were noted on most blood smears. The significance of these late maturation forms of the erythrocytes in the peripheral blood of our tamarins is still uncertain.

The total white blood cell count showed a wide range of values and a fairly high standard deviation. Comparison of these data to those reported by Krise (1960) on more than 700 observations of the rhesus monkey shows a surprisingly similar blood picture, particularly in the white blood cells. Krise reported a mean of 15.2×10^3 cells/mm^3, with a range of 1.2 to 43.1×10^3 and a standard deviation of 5.98, these values being in close agreement with those of the tamarin shown in Table II. In the differential analysis of the white blood cells, both the range and standard deviation for the percentage of lymphocytes and neutrophils were high (Table II), indicating that these two parameters are highly variable, an observation not too surprising in view of the heterogeneity of the animal in respect to chronic parasitism. Again, the differential analysis compares remarkably with the values obtained by Krise on his comprehensive studies on the rhesus.

The remaining set of values shown in Table II was obtained on single observations of individual animals, the number used for each shown in the left hand column. Body temperatures have been consistent among the members of the colony, and although not indicated in the table, have shown little variation over extended periods; indeed, we have found this to be an accurate prognosticator of the animal's well being, a drop in temperature below the

range shown often indicating a sick animal. As noted previously, body weights of the tamarins range widely; the standard deviation, however, shows that most of the animals weigh from 280 grams to about 350 grams. The serum protein concentration also appears to be uniform among the limited samples analyzed. The electrophoretic analysis (Spinco paper strip apparatus) revealed an unusually high percentage of protein migrating in the gamma globulin zone. This might be related to affliction with various parasites, which may also induce the high percentage of blood lymphocytes noted for this species.

Total-body Irradiation Studies on the Tamarin.

To ascertain the feasibility of using the tamarin in a study of radiation effects and the application of various therapeutic measures, a small group of animals was exposed to total-body gamma radiation to determine initially their radiosensitivity. The radiation facility used was the total-body irradiator designed at the Oak Ridge Institute of Nuclear Studies for the irradiation of patients at the Medical Division hospital (Brucer, 1960). The gamma radiation is from eight cesium-137 sources, one 500-curie source located near each corner of an 8-foot cubical room. The positioning of the sources provides a uniform total-body irradiation of the patient or the experimental animal placed on a platform located in the center of the room. The tamarins were irradiated individually or in pairs in a compartmented lucite cage placed on the platform, the cage turning once every five minutes on a rotating stand. The dose rate used was 4.1 r/min, with animals exposed to doses ranging from 100 r to 600 r. The dose rate was determined by measurement of the absorbed dose using the Fricke ferrous sulfate dosimeter system. Only tamarins maintained in our laboratory for at least three months were used in these studies, each animal being caged individually two weeks before irradiation and thereafter. The body weights of the animals used ranged from 280 to 355 grams. Blood samples for clinical

hematology were collected one day before irradiation and at intervals of 1, 4, 7 days and weekly thereafter until death.

The 30-day mortality of the tamarins exposed to 100 r, 200 r, 400 r, 500 r, or 600 r is shown in Table III. All seven animals exposed to 400 r or more died within 8 to 14 days after irradiation. Deaths also occurred at doses of 100 r and 200 r, although at a later date. Only two of five survived the 200 r dose and three of four survived the 100 r dose. The tamarins exposed to 500 r and 600 r showed distress shortly after irradiation; their appetites became poor within one to two days postirradiation and they were rather lethargic by the fifth and sixth day. Hemorrhage and vomiting were not observed in this group. The 400 r group showed anorexia within three days postirradiation and became listless within one week. Vomiting occurred in one on the fifth day. Three of the four animals hemorrhaged from the nasal and anal orifice beginning on the ninth day and continuing until death. Of the three animals dying in the 200 r group, only one showed nasal hemorrhage and this on the day of death, 22 days postirradiation. All showed significant loss in body weight within one week postirradiation, although food and water consumption was most often normal throughout the period of observation. The only animal dying in the 100 r group showed normal food consumption and activity until the twenty-third day, after which it became listless, uninterested in food, and started hemorrhaging shortly before death on the twenty-seventh day. Significant weight loss occurred within the first postirradiation week and recovery was noted thereafter, although this was usually irregular. Vomiting was not observed in animals receiving either 100 r or 200 r. Body temperature in all the irradiated tamarins showed little variation during the periods of observation, with the exception of a significant drop of about 2° to 3° C occurring in animals a few days before death. All animals showed a ruffling of the fur, characteristic when they are in distress.

Table III

Mortality of Tamarins after Total-body Exposure to Cesium-137 Gamma Rays

Radiation dose (r)	No. and sex of animals	Time of death after exposure (days)	No. of 30-day survivors
100	4 (2♀, 2♂)	27	3 (2♂, 1♀)
200	5 (♂)	18, 22, 24	2 (♂)
400	4 (1♀, 3♂)	9, 10, 12, 13	0
500	2 (♂)	8, 10	0
600	1 (♂)	11	0

The hematologic values in all the 400 r to 600 r animals showed a sharp drop within the first seven days, most striking of which was the white blood cell count, which fell to less than $1000/mm^3$. Differential analysis showed a decrease in the percentage of lymphocytes within the first four days postirradiation, followed by a decrease in segmented neutrophils, which reached their minimum on the seventh day. Figure 5 shows the changes in the red blood cells, white blood cells, platelets, and body weight in the tamarins exposed to 400 r, these being similar to the effects noted in the higher dose groups.

Of interest are the hematologic changes that occurred in the tamarins exposed to 100 r and 200 r. The total red blood cell, white blood cell, and platelet counts for these animals are shown in Fig. 6 and Fig. 7. Changes in hemoglobin concentration and hematocrit values paralleled the red blood cell counts and are not shown. No significant decrease in the red cell counts occurred until the fourteenth day postirradiation, reaching the minimum value among the survivors on about the twenty-first day. Recovery was apparent in the 100 r group beginning on the twenty-eighth day, although the preirradiation value thus far has been attained in only one and this on the fifty-sixth day postirradiation. In contrast, the survivors (greater than 30 days) in the 200 r group have not shown complete recovery, even at 70 days postirradiation. In the surviving animals of both groups, a large number of nucleated red blood cells appeared in the peripheral blood and reached maximum levels about five to six weeks postirradiation; a subsequent decline occurred by the ninth and tenth week. The number observed is empirically expressed as the number of nucleated red blood cells counted divided by the total number of nucleated cells in a differential of 200 white blood cells (NRBC/TNC on right hand ordinate of Fig. 6 and Fig. 7).

White blood cell counts reached their minimum in both groups by the seventh day. Recovery has been incomplete thus far in the two survivors of the 200 r group, and in two of the 100 r animals at 56 and 70 days. One animal in the latter group reached its preirradiation value on the thirty-fifth day. The white cell differential among these two groups again showed an early decrease in the percentage of lymphocytes reaching minimum values on the seventh postirradiation day followed by the decrease in segmented neutrophils, which often reached their minimum values on the fourteenth day. Significant changes in other cell types were not noted throughout the period of observation. Thus, eosinophilia as reported by others (Eldred, 1959; Newsome and Overman, 1960; and Pitcock and Melville, 1962) to occur in the rhesus monkey four to six weeks postirradiation was not observed in the tamarins. The radiation dose of 525 to 550 r used by these investigators, however, is considerably higher than that given to the survivors of the present study. Platelet values decreased significantly in the 200 r group of tamarins by the seventh day, when little change was noted in the 100 r animals. Both groups, however, showed a minimum platelet level at 14 to 21 days with recovery beginning on the twenty-eighth day. Recovery of this parameter of response was slow and not complete until five to eight weeks postirradiation.

Although the numbers of tamarins irradiated in these experiments is small, the mortality and hematologic data tentatively suggest this primate to be rather radiosensitive compared to others previously investigated. Thus, the 30-day LD_{50} for the rhesus (Macaca mulatta) is estimated to be approximately 550 r to 600 r (Eldred, 1959), which is in contrast to the 100% mortality of the seven tamarins exposed to 400 r to 600 r. Insufficient numbers in the present study prevent any estimation of an LD_{50} for the tamarins and additional studies are anticipated. That this radiosensitivity is a real phenomenon and not an apparent one is suggested by: 1) relation of

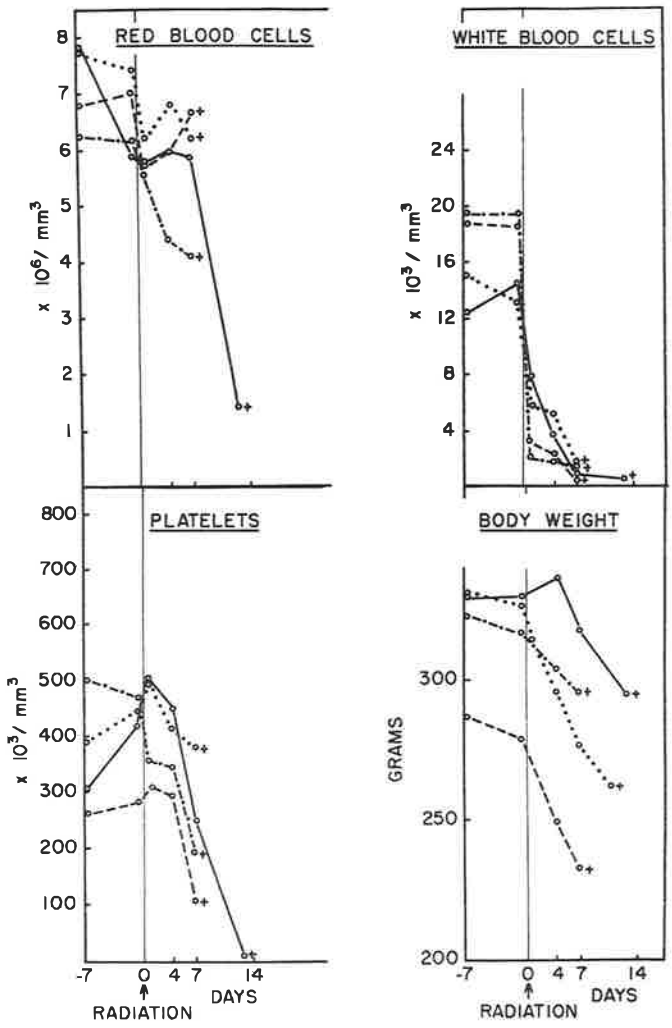

Figure 5. Hematologic and body weight changes in tamarins exposed to 400 r total-body gamma irradiation.

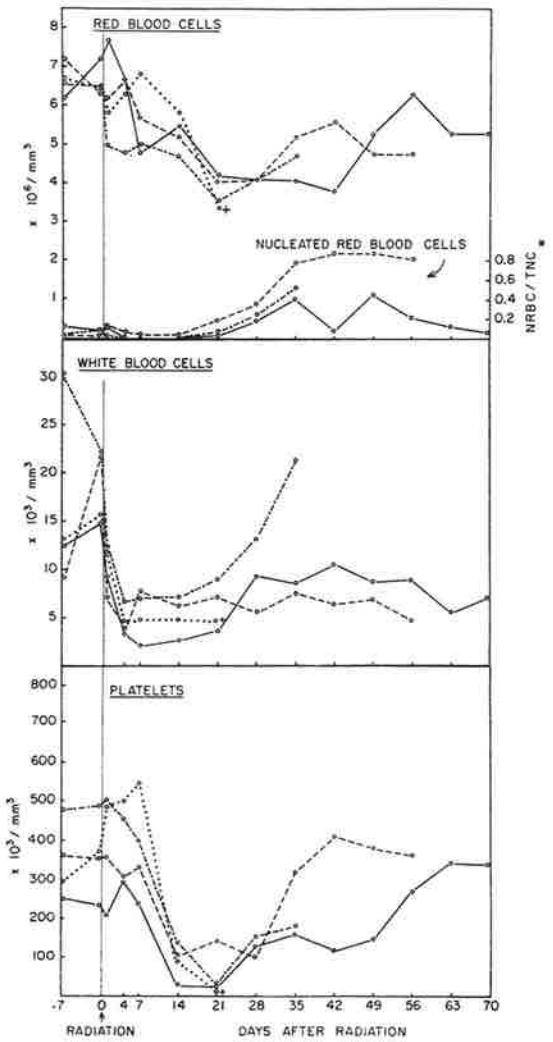

Figure 6. Hematologic changes in tamarins exposed to 100 r total-body gamma irradiation. *NRBC/TNC indicates the number of nucleated red blood cells per total number of nucleated cells counted in differential analysis of 200 white blood cells.

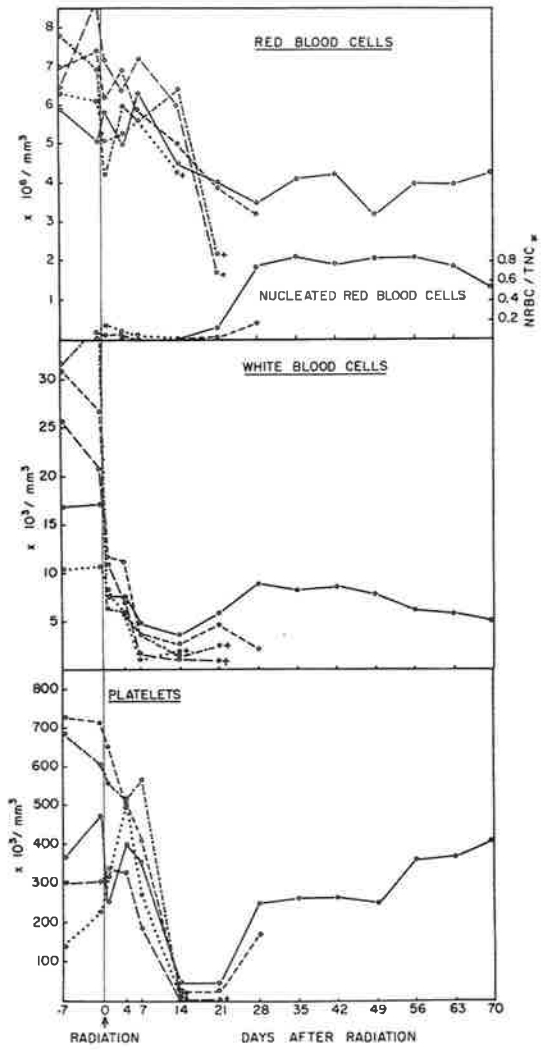

Figure 7. Hematologic changes in tamarins exposed to 200 r total-body gamma irradiation. *NRBC/TNC indicates the number of nucleated red blood cells per total number of nucleated cells counted in differential analysis of 200 white blood cells.

day of death to radiation dose (Table II); 2) differences in hematologic changes as a function of radiation dose (Fig. 5, Fig. 6, and Fig. 7); and 3) the incomplete recovery manifested by the few survivors for several parameters of hematologic response.

SUMMARY

We have presented preliminary studies permitting a tentative evaluation of the primate, Tamarinus nigricollis, as a laboratory research animal. Methods and procedures for caging, handling, feeding, and routine hematologic work with these animals have proved satisfactory throughout this one-year study. Although the parasitism among the tamarins is undesirable, it is not a deterring factor and effective control measures may be found. Indeed, the tamarin does not appear to present any more problems than has already been noted for the rhesus (Graham, 1960 and Ruch, 1959). These conclusions, however, are certainly not definitive and must await a more extensive period of study. Thus, reference is made to the mortality occurring in our colony of "acclimated" animals, the causes of which have yet to be determined. Similarly, although the broad variety of food given our primates appears satisfactory in terms of body-weight gain and maintenance, it is conceivable that the optimum diet has not been developed. On balance, however, the tamarin apparently represents a suitable laboratory primate, offering certain advantages not observed with other species. In particular, we note the feasibility of breeding these animals in the laboratory, the occurrence of twinning presenting excellent opportunities for experimental work in primate immunology and hematopoietic transplantation.

In the only experimentation done thus far in this laboratory, i.e., total-body irradiation, the tamarin appears to be more radiosensitive than other species of primates studied elsewhere. Possibly the damaging effects of radiation are enhanced by the parasitism prevalent in our animals, but such parasitism is ubiquitous among primates, particularly in the rhesus, in

which more extensive radiation studies have been performed (Crouch et al., 1961; de Vries et al., 1961; and Anderson, 1961). In the rhesus, however, the LD_{50} of about 550 r to 600 r appears to be considerably greater than that anticipated for the tamarins from our preliminary radiation studies. This observation tends to relegate the importance of parasitism to a minor role in the radiosensitivity of the tamarin. Additional studies are needed to verify this thesis.

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DISCUSSION

AMBRUS A group in our laboratory has maintained a Tamarin colony for several years now and they find that these animals can carry yellow fever so I think it is important that all personnel handling these animals should be vaccinated.

CROUCH Do you think, Dr. Gengozian, that the apparent radiosensitivity of this species could have been influenced by the trauma of handling and obtaining serial blood samples?

GENGOZIAN We are aware of this possibility and plan to study also animals that have not been handled.

KROHN I wonder if you have had an opportunity to recover the placentae from the pregnancies you report, to see if they were conjoint?

GENGOZIAN All of our tissues have been saved and are still being worked up by the pathologist.

KROHN I thought that this may provide a useful opportunity for doing skin transplantation in the free-martin situation in primates.

SCHOFIELD Could you give us an idea of the total amount of blood removed for sampling during the first 2 weeks post-irradiation?

GENGOZIAN This is a very small amount - just what was needed to do micro-hematocrit, hemoglobin and red and white cell counts.

SCHOFIELD In that case, the sensitivity of the bone marrow in this animal seems to be very high indeed. The effect of 100 r is tremendous as compared to that in other primate species.

GENGOZIAN We have tried to be cautious about this because we were surprised at the sensitivity of the marrow. I should point out that examination of one animal which died 27 days following exposure to 100 r showed that the

marrow was still hypoplastic.

NEWSOME Does this species provide a natural tagging system for detecting bone marrow graft "takes"?

GENGOZIAN Yes. The sex chromatin seems to be a satisfactory tag, but we have done no marrow grafting yet.

AMBRUS I understand that these animals are strictly monogamous and I wonder if the best way to breed them might not be to let them pair off spontaneously and then cage the naturally selected pairs together?

GENGOZIAN Yes, they are monogamous but we haven't tried the selection method you suggest. We did notice that the breeding pairs housed together and provided with a small, dark nesting area have done better than those caged in other ways.

A SYSTEMIC DERMATOSIS OF UNCERTAIN ETIOLOGY (X-DISEASE?) IN MONKEYS

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During the latter part of 1961 an outbreak of a peculiar disease which till then had not been observed occurred in our monkey colony. Thereafter an appreciable number of monkeys became affected. The main symptom of the disease was a patchy loss of hair of the scalp, trunk, extremities and the tail which in individual cases developed into a total shedding of the fur. The skin itself showed thickening, formation of coarse wrinkles, scaling and an unusual prominence of the hair follicles. At the base of the tail formation of abscesses was observed. Blepharitis was or had been present in the majority of the animals. The eyelids were red and edematous, while sometimes white dots could be seen at the margin of the lids.

A number of monkeys was found to be anemic (table I). The anemia was either of a normocytic or a macrocytic type and was often accompanied by reticulocytosis. In addition to these changes, the monkeys lost weight and a number of them became emaciated.

Lesions of the skin, stomach, intestinal tract, liver, pancreas and gall-bladder were regularly found at autopsy. Detailed findings of the autopsies of 21 of these monkeys will be reported (Table 1).

* This work was performed under contract with Euratom (European Atomic Energy Community) 51-53 rue Belliard, Brussels, Belgium.

Table 1

PATHOLOGICAL CHANGES IN MONKEYS WITH HAIR LOSS AND/OR BLEPHARITIS

Follicular hyperkeratosis	17/20*
Folliculitis	7/20
Hyperplasia of glands in stomach and duodenum	9/17
Hyperplasia of glands in colon	5/18
Atrophic gastritis	4/16
Dilatation of bile ducts	4/21
Cholangitis	3/21
Bile duct proliferation	4/21
Liver necrosis	3/21
Early liver cirrhosis	2/21
Periportal round cell infiltration	13/21
Cholecystitis	3/20
Dilatation of pancreatic ducts	6/18
Chronic inflammation of pancreatic ducts	6/18
Acinar atrophy of pancreas	1/18
Pancreatic fibrosis	1/18
Hyperplasia of lymphatic tissues	15/21
Micro-abscesses in lymph nodes	2/21
Infectious disease**	8/21
Esophagostomiasis	4/21
Anemia	6/12

* no. of monkeys with specified lesion/no. of monkeys examined for this lesion.

** Exclusive of esophagostomiasis. Shigellosis (2), staphylococcus infection (2), chronic interstitial nephritis (1), bronchopneumonia (1), lymph node abscess, agent not determined (1), granulomas in lymph node of unknown etiology (1).

SKIN

The epidermis and hair follicles showed acanthosis and hyperkeratosis. The changes were most prominent in the skin of the eyelids and base of the tail. At these sites excessive dilatation of hair follicles plugged with keratin often occurred. In a number of cases an inflammatory infiltrate consisting of lymphocytes, plasma cells and granulocytes surrounded the affected follicles (folliculitis). Sometimes follicular abscesses had developed. Micro-abscesses were occasionally found in the epidermis and keratin layer.

The mucous epithelium of the conjunctival mucosa often showed partial metaplasia to a stratified squamous epithelium. Multiple ulcerations of the conjunctival mucosa were occasionally seen.

STOMACH AND INTESTINAL TRACT

Hyperplasia of the mucosal glands of the stomach and duodenum was one of the most frequent findings in those monkeys. The glands were cystically dilated and filled with mucoid material. Proliferation of glands through the muscularis mucosae was characteristic, and resulted in the formation of nodular submucosal aggregates of glands. These glands were also partially dilated, displayed mucous degeneration and were surrounded by a mixed infiltrate of lymphocytes and plasma cells. Similar changes were seen in the colon of a number of animals.

In addition to the changes described above, disappearance of chief cells and parietal cells of the gastric glands was occasionally noted. These changes were accompanied by atrophy of the mucosa, increased numbers of mucous cells, metaplasia to an intestinal type of gland and a variable degree of round cell infiltration (atrophic gastritis). In 2 monkeys chronic esophagitis was also present, in one case there was a chronic glossitis.

LIVER

Dilatation of interlobular bile ducts occurred in a number of animals. Usually there was a certain degree of periductal fibrosis, the connective tissue being infiltrated with lymphocytes, plasma cells and occasionally eosinophils.

In 2 animals the distention of the bile ducts was excessive, the lumina containing large amounts of mucous substance. In these 2 animals multiple cholangiogenic abscesses as well as intrahepatic abscesses were present.

Except for bile duct ectasia, proliferation of smaller bile ducts in the periportal connective tissue was sometimes apparent. A lymphocytic and plasmocellular infiltrate was often present in these foci.

Foci of liver necrosis were found in 3 animals. In one case regeneration of liver lobules was also apparent. Early periportal fibrosis was found in

2 monkeys. In the majority of cases the periportal spaces were infiltrated with lymphocytes.

GALLBLADDER

Chronic cholecystitis as evidenced by fibrosis of the wall of the viscus accompanied by a chronic inflammatory infiltrate existed in 3 monkeys. At autopsy 2 of these gallbladders were found distended with inspissated bile.

PANCREAS

Slight dilatation of pancreatic ducts is difficult to evaluate, because of the wide variation in size of these structures in the normal animal. We believe, however, that duct ectasia was present in 6 monkeys. Usually this was associated with slight infiltration by lymphocytes and plasma cells in the surrounding connective tissue. Proliferation of duct epithelium was suggested in a few cases where budding of the epithelium of the larger ducts and increase of duct-like structures seemed to have occurred. However, the intricate pattern of branching of pancreatic ducts makes it difficult to establish such duct proliferation with certainty.

Atrophic changes of pancreatic acini was seen only once. Since this animal also suffered from disseminated vascular disease (see below), it is not known, whether these changes had developed independently. In this pancreas interstitial fibrosis was also present.

LYMPHATIC TISSUES

In most cases the lymphatic tissues in the spleen and lymph nodes showed hyperplasia, as evidenced by the presence of many follicles with prominent reaction centres.

In 2 animals abscesses were seen in the mesenteric lymph nodes. From one of these a staphylococcus was cultured. In another animal small granulomas were present in the peripheral connective tissue of a mesenteric lymph gland; some of these granulomas showed central accumulations of disintegrated neutrophils. Neither acid-fast rods nor mycotic organisms could be demonstrated.

INFECTIONS

Although in general our monkey colony is rather heavily infestated with the hookworm Oesophagostomum, only 4 animals of the group in question seemed to be affected with this parasite. From the feces of 2 monkeys positive cultures for Shigella were obtained. Staphylococci were demonstrated in the liver of 1 animal and in a mesenteric lymph node of another, as has been mentioned earlier. No cultures were made from a second lymph node, that showed abscess formation. Slight interstitial round cell infiltration was present in the kidney of 1 animal. As has been discussed already, granulomatous changes, probably of infectious nature, were encountered in the neighbourhood of lymph nodes in 1 animal.

MISCELLANEOUS CHANGES

One animal died with the classic changes of periarteritis nodosa. Since this same animal had obtained a homologous skin graft and was thereafter repeatedly tested with leucocytes from the same donor it is unwarranted to ascribe the development of these changes to the same agent, that may be responsible for the disease that is presently discussed.

The data presented may be summarized as follows.

The disease is characterized by hyperplastic epithelial changes in the skin, conjunctiva, gastro-intestinal tract, liver and possibly the pancreas. Stasis of the secretions of the liver and pancreas and duct ectasia of these sites may be explained by partial obliteration of the duct system of both glands.

In addition, inflammatory changes accompany the epithelial changes in a number of cases. In the skin, the folliculitis and abscess formation almost certainly must be ascribed to secondary bacterial infection.

The presence of infections is also suggested by the hyperplasia of the lymphatic tissues in the majority of the animals. The actual demonstration of infections in 8 out of 21 animals might point to an enhanced susceptibility to infectious disease. That the inflammatory changes are secondary to those of the epithelial tissues is suggested by the fact that, to our knowledge, no infectious agent is known causing a disease with the typical pattern and pathological characteristics described in the present report.

The occurrence of enhanced susceptibility to infection and anemia in conjunction with the described epithelial changes, are strongly suggestive of a deficiency disease and most likely a vitamin A deficiency.

Several arguments may be put forward, however, pleading against an extraneous vitamin A deficiency. The monkeys have an adequate supply of sources of this vitamin. Changes as desplayed by these monkeys can only be provoked by extreme deprivation of vitamin A.

An endogeneous vitamin A deficiency, as encountered in sprue-like conditions is also unlikely, since the animals did not produce copious fatty stools. Moreover, pancreatic lesions were not found with sufficient frequency and to an extent to allow the diagnosis of pancreatic insufficiency. Lastly serum determinations, carried out in a few cases by Dr. H. M. Klouwen of our laboratory, did not reveal decreased blood levels of vitamin A in the

diseased animals as compared with normal controls.

These considerations all lead to the tentative conclusion that the described pathological condition of the monkeys is most likely to be attributed to chronic exposure to a poison with anti-vitamin-A properties. A group of such poisons, the chlorinated naphtalenes are known to produce a disease in cattle (Smith and Jones, 1961), which duplicates most of the pathological findings in our monkeys (table II)

Table II

PATHOLOGICAL CHANGES IN CATTLE POISONED WITH CHLORINATED NAPHTALENES* (bovine hyperkeratosis)

Emaciation
Follicular hyperkeratosis, acanthosis
Squamous metaplasia of columnar epithelium
Nodular proliferations of epithelium of digestive tract, formation of cysts filled with mucus
Hyperplasia and dilatation of bile ducts, pancreatic ducts, and renal tubules; similar changes in gallbladder; fibrosis at these sites
Early biliary cirrhosis

* Smith and Jones (1961)

These compounds are added to commercial machine oils to improve their lubricating properties. The disease in cattle has been traced to food pellets contaminated with minute amounts of these compounds; contamination took place during the process of pressing the pellets by machinery which evidently was lubricated with such oils.

Although chlorinated naphtalenes are believed to exert their noxious influence by an anti-vitamin-A effect and decreased blood levels of vitamin A may be found, these never reach zero, as in the case in experimental deprivation of this vitamin. In addition, experimental intoxication with these substances demonstrated that supplying the intoxicated animals with large amounts of vitamin A did not result in a normal vitamin A content of the blood, when the amount of poison administered surpassed a certain limit. Lastly, treatment with vitamin A has a favourable effect in part of the animals only, while it has not been possible to duplicate the lesions even by complete deprivation of vitamin A from the food.

As to the possible sources of the poison in our case, the pellets which the monkeys are fed must be suspected in the first place. Although during the last 2 years the composition of their contents was made up in our laboratory, the pellets were actually pressed in a factory. Routine feeding of our monkeys with these pellets started in August 1961 and shortly thereafter the first symptoms of the disease were noted.

Another source could be the contamination of the monkey cages by oil during the process of cleaning of the cages with steam. Contamination of the steam by traces of oil from the condense water pumps might have occurred.

A systemic investigation of the mentioned possibilities will soon be started. Except for the deleterious influence of this agent on the health of the monkeys, it might also be a hazard to the personnel engaged in the care of the monkeys and the cleaning of the cages. Since the chief effect of this agent is excessive wide-spread epithelial proliferation, a possible carcinogenic action must be kept in mind, though we have no evidence for this last assumption as yet.

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DISCUSSION

VAN PUTTEN Certain pathological features of this disease remind me of descriptions of secondary disease following marrow transplantation. Can you point out the differences?

DE VRIES Yes. Several arguments may be presented to show that this is quite different from secondary disease. During the first series of homologous transplants in monkeys, X-disease did not occur in our colony so we were not troubled with interference at a time when secondary disease was present as seen by the fact that not a single animal, transplanted with homologous marrow, survived. Secondly, during that same 2 year period, none of the autologously treated animals displayed any of the clinical and histological signs shown by those given homologous marrow. Thirdly, although certain of the skin lesions may superficially resemble those of secondary disease, they are really quite different as are the lesions of the gastro-intestinal tract. Although hyperkeratosis occurs in "X-disease", dyskeratosis and vacuolar degeneration of the epidermis, which are characteristic of the dermatosis in secondary disease, are absent. In the gastro-intestinal lesions of "X-disease", the predominant finding is epithelial proliferation with secondary mucoid degeneration of glands. Stomach and duodenum are the predilected sites. In secondary disease destruction of epithelium appears to be the primary lesion, while colon and ileum are most frequently and stomach and duodenum only seldomly affected. Liver and pancreatic lesions such as encountered in "X-disease", have never been seen in secondary disease. I am, therefore, very confident that we have not confused these two diseases but nevertheless I am grateful that "X-disease" did not appear in the colony during our early experience with secondary disease.

GENERAL DISCUSSION
ON MONKEY COLONY MANAGEMENT

Chairman : R. E. Benson

BENSON Dr. Riopelle, as a result of your discussion of the good response of your animals to Sernyl, I wonder if you later found evidence of relectance of these animals to be re-anesthetized in view of the post-anesthetic hysteria?

RIOPELLE Remarkably enough, we have not, even though we have anesthetized some animals at weekly or monthly intervals for a period of a year and a half.

CROUCH I'd like to comment on Dr. Riopelle's excellent justification for the use of the chimpanzee in this kind of experimental work but I am wondering about its use in the fact of its slow maturation. If we must wait 9 years to obtain a post-pubertal animal from a breeding colony, will this not be prohibitively long?

RIOPELLE I would certainly be the last to recommend the chimpanzee for all our studies and breeding all the animals also it is not always necessary since the availability of adult chimpanzees - particularly from zoos - is rather good. And although, in purchased adult animals, you don't have the detailed background records, still such animals are healthy and are good strong animals to use.

CROUCH Can you give us an estimate of the price of an adult chimpanzee in the United States?

RIOPELLE Yes, the present price is about 600 dollars. When one compares this with the price of the rhesus monkey, it is not excessive. Another interesting animal that might be used is the gibbon which costs about 200 dollars. The presently limited availability of these larger animals makes them perhaps more suitable for special projects than as a replacement for the rhesus monkey.

BENSON Should we conclude, Dr. Riopelle, that you would not recommend a breeding program for chimpanzees but rather that animals of appropriate ages be purchased?

RIOPELLE No. I think whether you have a breeding program or not depends entirely on the problems you wish to study. We have a breeding program at the Yerkes Laboratories and will set up another at Tulane because if you want to study the development of, say, the immune reaction in prenatal or neonatal life, such studies can only be done in a breeding colony.

CROUCH I should like to hear some opinions regarding the feasibility of breeding rhesus or cynomolgus monkeys (since most of us are using these species) under specific pathogen-free (SPF) conditions for use particularly in studies on radiation, marrow transplantation and studies of chemical protection.

RIOPELLE Yes. I think the cost of maintaining a breeding colony of rhesus monkeys is staggering and does not compare too unfavourably with that of a chimpanzee colony.

CROUCH May I ask Dr. van Lancker the same question, since they have had a great deal of experience with this at the University of Wisconsin?

VAN LANCKER Although I have not been directly involved with our breeding program, I do know that we are producing about 40 monkeys a year and that this is rising by about 10 animals a year. Also we lose very few neonatal monkeys and I believe, as Dr. Riopelle suggested, that this is largely a matter of good sanitation.

CROUCH But do you believe this is excessively expensive when compared to purchasing animals?

VAN LANCKER I think it is more expensive than purchasing but it surely presents many advantages since the animals are essentially tuberculosis-free and have far less parasites than animals usually purchased.

CROUCH That is the point I am trying to make. It is more expensive to be raising healthy monkeys than to have to destroy an entire colony once in a while because of disease or to take months to condition animals at great expense only later to have experiments ruined by recurrence of infection? I still question the fact as to whether the breeding colony is really, in the long run, more expensive.

RIOPELLE Of course if you have a casual breeding program, it is relatively inexpensive since it goes piggy-back, as it were, on the rest of your research. But if you really set out to produce a fair number, say a specifiable number of rhesus monkeys every year, it is an expensive proposition.

VAN LANCKER I'd just like to comment that it may take a fair-sized colony to produce the number of offspring required every year. I know that the main problem has been fertility and we are now attempting to use artificial insemination.

GISLER One point that should be made, perhaps, is that the secret of a successful breeding program lies in culling out non-productive females. Since we have done this, we are getting about 50 per cent successful matings which is a pretty good figure.

CROUCH I believe that it is important to emphasize that although it is not, perhaps, necessary to breed <u>all</u> the animals that one intends to use for experimental purposes, the number of situations in our area of research where we need to know the age, the medical history, and to have healthy animals is sufficiently great to make breeding a mandatory affair in a number of laboratories.

KROHN The point in culling out unproductive females is a good one, I think, because in our experience the females either reproduce repeatedly and without difficulty or they don't reproduce at all.

WHITCOMB Relative to knowing the age of the monkeys we are using, I am wondering how much data is available to compare the adult and immature rhesus monkey. I believe most of us have been using the immature animals and perhaps we do not know much about infection or even much about radiation of the adult rhesus.

CROUCH Does anyone have evidence for a difference in radiosensitivity between the immature and mature monkey?

VAN LANCKER I believe that someone has studied the radiosensitivity of monkeys of various weights but I don't think this was correlated with age.

BANGHAM I wonder if it would be possible for us to organize some central collecting system for X-ray data on monkey foetuses of various ages? I think that for any one laboratory it would take a prohibitively long time to assemble adequate data on bone ages of the foetus. If this could be made a collaborative effort, it could surely be obtained much quicker.

VAN PUTTEN Do you mean X-rays of the isolated foetus or the foetus in utero?

BANGHAM It is very difficult to get pictures good enough for bone age determination in utero when the foetus is less than 15-16 weeks gestation, whereas the excised foetus of 9-12 weeks gives excellent pictures. I realize that foetuses of this age are rare and precious and thus that a collaborative effort is required.

VAN PUTTEN Would it been necessary to have exact information as to foetal age or would crown-rump length, combined with X-ray films be sufficient?

BANGHAM I am sure one would need as much details as possible about measurements as well as sureness of the length of pregnancy. I think this can only be obtained from breeding colonies that expose the females to the males for up to a week, perhaps.

HUMAN APPLICATIONS OF BONE MARROW TRANSPLANTATION

AUTOLOGOUS AND ISOLOGOUS BONE MARROW THERAPY IN MAN

By N. B. Kurnick, M. D., University of California, Los Angeles, California and the Veterans Administration Hospital, Long Beach, California

In this presentation I shall discuss the validity of bone marrow implantation in man and the indications for such implantation. Our experience with homologous bone marrow infusion has been small, but uniformly unsuccessful. We have been unable to detect any evidence of proliferation of the implanted marrow either by examination of the marrow space or by the peripheral blood picture. I shall therefore limit the discussion to our experience of the past six years with autologous and isologous bone marrow infusion, procedures which avoid the problem of histocompatibility.

The experience with the implantation of isologous bone marrow into an individual with long-standing bone marrow aplasia due to prolonged and extensive radiotherapy and chemotherapy appears to provide convincing evidence that bone marrow implantation in man can be successfully performed. We have previously published a case (Kurnick 1961) of a 21 year old male who had been treated for metastatic seminoma during the preceding 4 years. For over a year he had been known to have severe bone marrow aplasia with leukocyte counts below 1,000/ cubic milimeter, platelets between 10,000 and 20,000, reticulocytes 0, and anemia sustained at 9 to 10 grams by weekly blood transfusion. Figure 1 illustrates the prompt response to the intravenous infusion of 3×10^9 nucleated cells aspirated from the iliac bone marrow of his twin brother. The cellularity of the bone marrow, indicated in pluses, is based on 3+ for normal. The eosinophilia, which we

have not noted in other experiences with bone marrow infusion, is probably attributed to the presence of hay-fever and eosinophilia in the donor. The second experience in this patient following further therapy with Cobalt 60 teletherapy and P_{32} intravenously was also successful, although the response was slower on this occasion, probably due to the fact that the infusion was performed only 10 days after the last injection of 35 mc. of P_{32}, with, therefore, considerable residual radioactivity in the recipient. The reticulocytosis, return of white blood cell count, platelets, and hemoglobin to normal without further transfusions, the hyperplasia of the bone marrow, all in 3 to 4 weeks, could hardly be coincidental. This case, then, indicates that bone marrow implantation in man is valid and also provides one of the indications for doing so. Had the patient's own bone marrow been collected and stored prior to therapy, presumably the same result could have been obtained had no twin been available.

Further evidence for the validity of bone marrow implantation in man is obtained from a case of Hodgkin's disease of many years' duration in whom aplasia of the sternal marrow had resulted from radiotherapy to the chest (Figure 2). Marrow aspirated from the left ilium and stored by freezing in glycerol-tissue culture medium, was infused following 1.2 mg/kg of nitrogen mustard intravenously. Hyperplasia of the sternal marrow, following its long-standing aplasia, occurred simultaneously with repopulation of the other bone marrow sites, about 4 weeks after the autologous bone marrow infusion.

Patients who have received extensive and intensive radiotherapy to large portions of the body show only very slow recovery of the peripheral blood from the induced pancytopenia. Figure 3 illustrates the peripheral blood picture of a patient with seminoma who received approximately 2400 r tissue dose in

fractional doses to the entire torso, excluding the head and extremities. Incomplete recovery of the peripheral blood elements is noted at the end of 4 months. Figure 4 illustrates the persistent hypoplasia of this patient's iliac bone marrow two years after therapy. Only islands of nucleated bone marrow cells are found in a generally fatty bone marrow. One may contrast with this result, the hypercellular bone marrow (Figure 5) of a similarly treated patient who was infused with stored autologous bone marrow 3 weeks previously, following the completion of x-irradiation. Note, also, the rapid recovery of the peripheral blood (Figure 6).

We consider that the much more rapid recovery of the peripheral blood elements and induction of hypercellularity of the bone marrow, even when long-standing hypoplasia has existed, provide evidence for the effectiveness of bone marrow implantation in man. The long-standing aplasia of irradiated bone marrow, both following limited and widespread x-irradiation in man contrasts man from the mouse. In the mouse, the shielding of a single extremity provides sufficient circulation of repopulating elements for recovery of all the irradiated bone marrow sites. Similarly, we have found that the irradiation of a single extremity in the mouse is regularly followed by repopulation from the rest of the bone marrow, whether the therapy to the single extremity is 2000 r administered in a single dose or as much as 10,000 r administered in fractionated doses. This result indicates that continuous circulation of cell capable of repopulating the bone marrow occurs in mice. On the other hand, the long-standing, even permanent aplasia of irradiated bone marrow in man indicates that such circulation does not occur spontaneously in man. Therefore, the prevention of permanent severe bone marrow depression in man by

protection of limited bone marrow sites cannot be expected. On the other hand, the extraction of bone marrow cells from the bone marrow cavity and intravenous infusion of these cells does produce repopulation of irradiated bone marrow sites in pancytopenic individuals. Where only localized hypoplasia exists without pancytopenia, we have not succeeded in repopulating the hypoplastic sites by bone marrow infusion. We consider that this suggests that bone marrow repopulation from implanted uninjured cells requires stimulation by factors elicited in response to pancytopenia.

If we agree that bone marrow repopulation by infusion of autologous or isologous bone marrow is feasible, it remains to determine the indications. In our experience, acute bone marrow aplasia and pancytopenia produced by nitrogen mustard (up to 1.2 mg/kg), 5 fluorouracil, cyclophosphoramide, and amethopterin have regularly recovered in approximately two weeks after the nadir has been reached. It appears probable that acute medication with these chemotherapeutic agents permits the survival of sufficient numbers of resistant cells to repopulate the bone marrow. Bone marrow infusion in such patients would not be expected to promote or accelerate the recovery. Chronic medication with Busulfan has, however, produced long-standing bone marrow aplasia and pancytopenia. Since the patients we have seen with this syndrome had been treated for chronic granulocytic leukemia, suitable bone marrow was not available for storage and reinfusion during the aplastic stage; but we would anticipate that had suitable marrow been available for these patients, repopulation could have been achieved. Contrasted to the situation with chemotherapy, radiotherapy provides for predictable and definable indications for the infusion of autologous bone marrow. Patients who receive 2400 to 3000 r tissue dose administered to all parts of the torso will commonly show severe bone marrow depression. Such

depression may be predicted to be long-standing or permanent, and therefore presents serious problems in management and considerable risk to the patient from bleeding and infection. It is our practice to collect, and store by freezing, bone marrow from all patients who are to undergo such therapy, including primarily those with testicular tumors and lymphomata. Figure 7 lists the patients in whom this procedure has been performed. The majority of the patients have not been reinfused because alarming bone marrow hypoplasia did not result. Others were not reinfused because the tumors were radio-resistant and the patients succumbed to the tumors. Many of the patients who were not reinfused nevertheless profited from the fact that bone marrow had been stored because radiotherapy was not interrupted when the peripheral blood count began to drop rapidly, since it was known that bone marrow was available for reinfusion Most of the patients stabilized at low white blood cell and platelet counts (2000 to 3000 WBC/cmm and 40,000 to 60,000 platelets/cmm) while the therapy was continued, and therefore were not considered to require reinfusion of their stored bone marrow. When pancytopenia progressed to white blood cell counts below 2000 and platelet counts below 40,000, and did not show any tendency to rise at the completion of radio-therapy, infusion of bone marrow was performed. In addition to this indication for bone marrow infusion, we infused bone marrow into patients who were subjected to total body irradiation in the range 600 to 1200 r in a single dose, since we considered that this level is probably in the lethal range for man. Figure 8 illustrates the result in an individual given 800 r to the entire body for widespread metastatic disease.

We have had one individual who was treated with extensive and intensive radiotherapy for metastatic seminoma, who was not reinfused with his stored bone marrow, develop granulocytic leukemia 3 1/2 years after the radio-therapy

Up to the time of the sudden emergence of the leukemia, his bone marrow had been examined periodically and found to be continuously hypocellular, and his peripheral blood count revealed mild pancytopenia. It may be argued that if this individual had been infused with his stored bone marrow following the radiotherapy, the unirradiated infused cells would have repopulated his bone marrow with suppression of the remaining irradiated bone marrow cells from which the leukemia presumably evolved. Post-irradiation leukemia might therefore have been prevented in this patient. This possibility, plus the possible advantage of re-establishing normal bone marrow function rapidly in patients who have not suffered sufficient bone marrow depression from x-irradiation to qualify under the indications we have been using for reinfusion, suggests that it may be desirable to infuse the stored autologous bone marrow into all patients who undergo extensive radiotherapy. Reinfusion is simple, requires only dilution of the thawed marrow suspension with one-half volume 35% glucose. We do not remove the glycerol. No adverse reactions have been encountered except for two occasions of cerebral hemorrhage in severely thrombocytopenic patients.

A few words about the lymphomas and leukemia may be in order. We have treated one patient with widespread lymphosarcoma with 2400 to 3000 r tissue dose to all the node-bearing areas and spleen following bone marrow storage. This patient did not suffer reduction in his white blood cell count to below 2000 (lowest was 2100) or of his platelet count below 40,000 (lowest 90,000) and was therefore not reinfused with his marrow. He has been followed for three years without evidence of recurrence of his malignancy. Another patient with Hodgkin's disease was similarly treated six months ago. He was reinfused with his stored bone marrow, with resultant rapid repopulation. We are hopeful that an equally good result will be obtained in the suppression of his malignancy.

We consider that this approach with cancerocidal therapy to all the areas likely to be affected with the disease offers promise for improvement in the management of lymphomata. In the case of the leukemias we are less optimistic. We have stored bone marrow from patients with leukemia during hematological remission, induced by chemotherapy. During terminal relapse, two of these patients were subjected to total body x-irradiation, followed by reinfusion of stored bone marrow. Both patients succumbed to sepsis within two weeks, and the severely pancytopenic peripheral blood and hypoplastic bone marrow continued to contain myeloblasts.

We are of the opinion that storage of autologous bone marrow and subsequent reinfusion permits the extension of radiotherapy with increased chance of cure, particularly in the testicular tumors and lymphomata. Storage of bone marrow for individuals involved in occupational hazards, including perhaps space exploration, should also be of value. It is possible that the indications for reinfusion of the bone marrow, following radiotherapy should be liberalized.

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1. Kurnick, N. B. Autologous bone marrow in the treatment of severe iatrogenic myelo-suppression. Diagnosis and treatment of acute radiation injury, 1961, Geneva, World Health Organization, pp. 309-327.

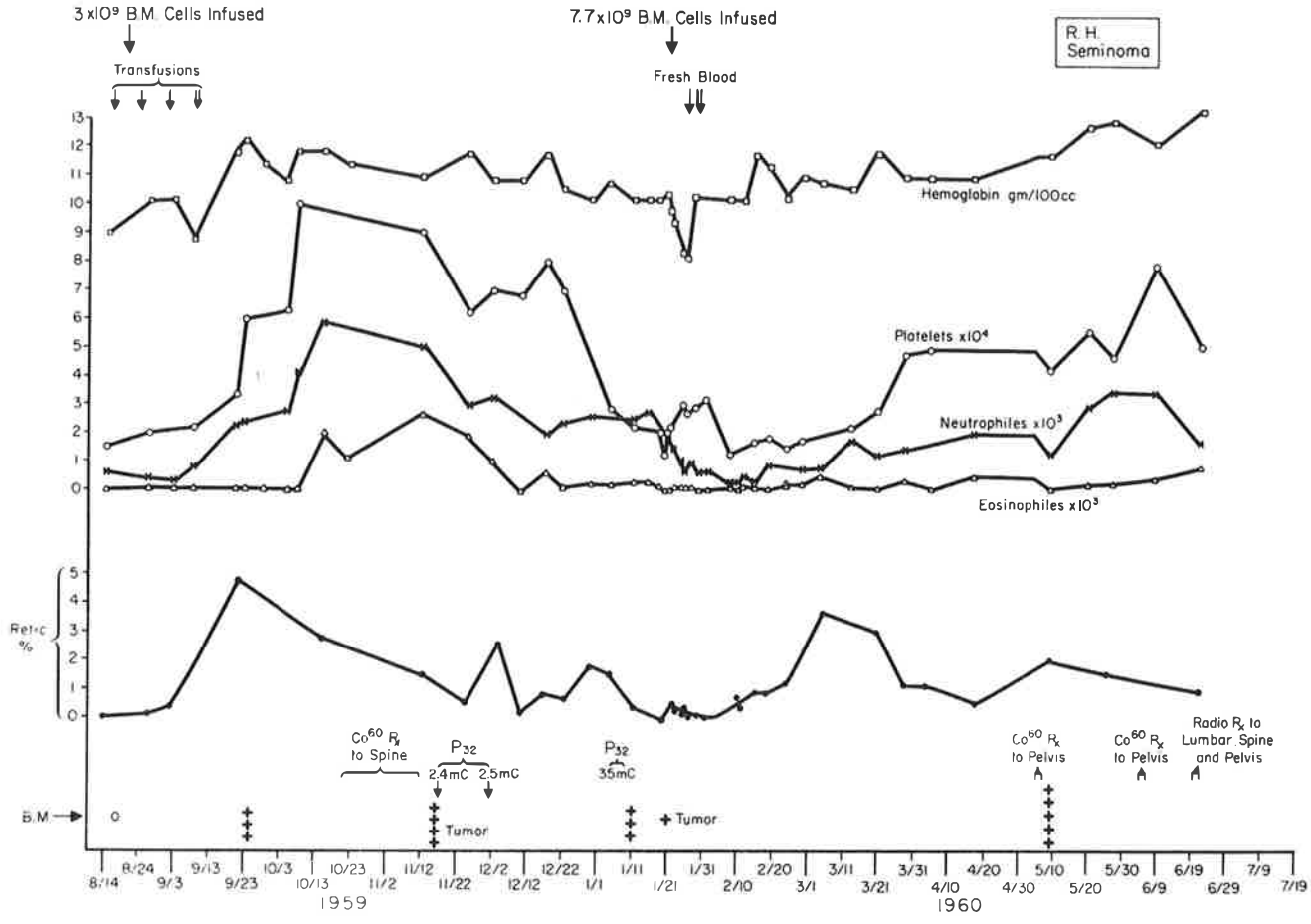

3 x10⁹ B.M. Cells Infused
Transfusions
7.7 x10⁹ B.M. Cells Infused
Fresh Blood
R.H.
Seminoma
Hemoglobin gm/100cc
Platelets x10⁴
Neutrophiles x10³
Eosinophiles x10³
Ret×c %
Co⁶⁰ Rx to Spine
P32 2.4mC 2.5mC
P32 3.5mC
Co⁶⁰ Rx to Pelvis A
Co⁶⁰ Rx to Pelvis A
Radio Rx to Lumbar Spine and Pelvis A
B.M.
Tumor
Tumor
8/14 8/24 9/3 9/13 9/23 10/3 10/13 10/23 11/2 11/12 11/22 12/2 12/12 12/22 1/1 1/11 1/21 1/31 2/10 2/20 3/1 3/11 3/21 3/31 4/10 4/20 4/30 5/10 5/20 5/30 6/9 6/19 6/29 7/9 7/19
1959
1960

Figure 1

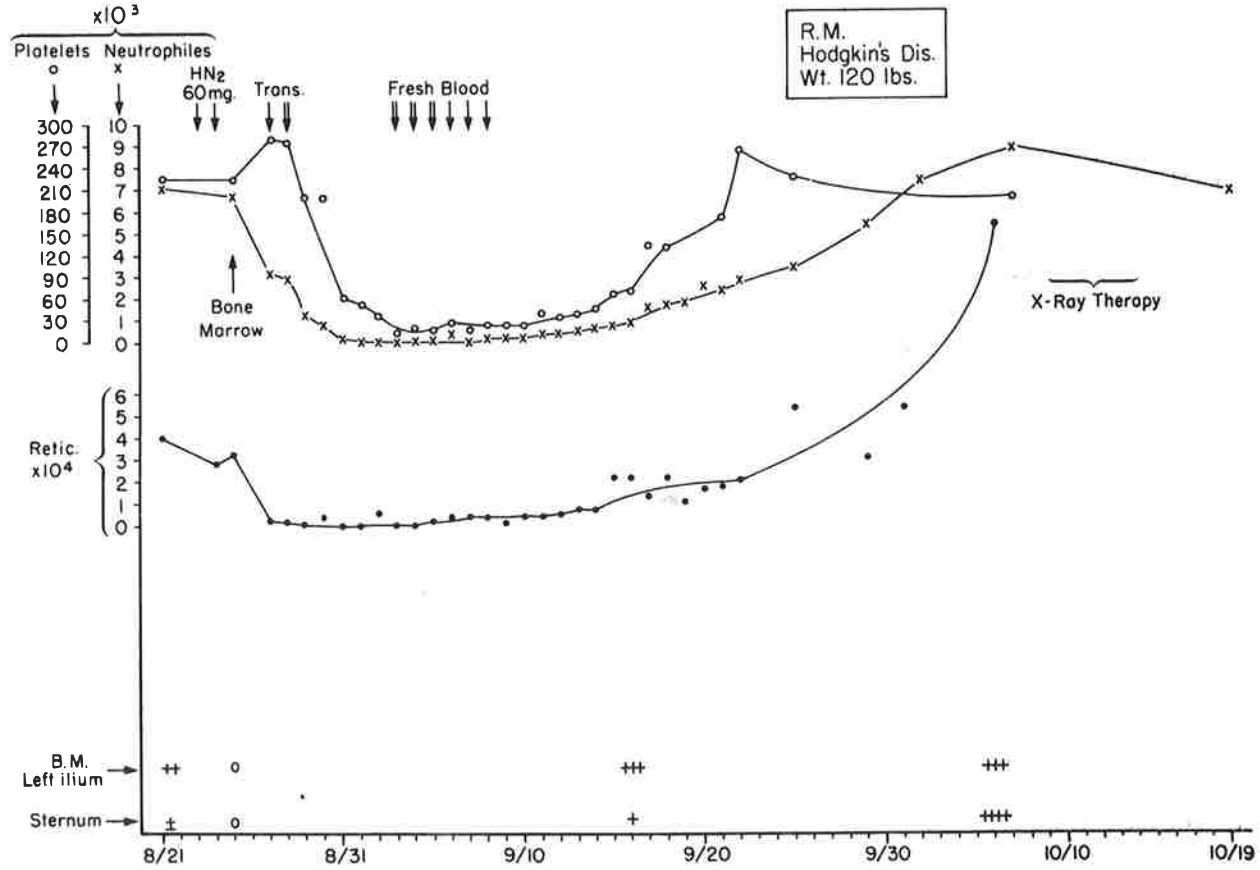

x10 3
Platelets
Neutrophiles
HN2 60mg.
Trans.
Fresh Blood
R.M.
Hodgkin's Dis.
Wt. 120 lbs.
Bone Marrow
X-Ray Therapy
Retic. x10 4
B.M. Left ilium
Sternum
8/21
8/31
9/10
9/20
9/30
10/10
10/19

Figure 2

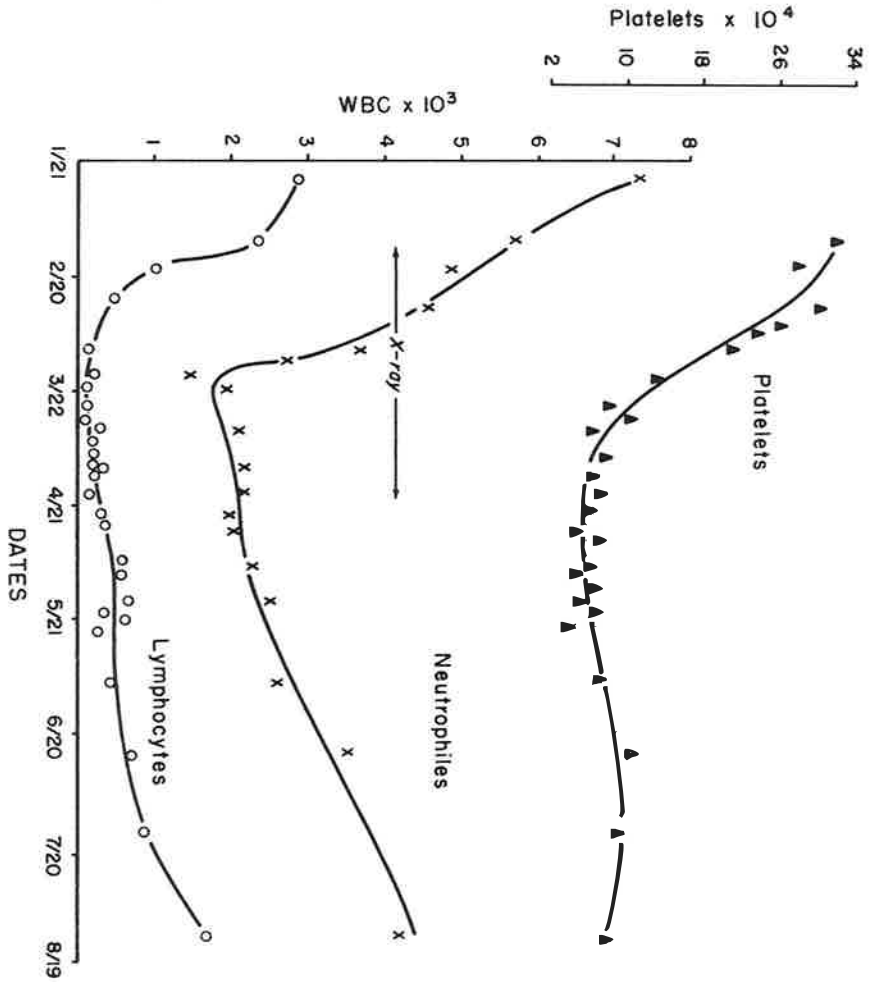

Platelets x 10⁴
2
10
18
26
34
WBC x 10³
1
2
3
4
5
6
7
8
X-ray
Platelets
Neutrophiles
Lymphocytes
1/21
2/20
3/22
4/21
5/21
6/20
7/20
8/19
DATES

Figure 3

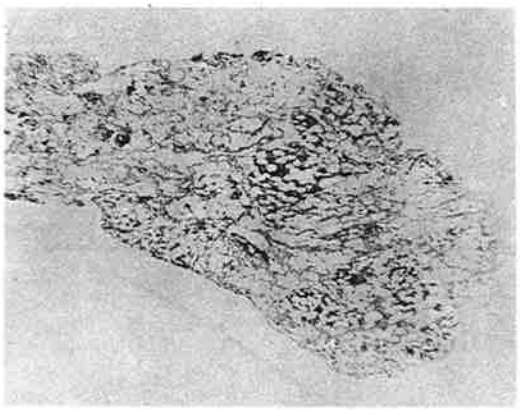


Figure 4

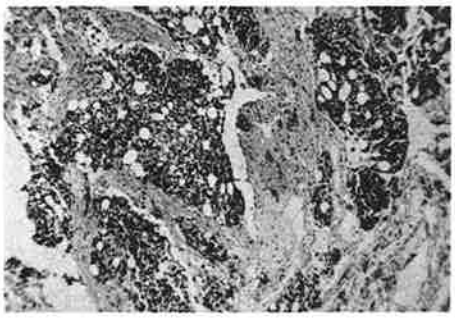


Figure 5

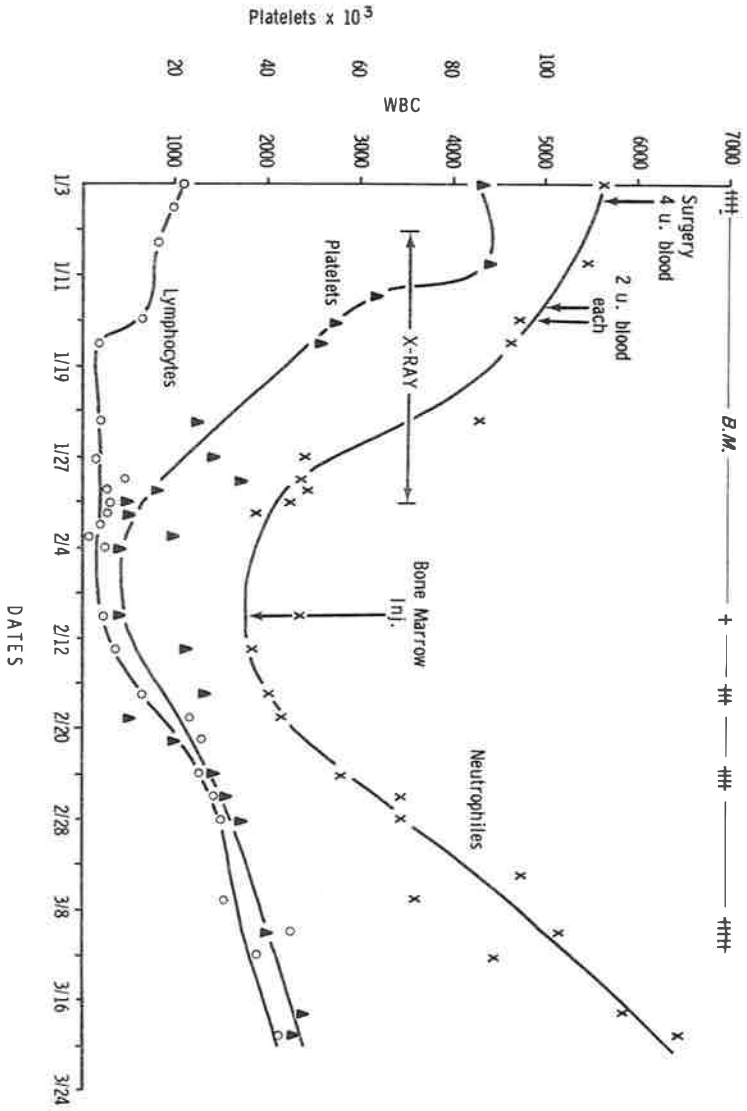

Platelets x 10³
WBC
20
40
60
80
100
1000
2000
3000
4000
5000
6000
7000
Surgery
4 u. blood
2 u. blood
each
X-RAY
Bone Marrow
Inj.
Platelets
Lymphocytes
Neutrophiles
B.M.
1/3
1/11
1/19
1/27
2/4
2/12
2/20
2/28
3/8
3/16
3/24
DATES

Figure 6

DIAGNOSIS	No. Collected	No. Infused
Seminoma	35	4*
Hodgkin's disease	13	3
Lymphosarcoma	10	
Embryonal carcinoma	9**	4**
Acute leukemia	7	2
Chronic granulocytic leukemia	5	1***
Bronchogenic carcinoma	4	
Renal carcinoma	4	3
Melanocarcinoma	3	2
Choriocarcinoma	2	
Adenocarcinoma of ovary	1	1
Breast carcinoma	1	
Carcinoid	1	2**
Esophageal carcinoma	1	
Ewing's sarcoma	1	
Fibrosarcoma	1	
Osteogenic sarcoma	1	
Reticulum cell sarcoma	1	
Sympathicoblastoma	1	
Tongue carcinoma	1	
Aplasia, Mesantoin, Tridione	1****	1****
Aplasia, etiology unknown	1****	1****
TOTAL	104	24

* Includes 1 patient treated twice with isologous marrow
** Includes 1 patient in whom the procedure was performed twice
*** Acute phase, terminal
**** Homologous bone marrow

Figure 7

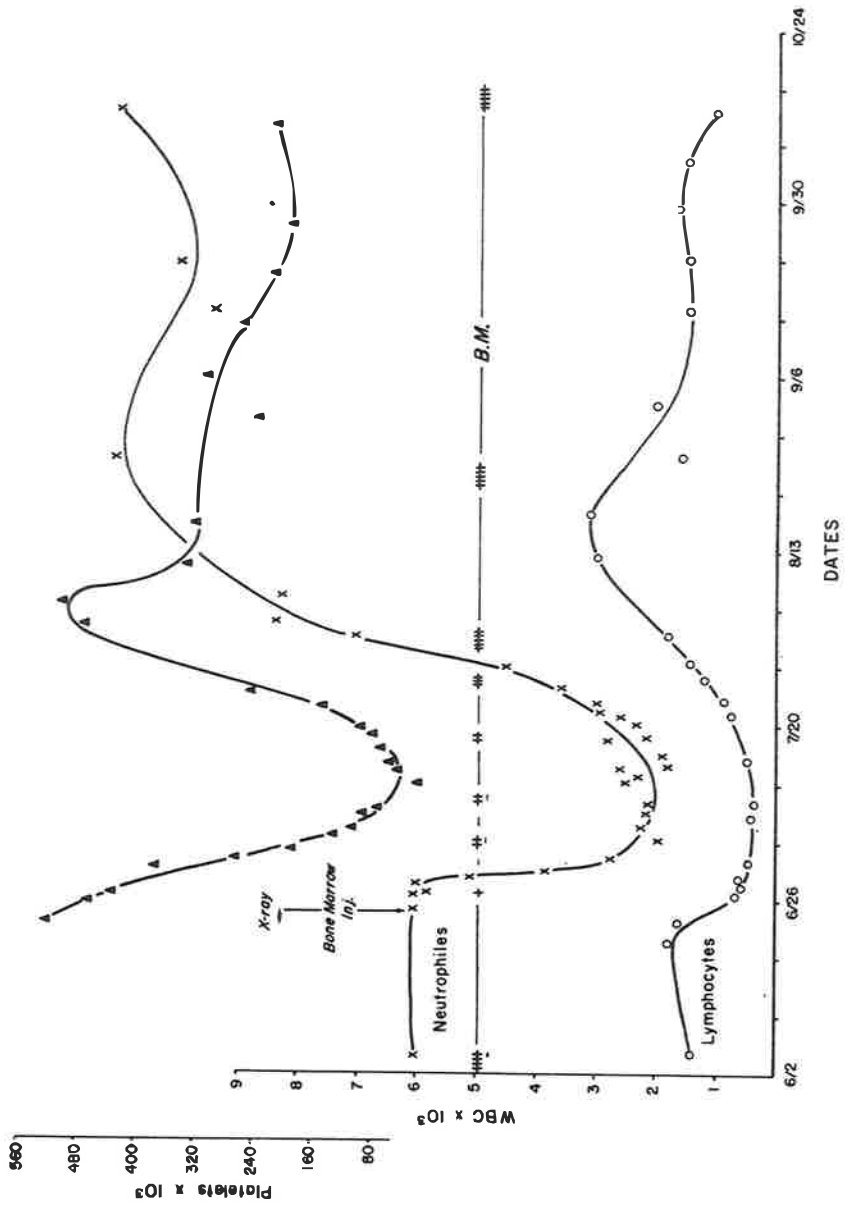

Platelets x 10³
560
480
400
320
240
160
80
WBC x 10³
9
8
7
6
5
4
3
2
1
X-ray
Bone Marrow Inj.
Neutrophiles
B.M.
Lymphocytes
6/2
6/26
7/20
8/13
9/6
9/30
10/24
DATES

Figure 8

DISCUSSION

HUMBLE I am very interested to hear from Dr. Kurnick that in cases where there was local hypoplasia following irradiation of part of the body without peripheral pancytopenia, he did not get repopulation of this area following the reinfusion of autologous marrow. We got repopulation of the sternal marrow following intensive irradiation of the chest.

KURNICK Dr. Humble, I do not think that there really is any discrepancy here. I think the difference is that in the acutely aplastic individuals where you have treated the chest massively you may have repopulation, but in those who have had an opportunity to stabilize with a long standing aplasia of 6 months to 2 years, our results have been negative as far as local repopulation is concerned.

KAY I question the validity of monkey experiments to test the mobility of the stem cell. How much of the marrow space is in fact hemopoietic? Of course in the human adult only quite a small portion is hemopoietic, whereas perhaps in the small animals there is much less reserve space available.

SCHOFIELD This is a question of comparing adults with children. Most of us were dealing with immature monkeys, where the situation is similar to that in children, but in adult monkeys the situation is just like in human adults.

KURNICK It would be interesting to know in monkeys, where better controls are possible, what the effects of local irradiation or of local shielding will be. If the monkey did happen to repopulate as the mouse, I suppose this would not prove anything about man. However if it failed to, it would probably give some indication that our conclusions in man have validity. Also, can buffy coat (peripheral blood) leucocytes repopulate bone marrow after lethal X-irradiation in the monkey?

BALNER What is the lowest dose where persistent aplasia does occur in man? At the International Symposium on the Reticulo-endothelial System, Gif sur Yvette, 18 - 22 June 1962 the role of the fixed reticulum cells was discussed. Could it be this is the dose where the rather radio-resistant reticulum cells are also killed? They might have a role in hematopoietic recovery.

KURNICK Complete local elimination of the stem cells even at very high doses in man does not appear to be the reason because even in the aplastic marrow 2 years later there were little islands of activity. As to what the maximum dose is in man, which will still permit repopulation, I do not really know.

VAN PUTTEN I think these studies on high dose local irradiation showing differences between mouse and man are very useful. However, there is another difference. Why do peripheral blood counts in man go down to very low figures after subtotal or even local high dose irradiation? I believe this does not occur in the mouse either.

KURNICK It does occur in the mouse. When you irradiated the lower extremities only of the mouse, you do get marked reduction in the cellularity of the marrow in the fore limbs.

VAN PUTTEN I think the crucial point is, why do you get aplasia in man after local or subtotal irradiation and I wonder whether anyone has got an answer for that?

KURNICK If you just irradiate locally you will not see aplasia. There may not be any indication for re-infusion in man and in fact we have not re-infused without what we consider dangerous pancytopenia. If you irradiate large volumes in man so that the remaining volume of bone marrow is such that it will be inadequate to maintain the need of the individual, then obviously there is indication for re-infusion.

MATHÉ I think there is evidence that the compensating power of the bone marrow of the human being may be less than in the mouse.
If we irradiate two times in the mouse the second irradiation will give the same effect as the first one. In man, we gave 100 r two months after 400 r and we had a more severe effect than the first one with 400 r and this may be an indication that the compensating power in man is much less than in the mouse.

CROUCH I think this indicates the recovery power of different species being quite different and in our laboratory now we are carrying on quite a large experiment using 11 different species and we see in larger animals Burros, goats and sheep) that the recovery time of the peripheral blood elements and the bone marrow is perhaps 20 to 40 times longer than that in the mouse, rat or hamster and this may be the same in man.

AMBRUS I do not really think that anybody has convincing evidence that if you apply only local irradiation even at high levels, you get generalized marrow aplasia. However, we all have evidence that patients who were treated locally with radiation and then later on subjected to systemic chemotherapy will respond with much more dramatic bone marrow depression to chemotherapy than you would expect in a previously non-irradiated population.

KURNICK I have not suggested and I do not believe that local irradiation in man produces generalized bone marrow depression. I have been concerned only whether there has been generalized bone marrow depression by generalized radio-therapy. The mouse has the advantage here I think, in the circulation of proliferative cells such that if there are a few proliferative cells at any place in the body, they can in time reseed all the marrow. Perhaps that is how the mouse can maintain its proliferative advantage in subsequent therapy more readily than man.

SCHOFIELD Perhaps some answers to these problems whether stem cells circulate in man may come out of the work of Lahjta (Lahjta, L. G., et al., Lancet 1, 353, 1962) in our laboratory on extra corporeal irradiation in man and from the Brookhaven group.

HUMAN APPLICATION OF BONE MARROW GRAFTING

J.G. HUMBLE. - Haematology Department
Westminster Hospital
England.

Haematological depression is well known to follow the treatment of humans by radiotherapy. In a previous study (Humble, Jayne, Pulvertaft and Wilson 1954) we showed that leucopenia and lymphopenia were much more likely to follow the radiation of the thorax and abdomen than the neck, the head and the limbs. In the case of the abdomen it has been possible to show that the physical nature of the radiation applied has a marked effect on the depression produced. The cases studied were all men undergoing prophylactic radiation to the pelvic and para-aortic lymph node fields after orchidectomy for seminoma or teratoma testis. The cases were only selected in that none showed evidence of palpable or radiologically obvious metastases elsewhere in the body. Two groups of 30 patients were studied. Group 1 were treated by 250KV X rays to a "centre dose" of approximately 2,500r to pelvic and lower abdominal fields followed 6-8 weeks later by a further course to a smaller upper abdominal field (Prossor 1950). Group 2 was treated by X rays generated by the 2 MeV. Van der Graaf generator. One treatment area covering the whole area desired was used. The centre dose here was approximately 4,500r. The results, (production of leucopenia) are shown in Table 1.

Table 1.

Production of Leucopenia.

Abdominal Radiation in two series of 30 cases.

A	250KV.	centre dose 3,000r 23.3%. (7 cases).
B	2 MeV.	centre dose 4,500r. 0.33%. (1 case).

It would seem therefore, that the type of radiation should be considered when the radiation of the human is being considered. What is clear, however, is the effect of radiation on red bone marrow. After 2000r of fractionated radiation to the skin local marrow hypoplasia was produced and recovery, sometimes incomplete was not found until 8-10 weeks later. (Stewart and Dische 1956, Stewart 1958) Denstad (1943) found that complete recovery was unusual when 3000r was exceeded. Sykes et al (1959, 1960) and also Stewart and Dische and Stewart (above) showed that after 4000r complete recovery is exceptional. With these results in mind, we have treated 10 cases with widely disseminated malignancy by radiation followed by autogenous (autologous) bone marrow therapy. 8 cases received whole chest radiation for multiple pulmonary metastases, 1 case, whole trunk radiation for widespread metastases and 1 case, whole body radiation for widespread metastases. In 8 of these cases, the radiation was preceeded by multiple aspirations of bone marrow cells and storage of these cells in 15% glycerol at - 80°C. In one case (whole body) the marrow was removed and held at room temperature until the radiation which was given the same day was finished (Case 9). In case 3, the radiation was followed

by intravenous injection of marrow freshly aspirated from the pelvis. The techniques used for aspiration, filtration and storage of bone marrow cells were those described by Pegg and Kemp (1960). In all cases the marrow suspensions were given intravenously after the radiation course was finished. These cases comprise the 10 cases treated by Autogenous (autologous) bone marrow infusions. Some of the data concerning these cases is given in Table 2. Note that radiation was given by the 2 MeV generator to all but Case 9 (200KV)

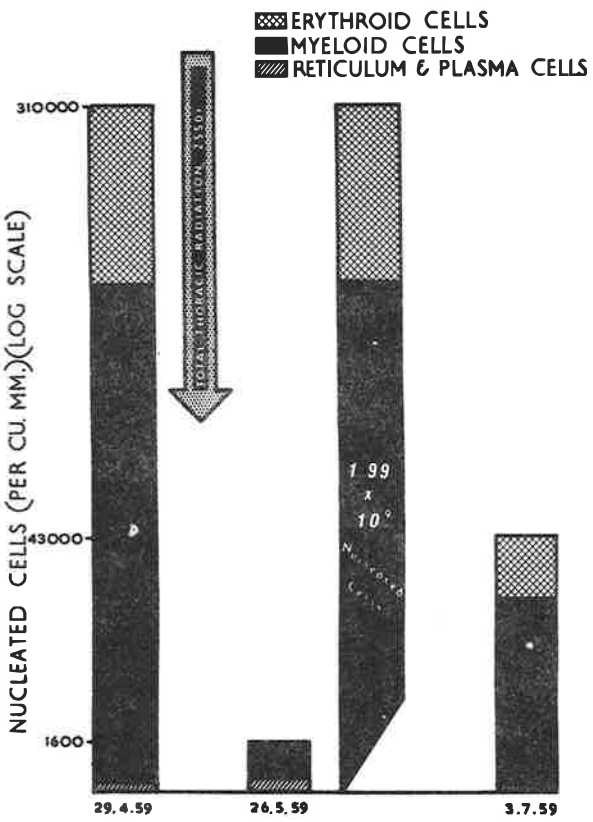
Table 2.

CASE	AGE	SEX	DIAGNOSIS	TOTAL RADIATION DOSE (r)	DAILY RADIATION DOSE (r)	TUMOUR REGRESSION	SURVIVAL TIME	CAUSE OF DEATH
1	21	F	Osteogenic Sarcoma	2,708	300	No	13 weeks	Radiation Pneumonitis
2	21	F	Ewings Tumour	2,900	300	Good	7 months	Radiation Pneumonitis
3	49	M	Seminoma	2,500	150	Fair	16 days	Broncho-pneumonia
4	19	M	Ewings Tumour	2,950	300	Good	6 months	Radiation Pneumonitis
5	20	M	Ewings Tumour	3,058	150	Good	2 years	Pulmonary Metastases
6	5	F	Nephro-blastoma	2,550	150	Good	Alive at 3 years	
7	52	M	Seminoma	2,550	150	Fair	12 weeks	Radiation Pneumonitis
8	8	M	Ewings Tumour	2,550	140	Good	8 months	Cerebral Metastases
9	17	M	Lympho-sarcoma	560	-	Good	9 weeks	Multiple Metastases
10	27	M	Reticulum cell sarcoma	300-500	-	Fair	20 days	Broncho-pneumonia

Haematological Phenomena.

In all cases the bone marrow as judged by previous sternal puncture was of normal cellularity. Where examined immediately afterwards (Cases 4-7) it was aplastic. Recovery had commenced after 2-3 weeks and was complete with the exception of Case 5. at 6-8 weeks (in cases where the marrow was examined). The dose of marrow cells infused varied from 3×10^9 to 133×10^9. The findings in case 6, a child of 5 are summarised in Fig. 1.

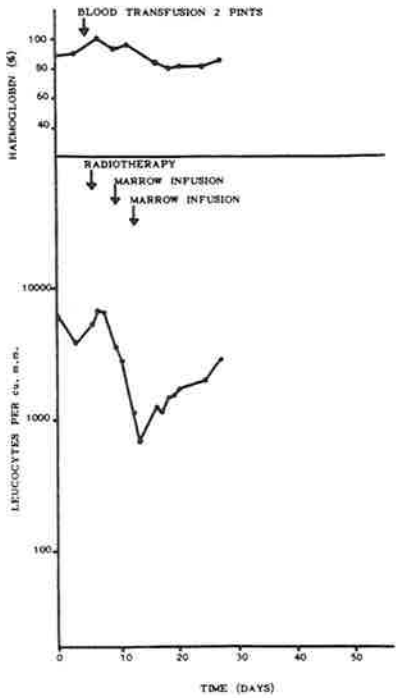
Fig. 1.



Bone marrow findings in Case 6. as judged by Sternal puncture. The pointed bar is the dose of cells re-infused.

Of the cases 1-8 only one showed mild leukopenia. Case 9. Whole trunk radiation to a centre dose of 560r (2MeV) showed severe leukopenia Fig. 2. but this recovered to normal limits in 18 days.

Fig. 2.



The blood counts in case 9

Case 10 receiving whole body radiation to a centre dose of 300-350r developed severe leucopenia which recovered in 18 days. According to the data of Mechanik (1926) and Custer (1932), the percentage of marrow radiated in Cases 1-8 was 32.6% of the total. In Case 9 it would have been 83.5%. We have concluded from the above cases that autologous bone marrow cells stored in the manner described are active in repopulating the marrow after considerable radiation damage.

Whole body radiation followed by Homologous Bone marrow in the Treatment of Acute Leukaemia.

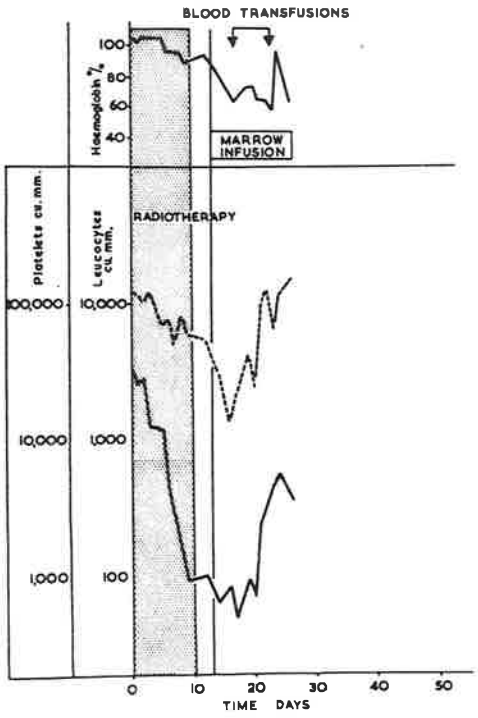
There would seem to be no doubt that the well known experiment of Barnes and his colleagues (Barnes, Corp, Loutit and Neal 1956) stimulated many workers to attempt to treat leukaemia in the human by radiation followed by homologous bone marrow cell infusions. Generally speaking it would be fair to say that no one has yet claimed to have eradicated human leukaemia by this method, although it has been shown that remissions of the disease sometimes lasting several months may be obtained. We have treated 5 cases by this technique. The first case in 1956, an adult of 48 years with monocytic leukaemia was given 1000r from the 2MeV generator, she was severely ill at the time of treatment and died 4 hours after its completion. A feature of some little interest was that the blood film showed numerous cells in mitosis before the radiation and after treatment chromosome abnormalities were readily seen in the blood film. The autopsy showed fluid blood throughout, massive submucosal small intestinal haemorrhage and a cerebral haemorrhage measuring 10 x 3 cms in the left parietal area.

Case 2. A boy of 8, with leucosarcoma was given uniform treatment to 500r in one treatment. He was given 40 x 10^6 marrow cells/Kg from his mother and at 14 days post radiation, he was given a foetal liver cell suspension by Dr. Kay. No improvement followed and he died at the 23rd day of bone marrow aplasia with active tumour tissue present.

Case 3. was treated for acute myeloid leukaemia to the same dose for another Hospital. He died 6 weeks later of haemorrhage without evidence of a graft.

Case 4. A girl, aged 7. Acute lymphatic leukaemia. Dosage, (centre dose) trunk 482r. Legs 616r. Treatment was stopped at this point because of shock, nausea and vomiting. 2 hours later she died after coughing up thick tenacious mucus. The autopsy showed much blood stained mucus in the bronchial tree with pulmonary collapse and blood stained pleural effusions. It seemed to us that it would be desirable to modify the treatment schedule bearing in mind the relatively high output rate (3r/min) of the 2 MeV generator at the target distance used (3 metres).

Case 5. A girl of 6 with acute Lymphatic Leukaemia. This patient was given 1,160r (centre dose) in 8 half body fractions, each half receiving 4 treatments at 2 day intervals. The equivalent single dose was calculated as about 860r. It was well tolerated. 3 days later 67 x 10^6 cells/Kg from the father were given. The progress of the blood counts can be seen in Fig. 3.



The blood count changes in case 5

It will be seen that the thrombocytopenia had recovered and the white cell count was rising when death occurred due to an overwhelming moniliasis. It was not possible to demonstrate leukaemic cells with certainty at autopsy. Thus, these cases, all of whom were only treated because conventional therapy no longer controlled the disease, demonstrate clearly the difficulties and danger of this method. At the present time we feel that not withstanding the inherent dangers of this type of treatment, the chief barrier to progress is the failure to eliminate the disease process. The work carried out by my colleagues, Dr. Hewitt and Dr. Wilson (Hewitt and Wilson, 1959 a) on a mouse leukaemia titrated in vivo shows clearly the response of these cells to radiation; a response shared by many other normal and malignant cells. These results show that there is a linear relation between the logarithm of the percentage of the number of cells which survive a given dose of radiation and the dosage administered. Furthermore if the cells treated are under anoxaemic conditions then more cells survive than if the cells are fully oxygenated. (Hewitt and Wilson 1959 b). These findings have led us to explore the prospects of treating widespread malignant disease by chemotherapy in large doses, a subject to be discussed at the later sessions.

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DISCUSSION

LOEB Have you ever used total body irradiation in acute leukemia in early cases?

HUMBLE No. All our cases were very late.

AMBRUS Dr. Humble, I am surprised about the large number of deaths you attribute in your series to radiation pneumonitis with doses as low as 2500 rads.

HUMBLE We considered this very carefully and we came to the conclusion that this may be a question of the type of radiation. These were very carefully iso-dosed and extremely uniformly irradiated in the chest with 2 MeV X-rays and as you notice the ones given 300 r a day uniformly got it, that is why we decreased the dose to 150 r a day.

KURNICK We also have irradiated the entire chest in doses of 400 to 600 r per siting using Co^{60} going to 6000 r. In the two cases in which we did this, we did not encounter radiation pneumonitis, although we also did not have a good effect on the bronchogenic carcinoma.

As regards the hemoglobinuria, the method of storage we use is not advantageous to the red cells. We have selected it to be advantageous to the nucleated cell rather than to the red cell; we therefore get marked hemolysis, and hemoglobinuria has been the rule rather than the exception and our urines are a good deal darker than the one that Dr. Humble showed. However, we have never seen rigor or any symptoms with our hemoglobinuria. Maybe it is where they buy their glycerol that makes the difference.

SCHOFIELD I wonder if Dr. Humble can give numbers of bone marrow stem cells rather than total bone marrow cells.

HUMBLE Dr. Schofield, what is a stem cell? Dr. W. Harrison (J. Clin. Pathol. 15, 254, 1962) who worked in our laboratory, found that cells which he clas-

sified as reticulum cells made up 0.7%; now include 1.3% of unidentified cells that's at most 2% stem cells. The total number of nucleated bone marrow cells per kg/body weight in man is about 12.8×10^9/kg.

INFUSION OF HOMOLOGOUS AND AUTOLOGOUS BONE MARROW IN THE TREATMENT OF A VARIETY OF MALIGNANT CONDITIONS

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The J. K. and Susie L. Wadley Research Institute and Blood Bank
Dallas, Texas

This paper is concerned with the infusion of homologous and autologous bone marrow into 44 cases of leukemia and other malignant conditions over a four-year period. Initially fresh homologous marrow transplantation into a group of cases of acute lymphocytic leukemia following preparation with isotopes was attempted. (Hill, Loeb, Smith, Speer, Denton, 1958) Unfortunately this isotope therapy and marrow transplantation had to be discontinued before completion of the planned series of clinical trials involving increasing dosages of isotope and later treatment of early rather than terminal cases. With this exception as a guide our investigation turned to the use of autologous marrow infusion, fresh and stored (preserved by freezing) in the treatment of various malignant diseases.

Since April, 1959 a total of 161 bone marrows from 32 cases of acute leukemia in remission and 129 other malignant conditions have been preserved by freezing.

Such stored bone marrow was returned to 38 patients and in 10 cases marrow was preserved a second time. In addition to observations on the clinical course and response of these patients, a preliminary report is included of the use of the in vitro stathmokinetic index as described by Astaldi (Astaldi, 1960) to give evidence of cell viability.

METHODS

In the earlier attempts to transplant marrow to 6 cases of acute lymphocytic leukemia fresh marrow was obtained surgically by curettement of ribs (2 cases) and ilium (4 cases) and given intravenously through a standard plastic transfusion set and filter. Preparation of these cases of acute leukemia with total body irradiation was done with P-32 (1 case), I-131 (1 case), and colloidal Y-90 phosphate (4 cases).

When preservation of autologous marrow was started, needle puncture of the ilium at several sites in the region of the posterior crest was adopted. The marrow was withdrawn into a series of 20 cc siliconed syringes containing 5 cc of Hanks solution plus 5% AB serum and E.D.T.A. 1% by volume. Ordinarily 2 bottles containing 100 cc of marrow and Hanks solution (ratio 3:1) were taken. Nucleated cells varied from 45 million to 2.5 billion.

Immediately after collection, the whole bottle was centrifuged at 1500 rpm for ten minutes and the supernatant aspirated and discarded. A mixture of 30% glycerol and 70% AB serum was added to the packed cells in equal volume and mixed. Refrigeration at 4° centigrade for one hour was followed by -20° degrees overnight. Storage was on dry ice or under liquid nitrogen.

For infusion the marrow was thawed at 37° centigrade, then 1/2 volume of 50% glucose in normal saline was added. Ten minutes later normal saline equal to twice the total volume of the marrow preparation was added. This final preparation was promptly given by vein.

For testing viability of cells, vital staining as described by Schrek (Schreck, Donnelly, 1961) and phase contrast studies were made. Because these studies were rather inconclusive, we adopted the technique of short term cell culture and use of a modification of the in vitro stathmokinetic index (Astaldi, 1960).

In the earlier series of cases of acute leukemia a new approach to total body irradiation prior to transplantation of homologous bone marrow was adopted at the Wadley Research Institute. Diffuse uniform irradiation of the body was attempted with P-32 and I-131 in the first two cases and in the other four cases a colloidal preparation of Y-90 was developed for intravenous use to provide great concentration of radiation with reticulo-endothelial distribution.

In the case of P-32 and I-131 a solution to the problem of removal of the irradiation prior to marrow infusion was attempted as follows. The patient treated with P-32 (25 Mc) received 8 to 10 litres intravenously of phosphate buffer daily and massive doses of parathyroid extract to accelerate elimination of the isotope from the body. The I-131 (500 Mc) was given intravenously to Case 2 after prior blocking of the thyroid with lugols solution. Elimination of the I-131 through the urine was held back by administration of pitressin for twenty-four hours and then rapidly eliminated by pushing fluids.

The four cases of acute lymphocytic leukemia receiving Y-90 were given 2-1/2, 3, 3-1/2 and 4 Mc of the colloidal phosphate per kilo of body weight respectively. With this colloidal preparation rapid elimination

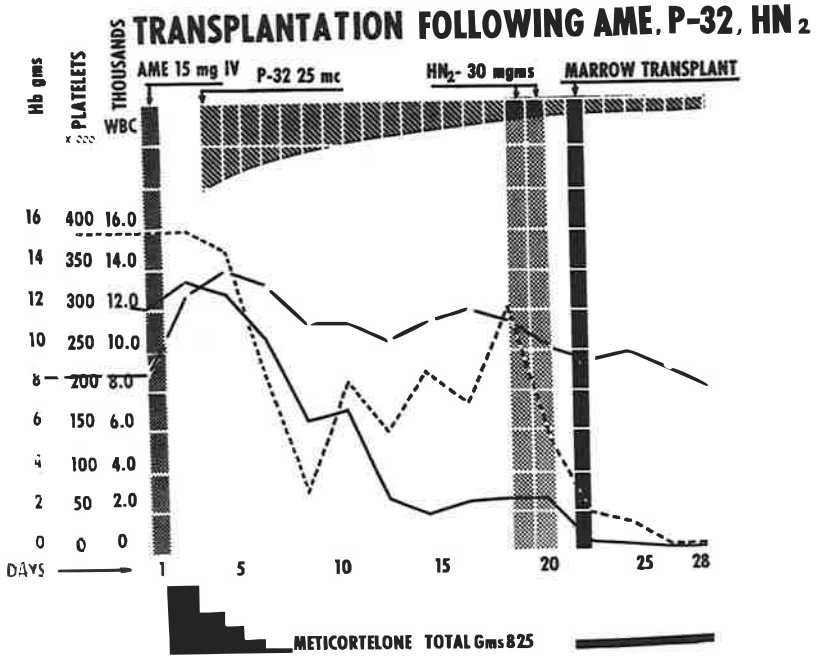
was impossible. However, rapid decay of this isotope was relied upon to reduce the radiation to negligible levels (?) at five or six half life periods.

In the second phase of our study when autologous marrow was stored, a variety of chemotherapeutic agents including amethopterin, alkylating agents, vinblastine, P-32 and AB-100 were employed, usually in super dosage. The preserved bone marrow was returned if the patient developed severe depression of any or all of the peripheral blood elements (e.g. platelet count less than 20,000, leukocyte count less than 2,000). Bone marrow aspiration was used frequently to confirm the hypoplasia, especially in acute leukemia. During the period of replant the patient usually received steroid therapy, blood transfusion and antibiotics as indicated.

RESULTS

In the transplant series of acute lymphocytic leukemia, despite the intention to use super dosage of isotopes there was doubt that lethal or sub lethal levels of irradiation were attained. In case 1 levels of peripheral corpuscles after reaching a low point were rising on the 14th to 18th day when nitrogen mustard was given and marrow infused as shown in Slide 1. Insufficient acceleration of P-32 elimination by phosphate loading and parathyroid extract resulted in too slow a diminution of radiation even though the total irradiation appeared insufficient for the preparation of patient for transplantation. In Case 2, the super dose of 500 Mc of I-131 yielded a total body irradiation equivalent to about 300 R. Surprisingly, this dose was followed by rapid enlargement of lymph nodes and

abrupt elevation of leukocyte count (lymphoblasts). Thiotepa 100 mgms was given intravenously on the thirteenth day after the irradiation followed by three marrow transplants as shown in Slide 2. Death occurred on the twenty-ninth day.



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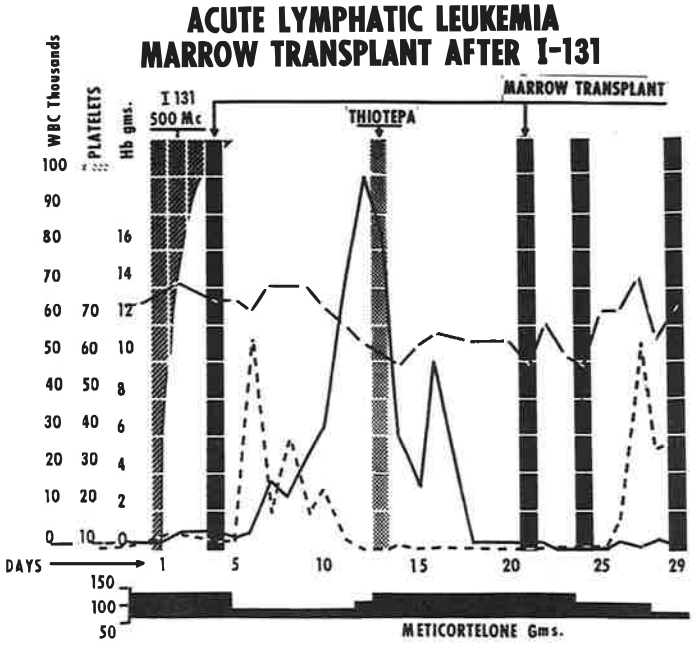
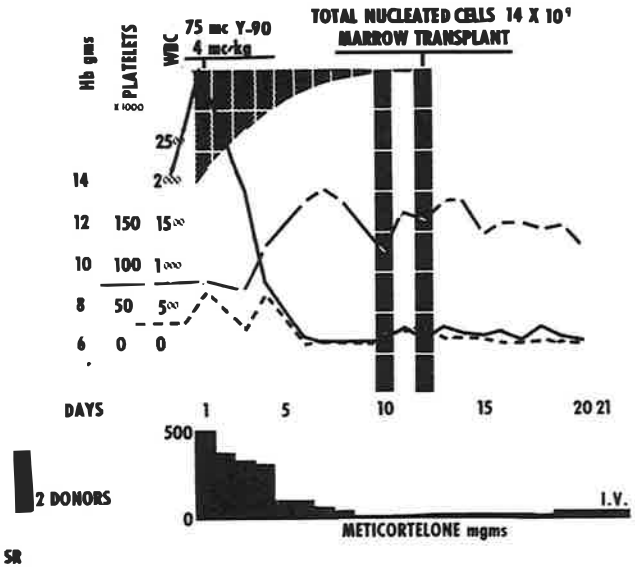

ACUTE LYMPHATIC LEUKEMIA
MARROW TRANSPLANT AFTER I-131
I 131
500 Mc
THIOTEPA
MARROW TRANSPLANT
WBC Thousands
PLATELETS
Hb gms.
100
90
80
70
60
50
40
30
20
10
0
70
60
50
40
30
20
10
0
16
14
12
10
8
6
4
2
0
DAYS
1
5
10
15
20
25
29
150
100
50
METICORTELONE Gms.

ACUTE LYMPHATIC LEUKEMIA MARROW TRANSPLANT AFTER Y-90



The 4 cases of acute lymphocytic leukemia, all small boys, who received the colloidal Y-90 failed to achieve definite transplant. Slide 3 illustrates the case receiving the maximum dose of 4 Mc per kilo. From autopsy the beta irradiation received by the principal tissues could be estimated. The dosage of irradiation in rads for this case was as follows: bone marrow -9480, spleen -6480, liver -5480, kidney -4320, lungs -208, muscle -68, brain -24. All died in 18 to 23 days but surprisingly none suffered any significant nausea or vomiting; all showed normal findings before, during and after irradiation with respect to blood urea, sugar, chlorides, sodium, potassium, carbon dioxide combing power, serum protein and urea (except in last case, urea became 67 mgs % post

irradiation. Serum transaminase and plasma electrophoretic pattern were normal before and after irradiation. Uric acid excretion was elevated 8 - 10 times normal in the 24 hours following irradiation.

Table 1 shows the condensed results of the autologous bone marrow replants in 38 cases of malignant disease. The criteria of recovery were elevation of platelets to 60,000 per cu. mm. or above, leukocytes 3,000 or higher, and survival 3 weeks or longer. In 10 cases bone marrow recovery was so complete that a second storage of the marrow was performed. One case not included in the recovery group because of the slow return of marrow function nevertheless lived 384 days after the replant. The period of time required for recovery of satisfactory levels of the platelets and leukocytes varied from 7 to 38 days with an average of 16.4 days, a figure approximating that given by Kurnick et al (Kurnick, Feder, Montano, Gerdes and Nakamura 1959). Length of storage varied from 6 days to 954 days and did not appear to correlate in any way with the success of the replants. Survival after the infusion of autologous marrow varied from 22 to 921 days with an average of 162 days. Especially interesting to us were the 5 cases of leukemia recovering apparently normal or near normal marrow function after the replant of cells taken for storage during a remission.

AUTOLOGOUS MARROW REPLANTS

DX	RECOVERED	NO MARROW RECOVERY	TOTAL
LEUKEMIA	5 b,c	4	9
OTHERS	16 a,b,c	13	29
TOTAL	21 b	17	38

a-10 CASES HAD 2nd PRESERVATION
b - LIVED 22 TO 900 DAYS
c - STILL ALIVE - 2 LEUKEMIA

Table 2 shows preliminary results of the use of an in vitro stathmokinetic index to give evidence of viability of stored cells. Our normal controls showed 0.9 to 1% mitotic figures after one hour incubation. This figure compares closely with the normal found by Fliedner et al (Fliedner, Cronkite, Bond, Rubini and Andrews, 1959) and Japa (Japa, 1942) on counting normal marrow without incubation in the presence of mitotic inhibitors. Studies of the stathmokinetic index with longer incubation are underway.

VIABILITY STUDY

STATHMOKINETIC INDEX - 1 hr. INCUBATION

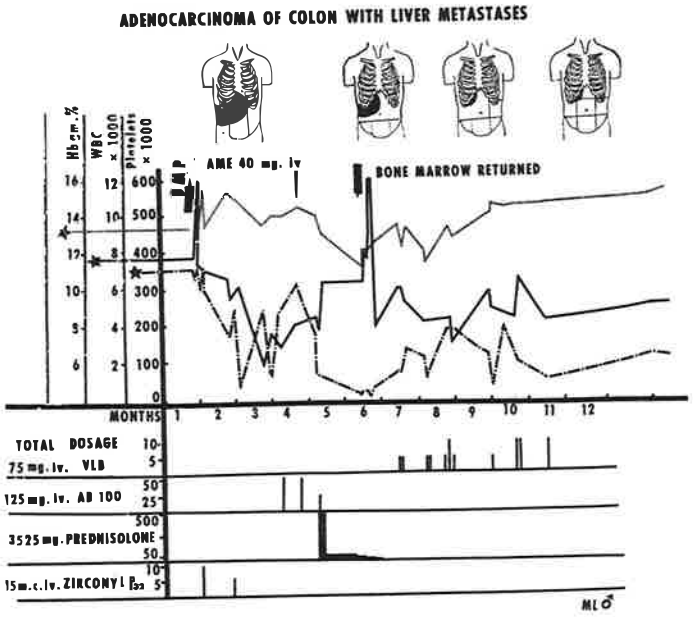
	%	No.
NO MITOSIS	27.7	5
NORMAL RANGE	11.1	2
MITOSIS (0.05-0.5 %	61.1	11
	100 %	18

BASKET CELLS 4 EOSINOPHILIA 8

Case 1 is illustrated in Slide 4. Mr. M. D. L., white male, age 54, had a diagnosis elsewhere of carcinoma of colon with extensive liver metastases and was considered inoperable and untreatable following failure of two courses of nitrogen mustard. This patient was first seen on our service April 4, 1960. Following storage of bone marrow the patient was treated with actinomycin D and zirconyl phosphate P-32 intravenously in two courses and in June, 1960 he received two courses of AB-100* and a month later he received Amethopterin. He developed thrombocytopenia

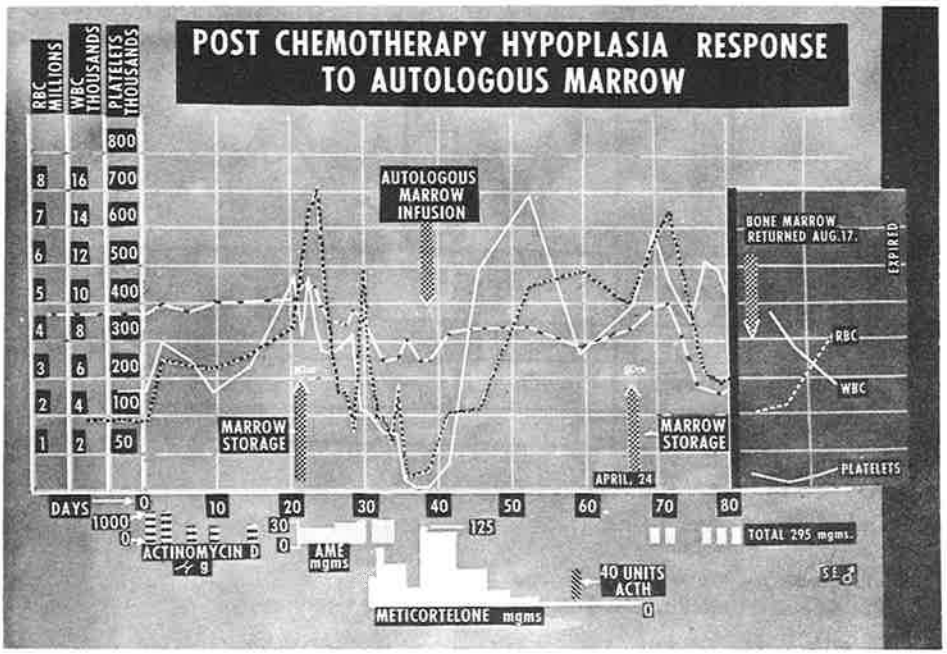
*Ethyl N-[Bis (ethylenimido)phosphoro]carbamate, Armour and Company

with hemorrhage and received Meticortelone* therapy. His stored bone marrow was infused, with platelet count returning to normal in about three weeks. Even though the therapy used caused marrow hypoplasia the patient lived on into the period when Velban** became available to us in September, 1960. On Vinblastine therapy this patient became symptom free, returned to work and only recently after 27 months has developed pain and enlargement of liver again.

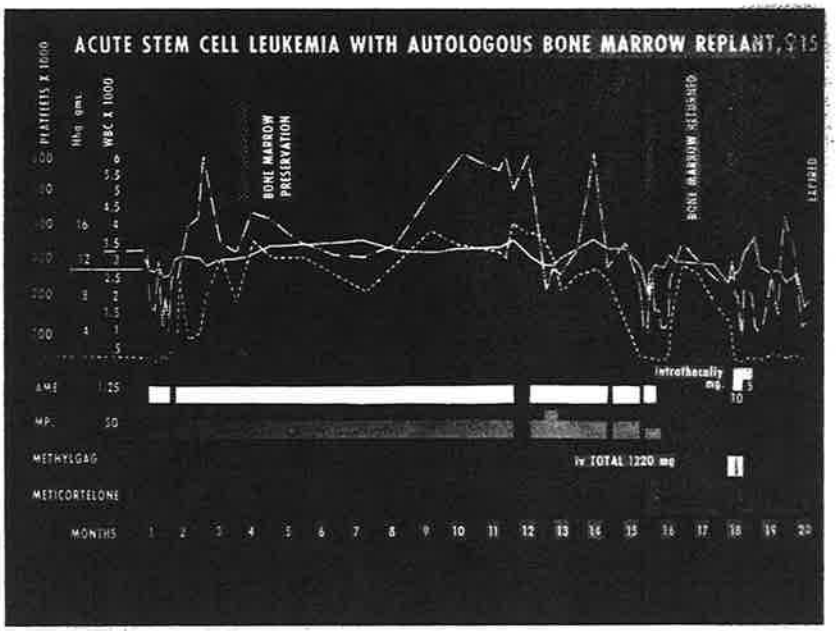


*Prednisolone, Schering & Corp.
**Vinblastine Sulfate, Eli Lilly & Company

Case 2 shown in Slide 5 was a 15 year old white male with rhabdomyosarcoma of the testicle with pulmonary metastases treated with massive Amethopterin following Actinomycin D. Although the extensive metastases in the lung cleared almost completely, infusion of the patient's stored marrow was required because of severely depressed platelet and leukocyte counts. Very rapid return of these counts to normal followed. A second marrow storage was done and again reinfusion was performed four months later with some elevation of platelet count but patient died three days after this second infusion and 161 days after the first replant. Amethopterin and nitrogen mustard proved ineffective in this terminal phase.



Case 3 illustrated in Slide 6 is of interest because it represents the potential benefit of marrow storage in the phase of good remission in acute leukemia. This 15 year old white female was diagnosed stem cell leukemia probably granulocytic type. The first remission was achieved on combination of amethopterin mercaptopurine and massive steroids and bone marrow was stored. After approximately 12 months in remission the bone marrow became hypoplastic with no apparent cause. Upon infusion of patient's bone marrow, platelets and leukocytes rose to satisfactory levels. Two months later the spleen had become very large, the peripheral leukocyte and platelet counts became very depressed but the bone marrow was full of leukemic blast cells. A second reinfusion of the patient's marrow was ineffective.



DISCUSSION

We believe the experience with autologous bone marrow replant has improved our treatment of malignant conditions, including leukemia. The more massive chemotherapy based in part on the additional sense of confidence insured by the stored marrow has given generally improved results. More aggressive therapy with newer agents has yielded surprisingly effective response in some cases, especially when the terminal nature of these cases is considered.

The data do not give any definite evidence of survival and successful take of replanted cells. Further study of viability of cells with various stathmokinetic techniques gives promise of determining whether stored cells survive but would not give proof of "take." Rate of recovery after hypoplasia of marrow, especially as emphasized by Kurnick (Kurnick, 1959) in the case of irradiation depression suggests "take" of stored marrow. Of course, there is also the possibility of a factor or factors from non viable cells (nucleic acid, etc.) may stimulate marrow recovery.

In acute leukemia the use of autologous marrow has more limited objectives. Certainly cells stored from even the most complete remission cannot be considered leukemia free. Furthermore, apparently only with hypoplastic marrow can any benefit from replant be expected. With the marrow full of primitive leukemic cells, no take is expected and none was seen in our experience. However, close clinical and laboratory observations lead us to believe that additional survival time was obtained for the five cases of acute leukemia considered as showing good response to infusion

of their stored marrow. We estimate that approximately 2-1/2 months survival may have been added to this small group. Although homologous marrow transplantation following total body irradiation has thus far failed to cure a human case of leukemia, continuing research concerning the conditions for a successful take (Thomas, Herman, Greenough, Hager, Cannon, Sahler, Ferrebee, 1961) (Billingham, Brent, 1959) and prevention of secondary disease may still make such treatment feasible. In this connection we regret that our planned sequence of increasing doses of radioisotopes especially colloid Y-90 had to be abruptly terminated when our license for this therapy of terminal cases of acute leukemia was not renewed. Differential tissue dosage of radiation with greater concentration on the basis of reticuloendothelial distribution still seems to us to offer a rational approach to eradication of leukemic cells as well as temporary depression of immune response. It is unfortunate that our studies of the radiation dosages in different tissues at still higher levels and correlation with clinical functional studies could not be completed. Whether or not such treatment should be tried in earlier phases of leukemia would depend on obtaining this additional data from terminal cases.

SUMMARY

1. An attempt of homologous bone marrow transplant following total body radiation in six patients with acute leukemia has been presented. The results have been disappointing but it is felt that further exploration of this method of treatment may be worthwhile.

2. A total of 38 autologous bone marrow replants were performed with satisfactory response in 21. There is still the question as to whether this is a replant or recovery. Regardless of this, autologous bone marrow preservation has given us more confidence in using more massive chemotherapy in malignant disease and leukemia.
3. A method of determining viability through the stathmokinetic index has been evaluated and will be investigated further by our group.

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DISCUSSION

BALNER Since you are in effect labelling the host very heavily, did you use autoradiography at any time to distinguish between host and donor cells?

LOEB No, we did autoradiographs to determine radiation doses only. The doses of irradiation were for instance for the bone marrow 9480 rads, the spleen 6480, the liver 5480, the kidney 4320, and the lung only 208, muscle 68 and brain 24 rads.

VAN PUTTEN Dr. Loeb, you use EDTA as an anti-coagulant. Is there a specific reason for this?

LOEB We found that EDTA did not injure the red cells as much as heparin but I cannot be definite because we did not study the two groups against each other; however, we did not have the hemolysis noted by others.

BONE MARROW TRANSPLANTATION AND CHEMICAL PROTECTION AGAINST ALKYLATING AGENTS IN THE THERAPY OF NEOPLASTIC DISEASES[1]

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From The Roswell Park Memorial Institute and the University of Buffalo Medical and Graduate Schools, Buffalo, New York

Bone marrow and chemical protection offer hope for the future in the treatment of radiation sickness. At present, however, immunologic barriers, the lack of radiation protective agents with a high therapeutic index in man and the necessity to administer chemical protective agents before radiation render these procedures impractical. However in the elective treatment of neoplastic disease autologous bone marrow protection may allow administration of higher doses of radiation or chemotherapeutic agents. Selective chemical protection of certain areas of the body may allow delivery of higher therapeutic doses of radiation or chemotherapy to the tumor. The present studies were undertaken to investigate these possibilities.

1. Supported by grants from the National Institutes of Health, U.S. Public Health Service and The American Cancer Society.

Table 1 shows the protective effect of bone marrow transplantation against intravenously administered nitrogen mustard in mice.

Table 1

EFFECT OF INTRAVENOUS HOMOLOGOUS BONE MARROW

(10×10^6 cells per animal) ON NITROGEN MUSTARD TOXICITY IN SWISS MICE

HN_2 DOSE	% DEAD	
mg/kg (i.v.)	HN_2 ALONE	BONE MARROW ½ HOUR AFTER HN_2
5.0	0	10
6.0	20	10
7.0	40	30
8.0	100	40
9.0	100	40
10.0	100	90

56174B

In this experiment marrow was injected half hour after nitrogen mustard. Table 2 shows a similar experiment in which bone marrow was transplanted 24 hours after nitrogen mustard. In both instances significant protection is apparent. Table 3 shows an experiment in which homologous bone marrow transplantation proved to be protective against and LD50 and LD100 dose of nitrogen mustard in monkeys.

Table 2

EFFECT OF INTRAVENOUS HOMOLOGOUS BONE MARROW

(10 x 10^6 cells per animal) ON NITROGEN MUSTARD TOXICITY IN SWISS MICE

HN_2 DOSE	% MORTALITY	
mg/kg (i.v.)	HN_2 ALONE	BONE MARROW – 24 HOURS AFTER HN_2
6.0	50	50
7.0	80	80
8.0	100	70
9.0	100	90
10.0	100	80

Table 3

EFFECT OF BONE MARROW TRANSPLANTATION ON LETHALITY OF NITROGEN MUSTARD IN 2.5 - 3.5 KG. RHESUS MONKEYS

Nitrogen Mustard Dose	1 mg/Kg.		2 mg/Kg.	
No. of homologous bone marrow cells transplanted	None	0.1 x 10^9	None	0.1-1 x 10^9
No. Survived/Total No.	2/4	3/4	0/6	5/6
Mean time of death of nonsurvivors	7 days	1 day	6 days	6 days
Survivors were observed for	24 months	24 months	-	24 months

Fig. 1 and Fig. 2 show the leukocyte and platelet levels respectively of control and bone marrow protected monkeys after the injection of lethal doses of nitrogen mustard. Leukocyte and platelet counts decrease almost indistinguishable in the control and bone marrow protected animals. However, control animals die when they reach low levels while part of the bone marrow protected group seems to be able to survive with low leukocyte and platelet counts and eventually recover. Hematologic values of bone marrow treated animals which died are not shown since their findings are almost identical with those of the controls.

Figure 1

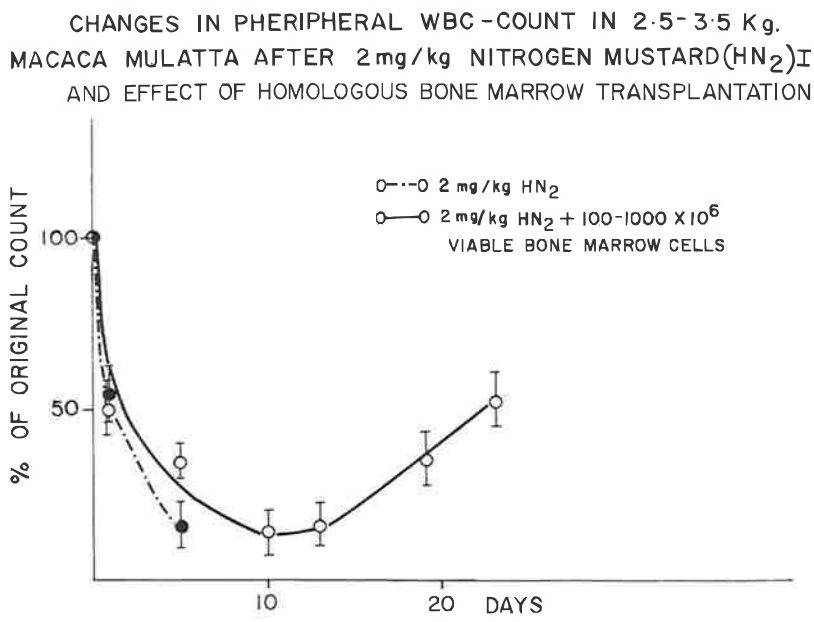
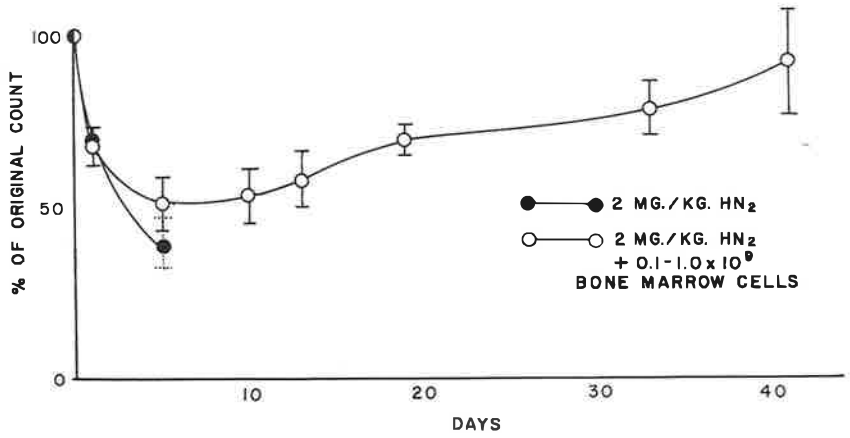


Figure 2

CHANGES IN PLATELET COUNTS IN 2.5-3.5 KG. MACACA MULATTA AFTER 2 MG./KG. HN_2, I.V., FOLLOWED BY HOMOLOGOUS BONE MARROW TRANSPLANTATION



A cautious clinical study was undertaken to investigate the possibility of autologous bone marrow protection in the treatment of neoplastic diseases with alkylating agents. A new alkylating carbamate (Bardos et al, 1959, Razis et al 1961) was used in these studies since this compound was shown to be effective against a number of solid tumors which seldom produce demonstrable bone marrow invasion. This agent has little gastrointestinal toxicity. Its chief side effect is hemopoietic depression. Bone marrow was removed from the iliac crest prior to the intravenous administration of a single large dose of AB 103. Marrow was stored in a tissue culture medium of 4^{o} C and reinjected usually 24-48 hours after administration of the drug. Attempts were made to determine the "takes" of autologous bone marrow transplantation by studying the leukocytosis promoting effect of Pyrexal, a commercially available bacterial lipo-

polysaccharide. Fig. 3 shows leukocytosis in response to Pyrexal in a normal individual.

Figure 3

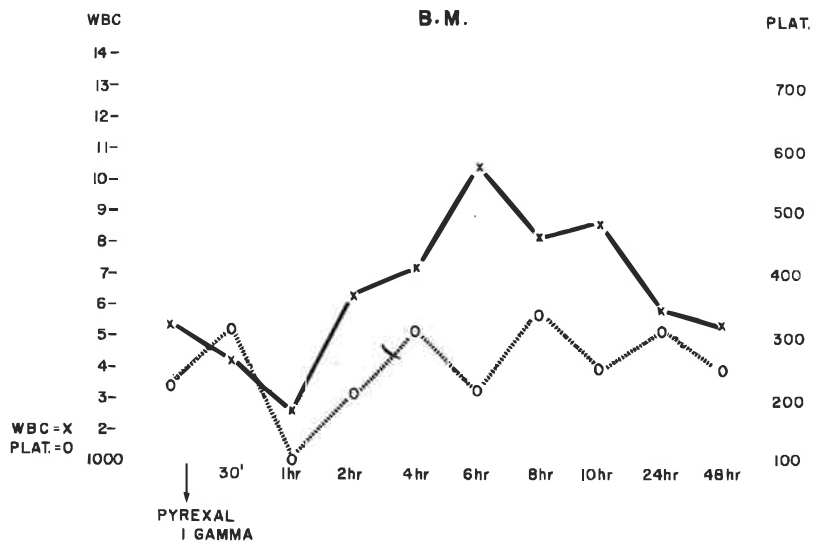


Fig. 4 shows data of a patient with inoperable squamous cell type bronchogenic carcinoma who recived a single large dose of AB 103 followed by reinjection of autologous bone marrow. There was marked objective decrease of tumor size and significant subjective improvement. After a period of thrombocytopenia and leukopenia, leukocyte and platelet counts returned essentially to normal.

Figure 4

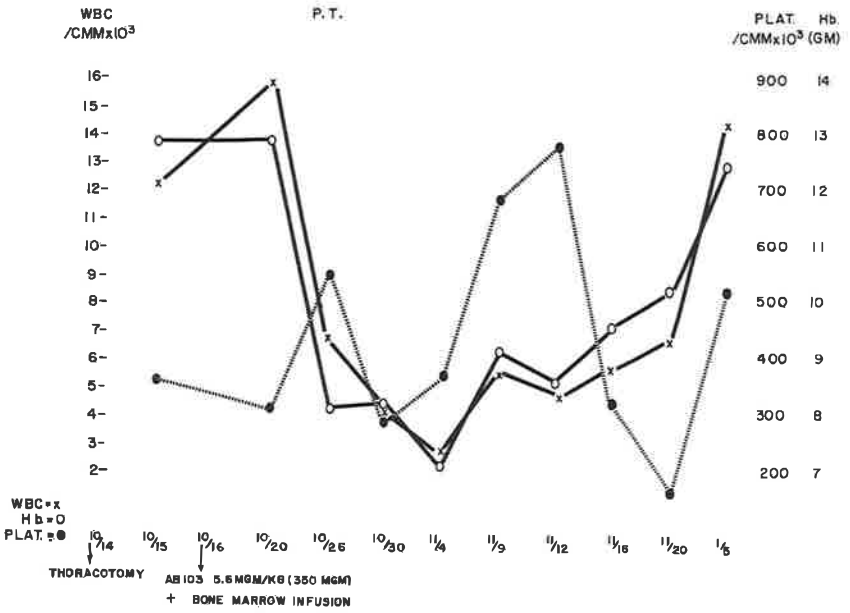


Fig. 5 shows the Pyrexal test in this patient after completion of nitrogen mustard therapy and bone marrow transplantation. This appears to be in the same order of magnitude as that of healthy individuals.

Figure 5

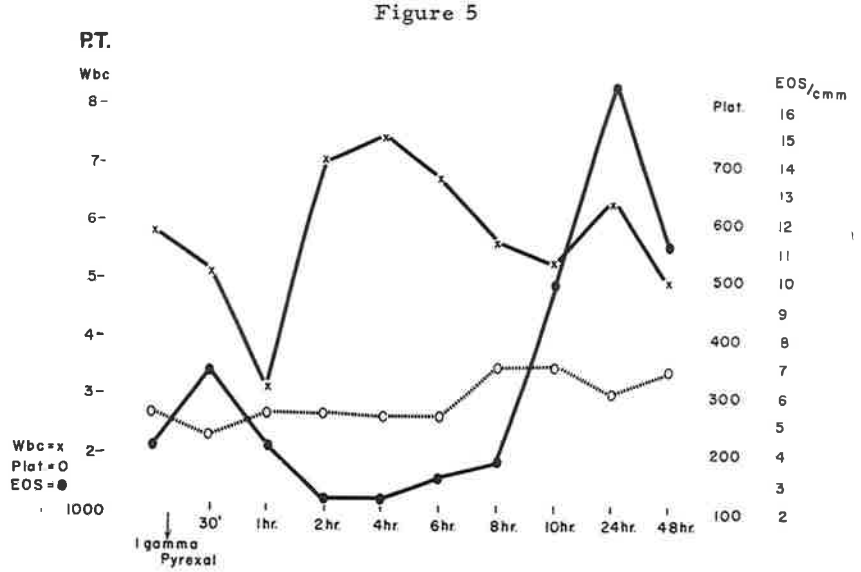
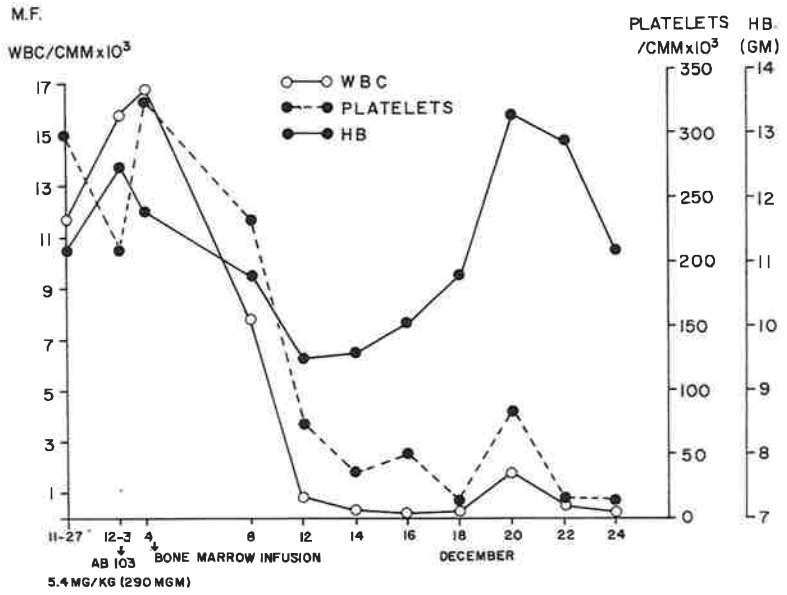


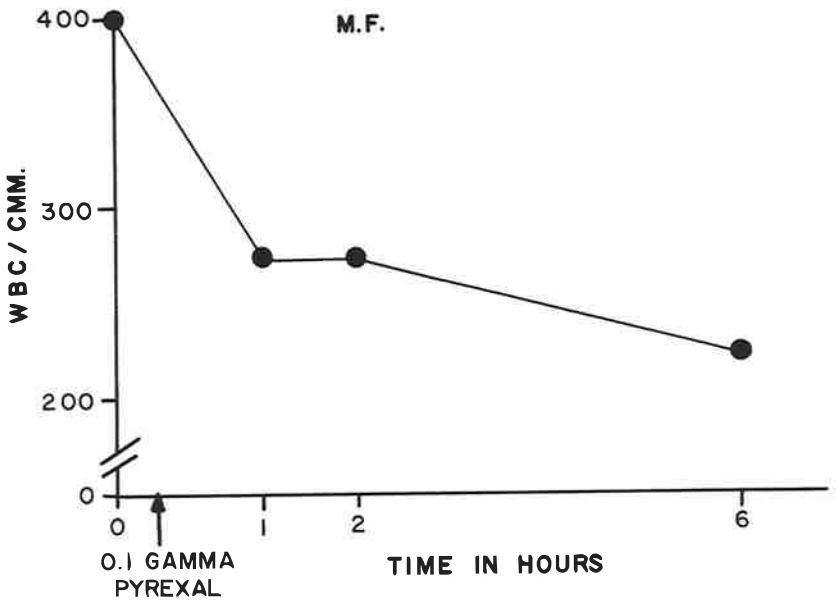
Fig. 6 shows a similar study in a patient who failed to exhibit hemopoietic recovery.

Figure 6



The Pyrexal test (Fig. 7) indicated no remaing functional marrow. We are presently exploring whether this test is suitable to estimate bone marrow reserves and to decide whether patients are ready for a second course of chemotherapy.

Figure 7



Chemical antagonists of alkylating agents may be used to localize alkylating effect into areas where this is preferred while protecting the rest of the body from toxicity. Table 4 shows an experiment in which Swiss mice were injected intraperitoneally with nitrogen mustard. Aminoethylisothiuronium (AET) given intravenously protected the host against alkylating agents injected intraperitoneally. Nitrogen mustard was injected intraperitoneally into mice with various types of ascites tumors while the animals were treated intravenously with AET.

Table 4

ALTERATION OF HN2 TOXICITY BY AET IN SWISS MICE*

HN2, mg./kg.†	% mortality			
	HN2 only (1)	AET adm. just prior to HN2 (2)	AET adm. 5 min. aft. HN2 (3)	AET adm. 15 min. aft. HN2 (4)
2.00	0	10	0	0
3.03	70	30	20	60
4.60	80	50	60	70
7.00	100	100	100	100

*HN2 was given intraperitoneally; AET was given intravenously in doses of 50 mg./kg.

†The median lethal dose of HN2, in milligrams per kilogram of body weight, was: (1) 3.03; (2) 3.90; (3) 4.06; (4) 3.30. For (1) vs. (2) the mean difference was 0.87; the standard error was 0.0579; t was 15.03; and $P<0.001$. For (1) vs. (3) the mean difference was 1.03; the standard error was 0.0529; t was 19.46; and $P<0.001$.

Table 5 shows that this procedure will prolong the life of mice inoculated with Ehrlich ascites tumor (Clone E-2).

Table 5

MEDIAN SURVIVAL TIME IN DAYS OF SWISS MICE WITH EHRLICH ASCITES TUMOR CLONE E-2

HN2 dose, mg./kg.	Days of survival							
	Treat. given 3d day after transplant.				Treat. given 6th day after transplant.			
	Control	AET	HN2	HN2+AET	Control	AET	HN2	HN2+AET
0.00	11.0	11.5	...	...	11.0	11.5	...	...
2.00	...	...	23.0	33.0	...	...	18.3	20.0
3.03	...	...	20.0	31.0	...	...	18.5	34.0
4.60	...	...	9.2	10.0	...	...	12.0	24.5
7.00	...	...	8.2	8.2	...	...	10.2	12.3

Table 6 shows a similar experiment with $6C_3HED$ Gardner lymphosarcoma. There is significant prolongation of survival time and in the group receiving 2 mg./Kg. nitrogen mustard together with AET, 90% of the animals survived for the observation period of 60 days.

Table 6

MEDIAN SURVIVAL TIME IN DAYS OF C3H MICE WITH LYMPHOSARCOMA 6C3HED GARDNER

HN2 dose, mg./kg.	Days of survival			
	Control	AET only	HN2 only	HN2 +AET*
0.00	14.0	13.0	...	...
2.00	...	...	14.0	>60.0 (90)†
3.03	...	...	10.0	21.3
4.60	...	...	8.0	8.3
7.00	...	...	7.5	7.5

*The HN2 was given intraperitoneally; the AET was given intravenously in doses of 50 mg./kg. Treatment was given on the fourth day after transplantation of the tumor.

†Per cent survival at 60 days.

Table 7 shows a similar experiment with Dalton Thymoma in DBA/2 mice without any apparent beneficial effect.

Table 7

MEDIAN SURVIVAL TIME IN DAYS OF DBA/2 MICE WITH THYMOMA DALTON

HN2 dose, mg./kg.	Days of survival		
	Control	HN2 only	HN2 +AET*
0.00	15.1	...	...
2.00	...	17.5	18.0
3.03	...	18.3	18.3
4.60	...	18.2	18.3
6.00	...	18.2	18.3

*The HN2 was given intraperitoneally; the AET was given intravenously in doses of 50 mg./kg. Treatment was given on the seventh day after transplantation of the tumor.

Summary

Bone marrow transplantation and aminoethylisothiuronium (AET) protected mice and monkeys against lethal doses of nitrogen mustard. Autologous bone marrow transplantation allowed administration of high doses of a new chemotherapeutic agent, AB 103. "Take" of autologous bone marrow can be determined by determining leukocytosis induced by Pyrexal, a bacterial lipopolysaccharide.

References

1. Bardos, T.J., Papanastassiou, Z.B., Segaloff, A. and Ambrus, J.L.: Dual antagonists; Alkyl N-(bis (ethylenimido)-phosphoro)-carbamates; a new series of antitumor agents. Nature 183: 399, 1959.

2. Razis, D.V., Ambrus, J.L., Ross, C.A., Stutzman, L., Sokal, J.E. and Rejali, A.M.: Use of AB 103, (a "Dual Antagonist") in the treatment of bronchogenic carcinoma. Cancer 14: 853, 1961.

OVERMAN 1. What was the form in which the AET was administered? 2. Was the AET given prior to the HN2 or afterwards, and finally I wonder whether AET perfused into the intestines in an aqueous solution could remain there, if the perfusion goes on?

AMBRUS The AET solution was always neutralized so we were actually dealing with MEG. In the therapeutic experiments in animals AET was always given prior to HN2. I haven't mentioned a number of alkylating agents, for example cytoxan whose toxicity is actually potentiated by AET rather than diminished. We don't quite know why and this is one phenomenon we are studying now. As to the last question we start a perfusion with AET and as soon as the perfusion is going we start to irradiate. At the time we stop irradiation, we switch to saline and within 15 to 30 minutes wash out all the AET remaining in the gut.

OVERMAN What was the total time involved in the perfusion and radiation?

AMBRUS It varied between 15 and 30 minutes.

OVERMAN This is a rather long time not to get sizable amounts of AET absorbed. How much of the sulfur labelled material was found in the tissues?

AMBRUS Well, all that we have done is to radioautograph organs from these animals following perfusion and we have found very low grain counts in the tissues and in the marrow and the only place where we have found high grain counts was in the intestines.

BALNER We have repeated in rats the experiments reported by Dr. Latarjet et al. (C. R. Acad. Sci. 252, 948, 1961) for mice, with AET dissolved in thiogel. The thiogel keeps the AET in close contact with the gut epithelium while resorption of AET is presumably low. Rats were irradiated with 1100 and

1200 r while the thiogel-AET was in the gut and we did not find any difference between these animals and controls, though Latarjet recorded success in mice with a similar procedure.

AMBRUS Is this conclusion based on survival or histology of the gut?

BALNER It was based on survival time. If intestinal death had been prevented the animals would have died much later.

MATHÉ Do you have a proof of the graft in mice and in monkeys when you inject homologous bone marrow after an alkylating agent?

AMBRUS No. These were preliminary experiments.

BALNER Did I understand you got permanent survival of mice treated with lethal doses of alkylating agents followed by homologous marrow transplantation?

AMBRUS Yes, mice were observed for 2 months.

BALNER I was never successful in getting long term survivors with homologous marrow after lethal doses of a number of alkylating agents and antimetabolites though I did get survivors with isologous marrow.

HUMBLE We have given humans relatively large doses of nitrogen mustard and other alkylating agents followed by autologous marrow, e. g., 1. 85 milligrams of nitrogen mustard per kilo in 15 hours and autologous marrow in a case of carcinoma of the esophagus with multiple secondaries. The patient survived 7 days but he became hallucinated and had pains in the limbs. He had very little diarrhea, or gastrointestinal upset and this was a feature of 3 other cases who were given doses of this kind as: 1. 3 milligram per kilo in 3 days, 1. 1 milligram per kilo in 11 hours and 1 milligram per kilo in 30 minutes. The last patient survived 80 days and the marrow recovered in two others. I must confess, though, that nitrogen mustard in that type of dose caused only slight tumour regression. Using other alkylating agents

particularly mannomustine HCl, although quite large doses were used, we had evidence of a quite rapid marrow recovery. With many drugs autologous marrow is very difficult to evaluate. After cyclophosphamide we were not able to demonstrate that giving autologous marrow improved the recovery. We gave one patient such a dose of cyclophosphamide, that the white count was reduced to one white cell/mm^3 but 10 days later the patient had a normal white count without any marrow treatment. We also used a compound called 3-ethylene-glycol-di-glycidyl-ether (TEG). We gave 300 milligrams of this compound in 5 minutes and the patient's marrow recovered following re-insertion of his own marrow at 12 days. We gave another infusion at 20 days. That patient survived 91 days and the tumour, a multiple metastatic malignant melanoma, was not greatly affected but there is no doubt that after this particular drug, which had produced aplasia, recovery was accelerated by autologous marrow.

MATHÉ I think one must underline that there is much variation from one patient to another as far as sensitivity to drugs is concerned.

HUMBLE I agree. The individual susceptibility of patients is very marked. The patient who looks ill is very sensitive to these drugs, generally speaking, whereas one that looks in a relatively good state of nutrition and health, whatever that means, tends to be on the whole capable of taking larger doses.

AMBRUS Of course this is one of the great problems that there are tremendous differences in tolerance of the patients towards chemotherapeutic agents and we are trying to develop a method to determine this beforehand. The procedure is to give Pyrexal to the patients. We determine the degree of leukocytosis we can provoke and see whether this can be used as a method of estimating functional marrow reserve. The way the experiment has been done is that in our out-patient-clinic a number of patients are seen and

chemotherapy where the people in charge of the out-patient-clinic simply use their clinical judgement. We do not interfere with this but we do a Pyrexal test and then we compare what would have been actually the better method to follow; their clinical judgment or our Pyrexal test. In contrast, in the in-patients where we have control over the patients, we are using the Pyrexal test and we are treating them accordingly, so we hope that in a few years we will have enough cases that we can make a statement as to whether the Pyrexal test is a useful and reliable method or not.

GENERAL DISCUSSION

HUMAN APPLICATIONS OF BONE MARROW TRANSPLANTATION

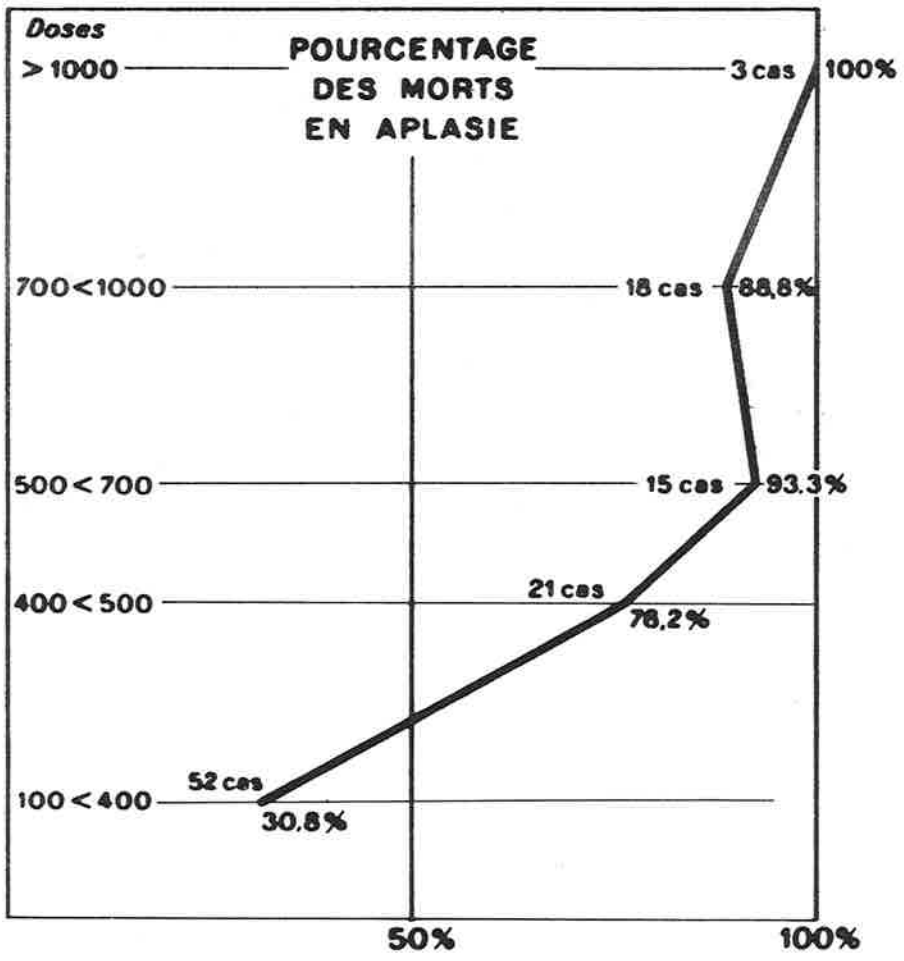
Chairman : G. Mathé

VAN BEKKUM Dr. Mathé since you did not give a paper and some people here are not familiar with your publications (Mathé et al., Rev. Franç. Et. Clin. Biol., 4, 226, 1959; ibid. 4, 675, 1959; Rev. Hémat., 15, 115, 1960) could you possibly give us a short review?

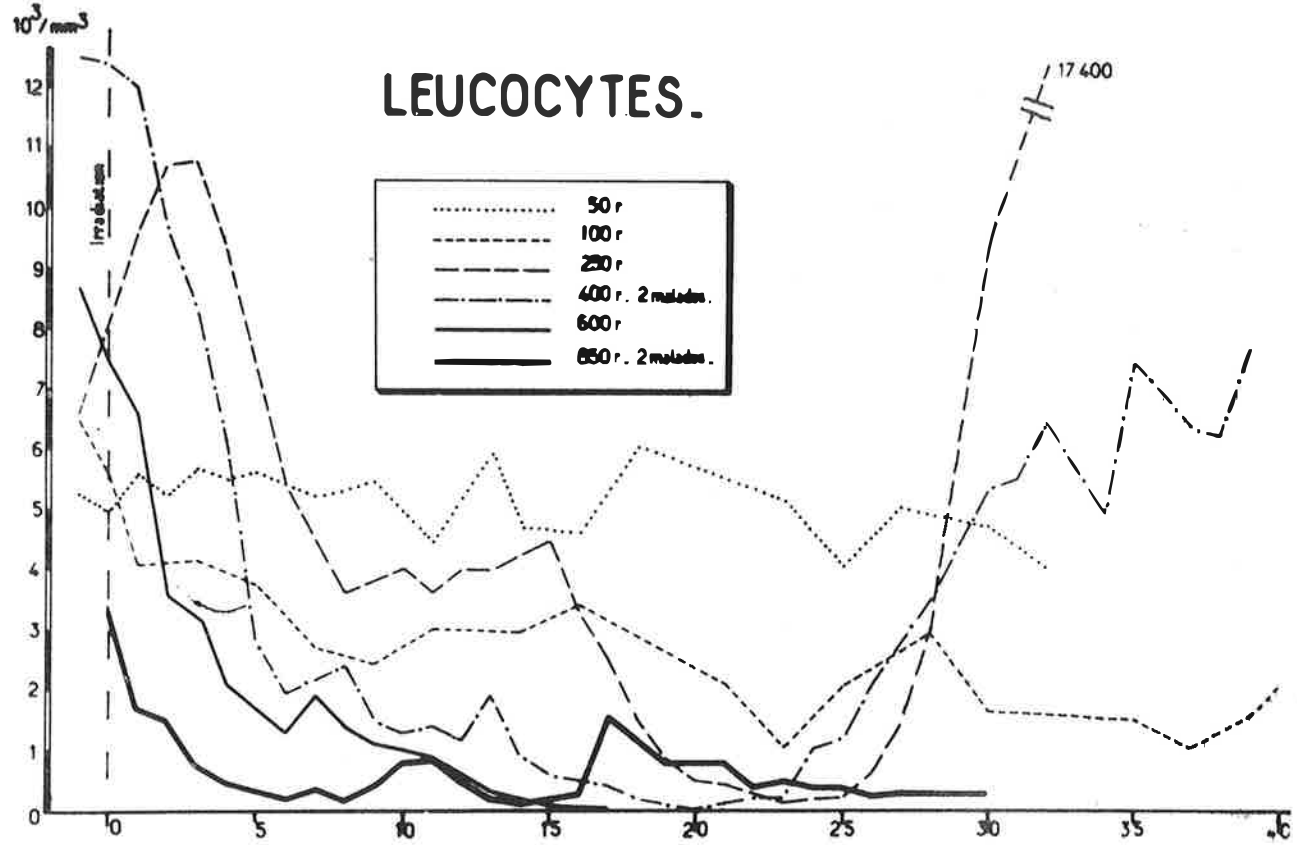
MATHÉ In the first place we have been interested in the radiosensitivity of man. Enquiries from different friends who have irradiated patients, produced the data collected in slide 1. The LD50 is between 400 and 500 r. Thus the human being seems very sensitive, even if very carefully treated symptomatically. These patients had of course different diseases: some were irradiated for leukemia or cancer, some for kidney transplantation, some for a severe glomerular syndrome. The mortality is similar in these 4 groups; so these data seem to give a good approximation of human sensitivity.

My own experience confirms this sensitivity and the blood count after 50 or 100 rads reflects this marked sensitivity (slide 2). After 250 rads we saw severe aplasia. Another patient died after 600 r after doing quite well on platelet transfusions and asepsis. Autopsy gave no cause of death in this case. Finally the curves after 850 rads in 2 patients with leukemia treated unsuccessfully with bone marrow will complete our data correlating hematological effects in man with radiation dose.

Our symptomatic treatment is shown in slide 3. The effectiveness of platelet transfusions is shown in slide 4. Although the platelet numbers do not increase, their function in hemostasis improves and remains above dangerous levels. We have not lost a patient from hemorrhage.


Doses
POURCENTAGE
DES MORTS
EN APLASIE
> 1000
3 cas
100%
700 < 1000
18 cas
88,8%
500 < 700
15 cas
93,3%
400 < 500
21 cas
78,2%
100 < 400
52 cas
30,8%
50%
100%

SLIDE 1

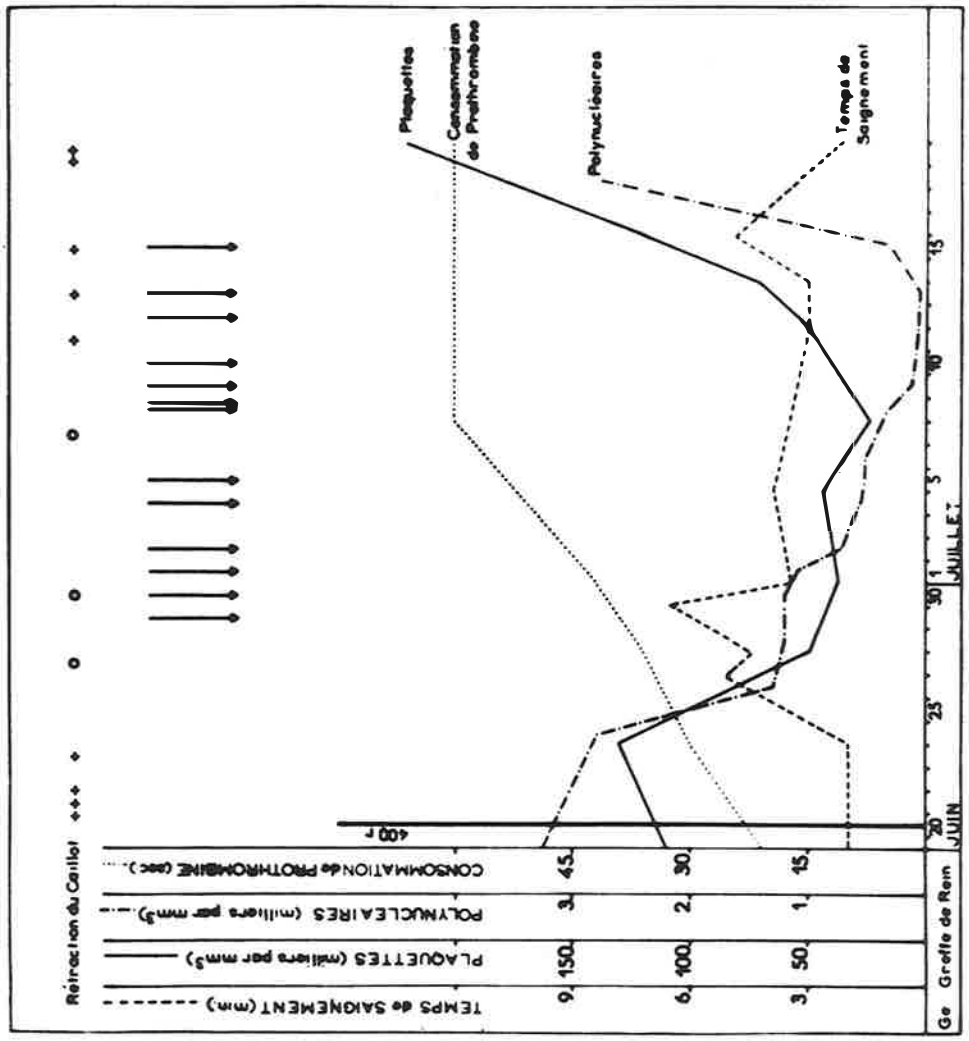

LEUCOCYTES.
$10^3/mm^3$
17 400
50 r
100 r
250 r
400 r. 2 maladies.
600 r
650 r. 2 maladies.
0
1
2
3
4
5
6
7
8
9
10
11
12
0
5
10
15
20
25
30
35

SLIDE 2

INDICATIONS	TREATMENT METHODS
Anemia (R. B. C. $<2.10^6$):	Transfusions of packed cells
Thrombopenia ($<10^5$):	a.) Platelet transfusions
	b.) Δ_1-cortison?
Leukopenia	
A) routine:	a.) Isolation in aseptic room with UV-irradiation Sterilisation of all objects and foods. Personnel under close bacteriological supervision
	b.) Gamma globulins
B) with fever	Antibiotic chosen according to sensitivity of isolated microorganism
1. with identification of microorganism	Broad spectrum antibiotic (preferably no chloramphenicol)
2. without identification of microorganism or cause of fever	Mycostatine Symptomatic treatment of fever

SLIDE 3

The next point under discussion is the Yugoslave reactor accident dosimetry (slide 5). This was first worked out in France by Jammet et al. (Rev. Franç. Et. Clin. Biol., 4, 210, 1959) and co-workers in rems and this resulted in 600 to 1000 rems, which is in the 90-100 % lethal range. Savić (Bull. Inst. Nucl. Sci. Boris Kidrich, 9, no. 167, 1959) estimated 683 rads average which is in the same range and finally the accident was reproduced by an international team using phantoms (Hurst et al., Health Physics, 5, 179, 1961). Their data are given in rads and the doses seem now to be just over 400 rads, thus


Plaquettes
Consommation de Prothrombine
Polynucléaires
Temps de Saignement
Réaction du Caillot
TEMPS de SAIGNEMENT (mn)
PLAQUETTES (milliers par mm³)
POLYNUCLEAIRES (milliers par mm³)
CONSOMMATION de PROTHROMBINE (sec)
400 r
Ge Greffe de Rein
JUIN
JUILLET

SLIDE 4

1) PHYSICAL DOSIMETRY VARIOUS ESTIMATES		% MORTALITY DUE TO APLASIA FOR THESE DOSES
JAMMET ET AL. (REMS)	: 600 - 1000	90 %
SAVIC (REMS)	: 683 ± 15 %	90 %
HURST ET AL. (RADS)	: 436, 414, 426 419 323	 76.2 % 30.8 %
2) BIOLOGICAL DOSIMETRY AND NEUTRON PROBLEM		400 RADS (NON HOMOGENEITY)
HEMATOLOGICAL EFFECTS		600 AND 875 RADS IN DIGESTIVE TRACT
HISTOLOGICAL LESIONS		600 RADS AND 875 RADS
GENERAL CONDITION AND FAILURE OF SYMPTOMATIC TREATMENT		

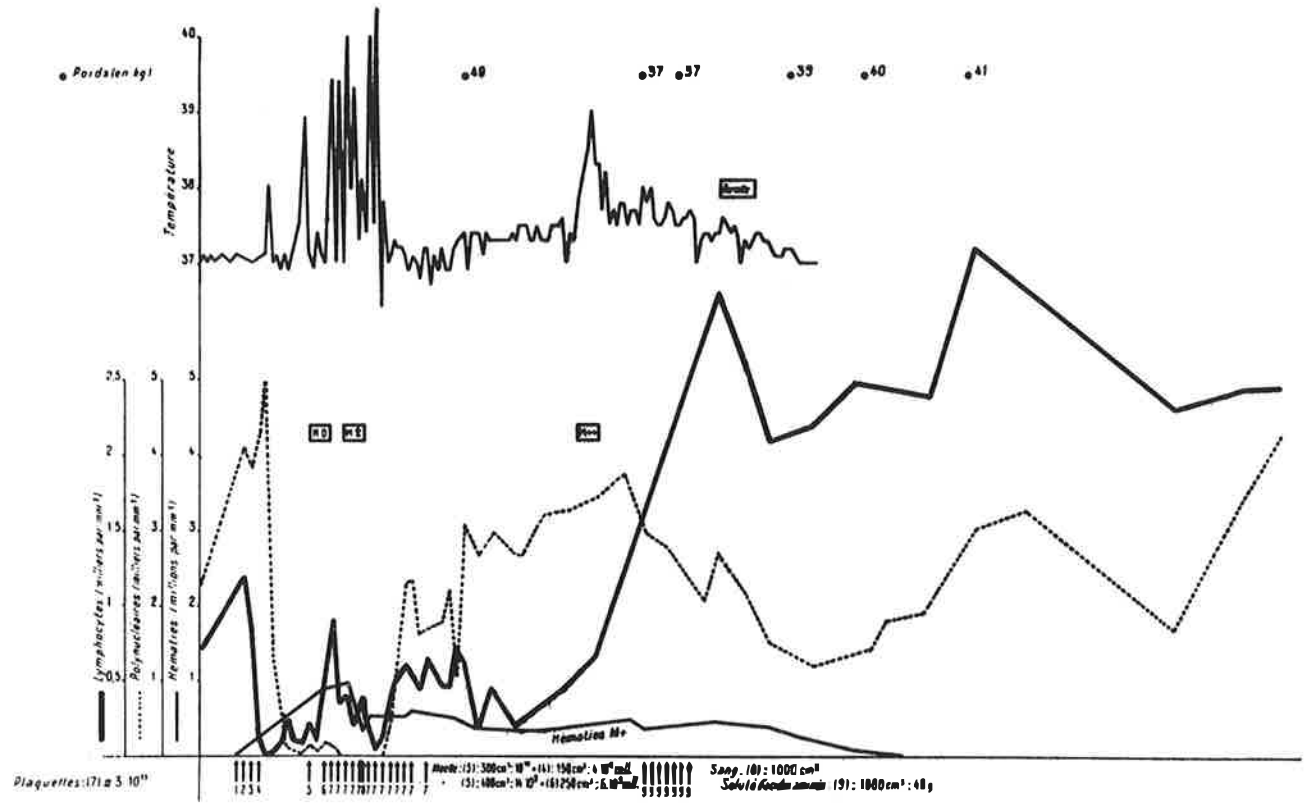
Jammet, H. et al. Rev. Franç Et. Clin. Biol. 4, 210, 1959.
Savic, P. P. Bull. Inst. Nucl. Sci. Boris Kidrich 9, no.167, 1959.
Hurst, G. S. et al. Health Physics 5, 179, 1961.

SLIDE 5

in the 76 % lethal range. There is a difference that the accident patients were not irradiated as homogeneously as our patients, but the biological criteria seem to point at a higher equivalent dose. We had more intestinal lesions in these people than we usually see in patients irradiated with 875 rads. Thus we would think that neutrons are probably worse than gamma radiation.

Of the patients with leukemia we irradiated, 6 were in remission. Two of these died after 1 month without success (aplasia). Two had temporary evidence of donor cell proliferation and then they had fever, cough, lymphopenia, skin infections and diarrhea. This disappeared when the donor cells disappeared and we think this may have been a transitory secondary syndrome (slide 6).

Those two patients were in their third remission which we expected to last 1 or 2 months, but after this treatment they were in remission for 9 months and 1 year respectively.

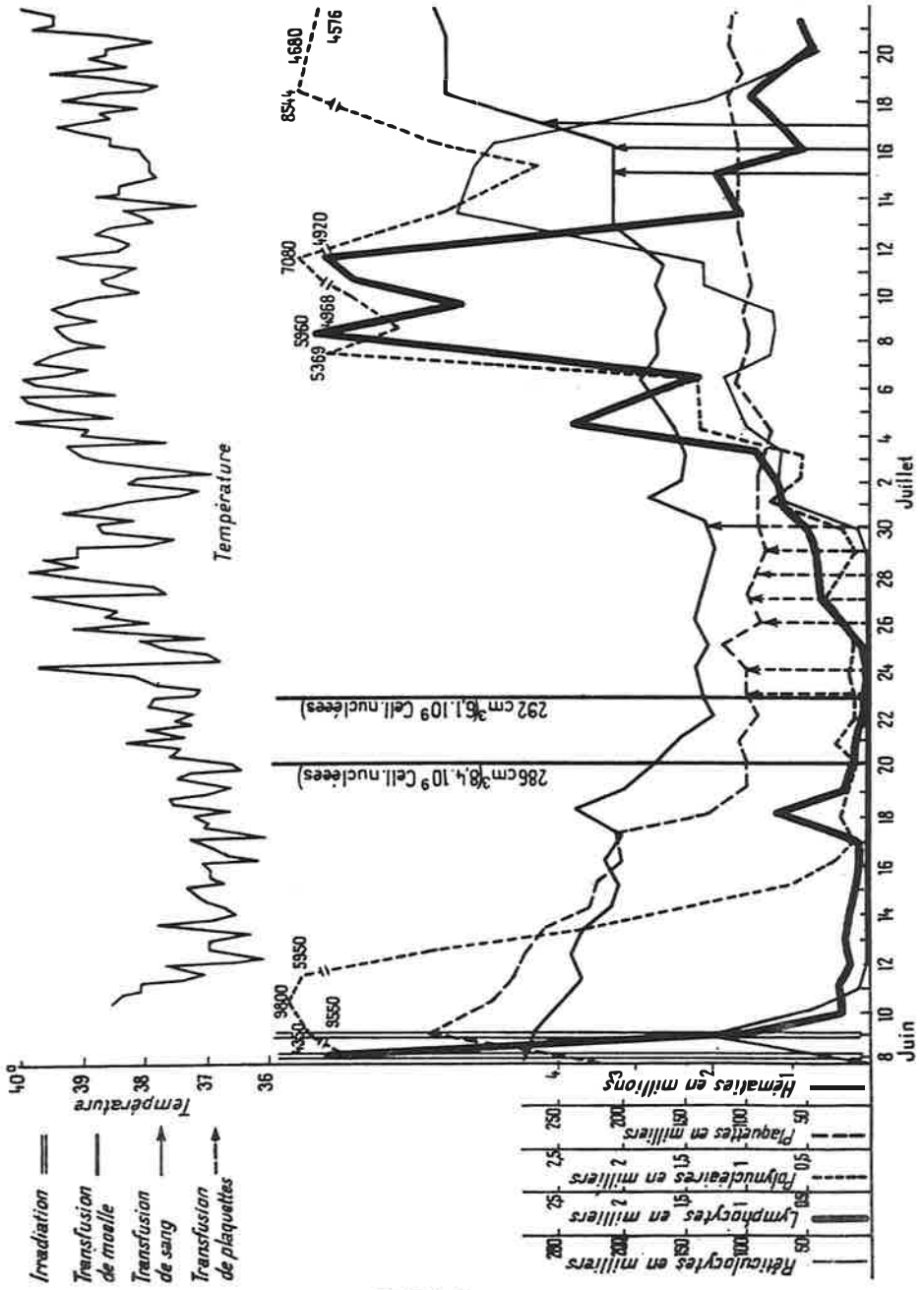

Poids en kg
40
39
38
37
Température
Lymphocytes (unités par mm³)
Polynucléaires (unités par mm³)
Hématies (millions par mm³)
Hématies M+
Plaquettes : (7) ± 3. 10¹¹

SLIDE 6

The two other patients received the bone marrow from their mother and in the boy we could use the sex-linked chromatin in the leukocytes in addition to the red cell antigens to study the proliferation of the donor cells.
These children both had a very early secondary syndrome after 20 days with a severe dermatosis. Dr. de Vries yesterday showed you the histological aspects of the skin. These children had fever, diarrhea, lymphopenia, infections and one of them had a very severe hepatic necrosis, probably due to a virus.

Slide 7 shows that initially the lymphocytes increased together with the other cellular elements but characteristically disappeared in the secondary syndrome.

Finally an adult patient with lymphoblastic leukemia was irradiated recently, not in remission but in relapse. In this patient a skin eruption was evident before hematological recovery occurred and he died probably from this very rapid secondary syndrome. His wife was the donor and she could have been immunized in pregnancy against the antigens of their children which were of course his antigens., Thus it may have been a secondary immune response, causing this early secondary syndrome.


Irradiation
Transfusion de moelle
Transfusion de sang
Transfusion de plaquettes
Température
40°
39
38
37
36
Réticulocytes en milliers
Lymphocytes en milliers
Polynucléaires en milliers
Plaquettes en milliers
Hématies en millions
Juin
Juillet
8
10
12
14
16
18
20
22
24
26
28
30
2
4
6
8
10
12
14
16
18
20
9800
9550
5950
7080
4920
5960
4968
5369
8544
4680
4576
286cm³(8,4.10⁹ Cell.nucléees)
292 cm³(6,1.10⁹ Cell.nucléees)

SLIDE 7

AMBRUS Dr. Mathé is there any rationale why a homologous graft should react more against the leukemic tissues of a patient than against his normal tissue?

MATHÉ No, but you cannot eradicate leukemia with radiation alone or with isologous bone marrow as you can with homologous bone marrow due to the histocompatibility difference. In the mouse you need for instance with L. 1210 leukemia 5000 cells to transplant leukemia in 50 % of cases, so one single cell does not always develop into leukemia. Maybe the leukemia can somehow be eradicated before the normal cells are.

AMBRUS These rodent data may not apply to man, where there is not the long history of serial transplantation in which subtle antigenic differences may develop. In man antigenic differences between tumour and host may not exist at all or if they do they are much smaller.

MATHÉ Even if there are antigenic differences, as has been claimed by some people in Sweden, these differences may be no good, since they may lead to enhancement of tumour growth.
Such an enhancement was seen by us after transplanting women suffering from chorion epithelioma with skin of their husbands. We found no quick rejection of these transplants although there were positive leuko-agglutinins and thrombocyte agglutinins against the husband. There was a longer tolerance for those transplants which might be due to the enhancement phenomenon.

DE VRIES Our experiments with treatment of lymphosarcoma in mice with irradiation and homologous bone marrow were done in various combinations and the general conclusion is, that in those combinations in which secondary disease is severe, tumour growth is inhibited, but where secondary disease is mild there is no influence on tumour growth, so we feel pessimistic about this approach.

MATHÉ If this is late secondary disease we can treat it with antimetabolites like amethopterin or alkylating agents like cyclophosphoramide and we can save some mice from the syndrome.

DE VRIES But in that case will the tumour not be less affected?

MATHÉ No, we found a dose where there is also a direct effect from the agent on the tumour.

VAN PUTTEN I would appreciate an opinion on the question whether homologous bone marrow infusions may be useful when no permanent take is obtained.

C. AMBRUS This is about the only thing we are sure of in monkeys, since only the monkeys reversing to host type blood cells seem to have survived.

MATHÉ In addition I think in man the acute effect is important: an improvement in bleeding time and prothrombin consumption for a few days.

HUMBLE I agree and in addition reticulocytes may increase for a while also. Occasionally even longer lasting effects are noted.

F. E. NEWSOME In our rhesus monkeys where we found a harmful effect of marrow suspensions after early treatment with sublethal doses, I assume I had graft rejection and I assumed this rejection is harmful somehow. This harmful effect does not seem to occur when injection is given 48 hours after irradiation. I would welcome repitition of this work. Since however an interval of 48 hours after irradiation seems to make this treatment safe, its application at this interval must presently be recommended. This delay would be no problem in radiation accidents.

WHITCOMB Dr. Mathé what percentage donor cells did you see in your patients that received 850 rads?

KURNICK And in the Yugoslaves?

MATHÉ After 850 rads the maximum was 30 %, 3 months after irradiation. But in theYugoslaves the donor red cells increased only for 3 weeks. The estimation is done by a special accurate technique, originally described by Wurmser (Rev. Hemat., 9, 291, 1954) and modified by Salmon (Rev. Franç. Et. Clin. Biol., 4, 239, 1959).

DE VRIES If it is concluded that in these conditions; radiation accidents, sublethal irradiation and overdosage of chemotherapeutics, a temporary take of foreign bone marrow is better than ground glass, it seems important to study factors that promote such a temporary take, rather than a permanent take.

MATHÉ Is not the radiation dose and its homogeneous distribution most important?

AMBRUS What we really must study is the smooth reversal of donor type to host type cells before secondary disease occurs.

BALNER Dr. Mathé, you suggested that in accidentally irradiated people you would rather depend on clinical symptoms than on dosimetry. At what point would you then take the risk of a homologous graft?

MATHÉ I think I would do as with the Yugoslaves, wait under aseptic conditions, without antibiotics, without blood transfusions but with platelet transfusions if these go below 50,000.
I would give bone marrow only if there is "decompensation", if there is the clinical impression that for the patient this treatment is not enough.

VAN BEKKUM Is it the opinion that homologous bone marrow transplantation offers still enough possibility for any clinical application to warrant its continued or even extended study in primates?

MATHÉ First this question: are you sure that primates give you better information than mice or dogs? Man has more granulocytes than mice or

monkeys and further man has maybe less migration of stem cells than the mouse. Are monkeys better than mice, and would dogs not give the same information?

VAN BEKKUM I would prefer information on monkeys over data on mice on the basis of the differences that have been reported between rodents on the one hand and primates on the other hand. I am not so sure about the similarities between monkeys and primates but because of that uncertainty, I would prefer monkeys.

MATHE The alternative object of study is human leukemia. The patients who have acute leukemia have nothing to lose. This is a moral problem but maybe it is a very important one. Do you think we can continue to try to treat acute leukemics and use them as material for study?

VAN BEKKUM I am not a clinician so I cannot give an opinion. I would feel, however, that it is not very wise to continue treating patients unless we have much more information about the eventual effects of homologous bone marrow transplantation in monkeys. But I am still not satisfied about the applicability of homologous bone marrow transplantation in humans. Are the results so poor that these trials would better be discontinued for the time being?

MATHÉ No, it may eventually be applied in leukemia or for kidney transplantation.

KURNICK As a clinician I feel that <u>first</u> it is justified or even mandatory to continue this work in primates or other species in the hope of getting a solution whereby homologous transplantation may be a means of preserving life, of treating leukemia and getting around the problem of secondary disease. Secondly, I would not agree with Dr. van Bekkum in questioning the validity of continuing experiments such as Dr. Mathé's with patients since

from a moral point of view these patients once they have had one or two remissions, have nothing to lose and may conceivably gain something.

HUMBLE I agree entirely; on both moral and practical grounds these experiments must continue. No one has complained because we treated these patients. A lot of people complained because we weren't very keen to treat the patients.

OVERMAN Speaking as a non-clinician, in the first place I think we have only scratched the surface of trying to see what can be done with homologous marrow in the monkey and this work will continue.

F. E. NEWSOME We have been wondering whether the hemoglobin first produced by an autologous graft was foetal-type (i. e. alkali-resistant) or normal adult. If it is adult, this would be different from spontaneous recovery, when a foetal-type hemoglobin always seems to be the first to be produced. In that case it might be used as an indication for success of an autologous graft.

Then followed an exchange of information on the technical aspects of bone marrow procurement and storage. This information has been presented in the following table.

BONE MARROW TRANSPLANTATION TECHNIQUES USED FOR HUMAN APPLICATION AS DESCRIBED BY THE FOLLOWING PARTICIPANTS TO THE SYMPOSIUM:

	Dr. J.L. Ambrus	Dr. J.G. Humble
Diluent for the bone marrow cell suspension	modified Eagle's*	TC 199
Final heparin concentration (U/ml)	10	10
Filtration of suspension	+	+
Centrifugation of suspension	-	+
Removal of fat	+	+
Partial removal of erythrocytes	-	-
Cell counts, as presented have been corrected		
for peripheral blood nucleated cells	+	+
for dead cells	+	-
Dead cells estimated in: fresh suspension	Schreck	D.E. †
in: frozen suspension		D.E.
Complications noted after injection of fresh marrow suspension	-	-
Additive for freezing (D = dimethylsulfoxyde; G = glycerol)		15% G
Size of individual freezing ampoules		30 ml
Freezing procedure at 1°/min down to:		-15° C
then at higher speed to:		2-3°/min -63°
storage at:		-79° C
Thawing rapidly in water at 37° C		+
slowly in air at 0°- 2° C		-
Dilution with:		-
Complications seen after infusion of frozen suspension		Hb-uria; Venous spasm; rarely rigor

† D.E. = Dye exclusion method.

* Modified by addition of: ATP, streptokinase, streptodornase, penicillin and streptomycin.

BONE MARROW TRANSPLANTATION TECHNIQUES USED FOR HUMAN APPLICATION AS DESCRIBED BY THE FOLLOWING PARTICIPANTS TO THE SYMPOSIUM:

Dr. H.E.M. Kay	Dr. N.B. Kurnick	Dr. E. Loeb	Dr. G. Mathé
TC 199	TC 199 or Osgood's	Hanks + 5% AB serum	none
10-20	2-5	none: 0.002% EDTA	10
-	-	-	-
for freezing only	-	+	-
-	-	+	-
for freezing only	-	-	-
-	+	-	-
-	+	-	+
-	D.E.	-	D.E.
Acridine orange staining	D.E.	Stathmokinetic test ●	
-	- ▲	-	cough ▲
12.5% D	15% G	15% G	
4 ml round or 20 ml flat	10 ml	250 ml	
-15° C	-40°C		
5-10°/min -60°	immediately in refrigerator	■	
-79° C	-80° to -95° C	-79° or -190° C	
+	-	+	
-	+	-	
-	½ Vol. 35% glucose	½ Vol. 50% glucose + 10 min later 2 Vol. saline	
Hb-uria ▲▼	Hb-uria; if infused rapidly: headache and sternal pain; once cerebral hemorrhage	No Hb-uria; twice pulmonary embolism	

▲ Injected immediately from aspiration syringe.

● See text of Dr. Loeb's paper.

■ Polge, Smith, Parkes technique: Nature, 164, 666, 1949.

▲▼ Effectiveness of freezing method evaluated by:

1) mouse radiation protection: 10-50% efficacy as compared with fresh marrow (cell dose range 10^4-10^5 cells/mouse).

2) human cells: 80-100% survival of HeLa cells using Puck plating technique.

3) similar acridine orange staining in mouse and human suspensions.

CHEMICAL PROTECTION OF PRIMATES

PRACTICAL APPROACHES TO CHEMICAL RADIATION PROTECTION IN PRIMATES[1)]

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One major facet of the work of our laboratory for the past six years has been a productive inquiry into the subject of chemical protection against both lethal and sublethal acutely delivered whole-body x-irradiation in mammals. Although most of the preliminary studies of potentially radioprotective compounds were carried out in the dog, these investigations had as their goal the development of a safe and reliable preparation of the drugs for their ulitmate use in primates, including man. These dog experiments will be summarized in order to demonstrate the rather step-wise manner which has lead to the development of what the authors feel is one of the best radio-protective preparations to date. In addition, the results of preliminary studies of chemical radiation protection in the rhesus monkey (Macaca mulatta) and purposed investigations in this primate will be presented.

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1) This study was supported by research funds from the UNITED STATES ATOMIC ENERGY COMMISSION, CONTRACT NO. AT (40-1) 1642.

2) Present address: U.S. NAVAL RADIOLOGICAL DEFENSE LABORATORY, SAN FRANCISCO, CALIFORNIA.

The concept of chemical radiation protection is based on the premise that, in some manner, the presence of a chemical substance, or substances, in the biological system at the time of irradiation either reduces or prevents the damaging effects of ionizing irradiation. Inherent in this concept are many problems since it implies the administration of an exogenous substance in somewhat high doses. Some, but certainly not all, of these problems are (1) selection of the compound or compounds and the animal in which they are to be studied, (2) drug dose, including methods of administering a completely protective dose without excessive toxic effects to the animal. Also related to the drug dose are the problems of (a) the route of administration, (b) getting the compound into the animal at the onset of irradiation and (c) maintaining a constant and protective drug level during the entire period of exposure. One other problem might be the selection of the irradiation factors such as acute or chronic, lethal or sublethal, and the source.

Since the reports by Patt et al. (1) and Bacq et al. (2) on the radioprotective activity cysteine and cyanide, respectively, there have been many diversified substances tested for protective activity against radiation damage. We selected what was considered to be the most promising chemical representatives of two major classes of radioprotective agents, aminoethylisothiuronum (AET) as a sulfhydryl or potentially sulfhydryl compound and para-aminopropiophenone (PAPP) as a hypoxic (methemoglobin-producing) agent.

The dog was selected for these preliminary studies for several reasons. (1) The radioprotective ability of the drugs in mice had already been well documented in the literature (3, 4, 8). (2) The prohibitive financial burden in obtaining and housing the large number of monkeys necessary to conduct these experiments since 400 dogs were used. (3) Pilot studies in our laboratory as well as the report by Benson et al. (5) indicated that the dog is hypersensitive to the toxic effects of AET and PAPP. (4) The dose of acute irradiation lethal for the dog is less than that for the monkey and, presumably, man. One might expect then that techniques resulting in a reliably safe procedure in the dog might also be successful in the primate.

AET and PAPP have been intensively studied both alone and in combination with regard to toxicity, pharmacology and radioprotective abilities. Knott and Overman (6) and Blouin and Overman (7) have recently reported on the pharmacology of AET. Since time is short this aspect of these studies will not be discussed. The toxicity studies of AET were carried out using five routes of drug administration: intravenously, intraperitoneally, orally, intramuscularly and subcutaneously. In addition, for each of the routes AET was prepared in various combinations of the following: (1) in saline or distilled water, (2) in saline or distilled water with varying concentrations of dextran and/or glucose, (3) in an oil solution or oil emulsion, (4) in gelatin or enteric coated capsules and (5) neutralized or not neutralized. In addition, the concentration of AET in the different solutions was varied.

The intravenous administration of AET as well as the intraperitoneal route was found to be severely toxic to the dog. However, one significant fact was found in these studies, that is, the rate of injection is more important than the total dose given. When 100 mg/kg AET is given in a rapid (15 sec.) intravenous injection, all the typical signs of AET toxicity are observed. However, when the same drug dose is slowly infused into the venous system at a rate of 3 mg/kg/min, only the milder of toxic effects are seen. In all cases being presented here, AET was given in a 2 to 3 per cent neutral aqueous solution.

Although the dog was able to survive oral doses of an AET solution up to 250 to 300 mg/kg, the toxicity was considered too high to be of practical valve. Emesis with loss of the drug solution was a constant finding. This was attributed to the reaction of the acid gastric contents with the AET to form the more toxic and less protective derivative 2-AT or 2-aminothiazoline. The results were the same when the drug was administered in gelatin capsules. In order to by-pass the gastric environment, AET was obtained and studied in enteric coated capsules. Again, however, some toxic effects including mild emesis and varying degrees of hemorrhage in the intestinal mucosa were observed. This time the harmful effects were due to the sudden release of AET in

the intestine. It must be pointed out that in most all cases the toxic effects of AET when given orally, either in solution or capsules, were less severe than even lower doses given intravenously or intraperitoneally.

A number of points of importance have emerged from these studies of AET in the dog. When AET is prepared in an aqueous solution, careful neutralization is essential to provide for maximal conversion of AET to MEG (mercaptoethylguanidine), the alleged protective form of this compound (8). This point is mandatory in using a solution of AET by any route of administration. Lack of the complete conversion of AET to MEG at a neutral or near neutral pH allows the formation of other intermediaries, especially 2-aminothiazoline at acidic pH's, which are more toxic and less protective than MEG.

The rapid administration of an AET solution is also undesirable. Any method or route which allows for a slow rise in the blood drug level makes possible the administration of completely protective doses which are otherwise severely toxic. The oral route of administration is the most desirable, if extrapolated to potential use in man, and fortunately, the most successful. It should be added, however, that nine of ten dogs given 125 mg/kg AET by rapid (15 seconds) intravenous injection survived a whole-body x-irradiation dose of 500 r whereas all the control animals died.

Enough experience had been gained from these early studies to establish beyond question that in order to employ AET as a radioprotective agent in the dog we must use a preparation which (1) could be given orally, (2) would be enteric coated so that the acid gastric contents could not reach with the AET and thus form 2-AT, (3) provide a sustained or an intermittent intraintestinal release in order to (a) assure conversion of AET to MEG in the alkaline environment of the intestine and (b) to avoid a sudden, high local concentration of the released AET which is highly irritating to the intestinal mucosa.

Concurrent with the AET toxicity studies, PAPP toxicity in both the dog and monkey was investigated. The dog shows a much greater toxic sensitivity to PAPP than does the mouse or monkey. Whereas a PAPP dose sufficient to provide for a 60-80 per cent methemoglobinemia in mice is of the order of 30-40 mg/kg, doses of 3-4 mg./kg produce the same levels in the dog. However, in both the mouse and dog the methemoglobinemic response to PAPP is exceedingly well related to the total dose both as to peak levels of methemoglobin attained and the rapidity of return toward normal. The optimal PAPP dose that produces a high, but safe, level of methemoglobin (about 70 per cent) in the dog was found to be 3,5 mg/kg. If given orally, the drug was administered in gelatin capsules. A 2 per cent solution

of PAPP in propylene glycol was used when the drug was given intravenously or intraperitoneally. Although either of these two preparations proved satisfactory in the dog, this drug was also obtained in the enteric coated, intermittent release form.

To determine the effect of PAPP on the rhesus monkey (M. mulatta) various doses up to 50 mg/kg as a 2 per cent solution in propylene glycol were administered intravenously and intraperitoneally. Important differences in the reponse of the dog and monkey to PAPP were found. While in the dog the methemoglobin level was dose related, raising the PAPP dose from 25 to 50 mg/kg in the monkey produced little additional methemoglobin. In addition, in this species the overall effects of the degree of methemoglobinemia were mot as marked as in the dog. A PAPP doge of 25 mg/kg produced methemoglobin levels of approximately 30-40 per cent.

The investigation of the enteric-coated, intermittent release preparation of AET and PAPP in the dog revealed the following : (1) this form of AET could be given in doses up to 175 mg/kg without the appearance of severe toxic effects, (2) PAPP, 3.5 mg/k produced appraximately the same degree of methemoglobin as when given in gelatin capsules or propylene glycol, (3) when the two drugs were given simultaneously (a) the presence of AET did not alter the time of attainment of maximum methemoglobin levels, (b) nor did AET change the level of methemoglobinemia and (c) the simultaneous presence of PAPP appeared to have no effect on either

the time of appearance or the severity of the signs of AET action. It might therefore be concluded that these compounds have no influence on each other's solubility, absorption rate, distribution time and have, indeed, independent toxicities.

The first radiation protection studies employing the enteric forms of AET and PAPP revealed that neither drug alone, when given in maximal safe doses, could fully protect dogs against 550 r whole-body x-irradiation (Table I). Nevertheless, the mean survival time of both groups were significantly increased over that of the control animals. It can be seen, however, that a combination of these two compounds did provide excellent protection against 550 r. Further evidence of the radioprotective activity of the combination of 175 mg/kg AET and 3.5 mg/kg PAPP was demonstrated at 650 and 800 r. All six dogs which received the drugs prior to 650 r survived thirty days. Five of the six animals were long-term survivors whereas one died on day 31 post-irradiation. Although there were no thirty-day survivors out of six dogs given 800 r and the drug combination, the mean survival time was more than 100 per cent increased over that of the 800 r controls (17.2 vs. 8.1 days).

Prior to the development of the highly effective enteric coated preparations of AET and PAPP, preliminary studies of AET toxicity and its radioprotective ability were carried out in the monkey (M. mulatta). Our experience has been that AET administered intraperitoneally appears to be less toxic in the monkey than in the dog. Furthermore, we have evidence that drug tolerance can be

TABLE I

AET AND PAPP IN ENTERIC CAPSULES, 550 r

GROUP LR-II

Group	Number of Animals	PAPP Dose (mg./kg.)	AET Dose (mg./kg.)	Per cent 30-Day Survivors	Per cent Long Term Survivors	Mean Survival Time[a] (Days)
Controls	6	0	0	0	0	9.6
LR-IIa	6	3.5	100	0	0	21.5
LR-IIb	6	3.5	125	33	33	25.6
LR-IIc	6	3.5	150	100	66	39.5
LR-IId	6	3.5	175	100	100	——
LR-IIe	6	0	175	0	0	26.0
LR-IIf	6	3.5	0	0	0	19.3

[a]Non-survivors only.

induced in primates by the daily administration of gradually increasing doses of this compound. Utilizing doses ranging from 100 to 525 mg/kg AET intraperitoneally as a neutral solution in 40 cases, 25 showdd no ill effects, 7 displayed but moderate illness and 8 expired between one hour and 5 days after the compound was given. No animals died at dose levels below 200 mg/kg. In animals displaying toxic signs, these were generally less severe than those which followed AET administration in the dog.

Since the toxicity studies indicated that the rhesus monkey could survive rather high doses of AET administered intraperitoneally, the radioprotective activity of the drug was investigated. Table II shows a series of 14 monkeys which served as controls for the animals treated with AET and irradiated. These controls were radiated with 650 or 700 r and were left untreated until death occurred. The range of survivals in the 700 r group was 4-14 days post-irradiation with a mean survival time of 9.14 days. In the 650 r group the survival range was 7-13 days with a mean of 9.86 days.

Table III shows a series of 7 animals which received one or more doses of AET prior to administration of whole-body x-irradiation. The actual protective doses of the compound ranged from 150 to 250 mg/kg body weight. It is clear from this data that lower than lethal doses of AET are quite capable of protecting monkeys from whole-body radiation death for extended periods of time.

TABLE II

CONTROL MONKEYS

RADIATED - UNPROTECTED

ANIMAL NUMBER	RADIATION DOSE	SURVIVAL TIME IN DAYS POST- IRRADIATION
C-45	700r	6
C-43	700r	4
C-44	700r	11
C-47	700r	7
C-50	700r	11
C-48	700r	11
C-52	700r	14
Mean Survival Time		9.14
C-63	650r	8
C-64	650r	13
C-7	650r	9
C-69	650r	13
C-74	650r	7
C-76	650r	8
C-80	650r	11
Mean Survival Time		9.86

TABLE III

MONKEYS

ABT - RADIATED

ANIMAL NUMBER	DRUG DOSAGE mg/kg body wt.	RADIATION DOSE	SURVIVAL TIME IN DAYS POST-IRRADIATION
C-16A	100		
C-16B	150		
C-16C	200		
C-16D	250	650r	373
C-18A	100		
C-18B	150		
C-18C	200		
C-18D	250	650r	370
C-25A	100		
C-25B	150		
C-25C	200	650r	124*
C-78	150	650r	148
C-79	150	650r	42*
C-81	150	650r	35
C-83	150	650r	134

* Sacrificed

Animals C-16, C-18 and C-25 each received repeated I.P. doses of AET prior to irradiation in an effort to lessen the chances for toxic reactions. Two of these animals survived for more than one year post-irradiation. Monkey C-25 was sacrificed on day 124 post-irradiation for histological examination. The last 4 monkeys were each given a single I.P. injection of 150 mg/kg body weight AET prior to irradiation. Three of these animals were long-term survivors, while the other was sacrificed for examination.

In another early study, the protective effect of a combination of AET and PAPP in the rhesus was investigated. Prior to 700 r, 100 mg/kg AET and 20-25 mg/kg PAPP were administered intraperitoneally to nine monkeys. The PAPP was given as a 2 per cent solution in propylene glycol 30 minutes before radiation and the AET was administered as a neutralized aqueous solution 20 minutes before radiation.

Four of the monkeys died but three of the deaths occurred within a few hours of medication and radiation and were quite possibly drug deaths. These particular animals were extremely lethargic during radiation and showed signs of hypoxia for some time following radiation. We later learned to administer an antidote (methylene blue) for protracted PAPP activity. The other death was accidental occurring on the day after radiation. This animal caught his neck chain on the cage and strangled. None of these can properly be called radiation deaths.

In 2 of the 5 survivors the methemoglobin level rose to about 50 per cent during radiation. As this was somewhat higher than expected, these monkeys were given 1.5 mg/kg of methylene blue as a 0.1 per cent aqueous solution immediately after radiation. The methemoglobin levels of the other 3 survivors ranged from 17 to 43 per cent.

The peripheral blood picture was followed by-weekly for 4 weeks and after that less frequently. Figure 1 illustrates the more surviving monkeys. The early reticulocytosis possibly alleviated the anemia due to loss of erythrocytes which usually occurs after the third week. The slow decline of the leucocyte count is also noticeable. Other signs of radiation damage were either reduced or absent. A few petechiae and some moderate diarrhea appeared together with a slight hyporaxia. Rectal bleeding or serious weight less was not observed.

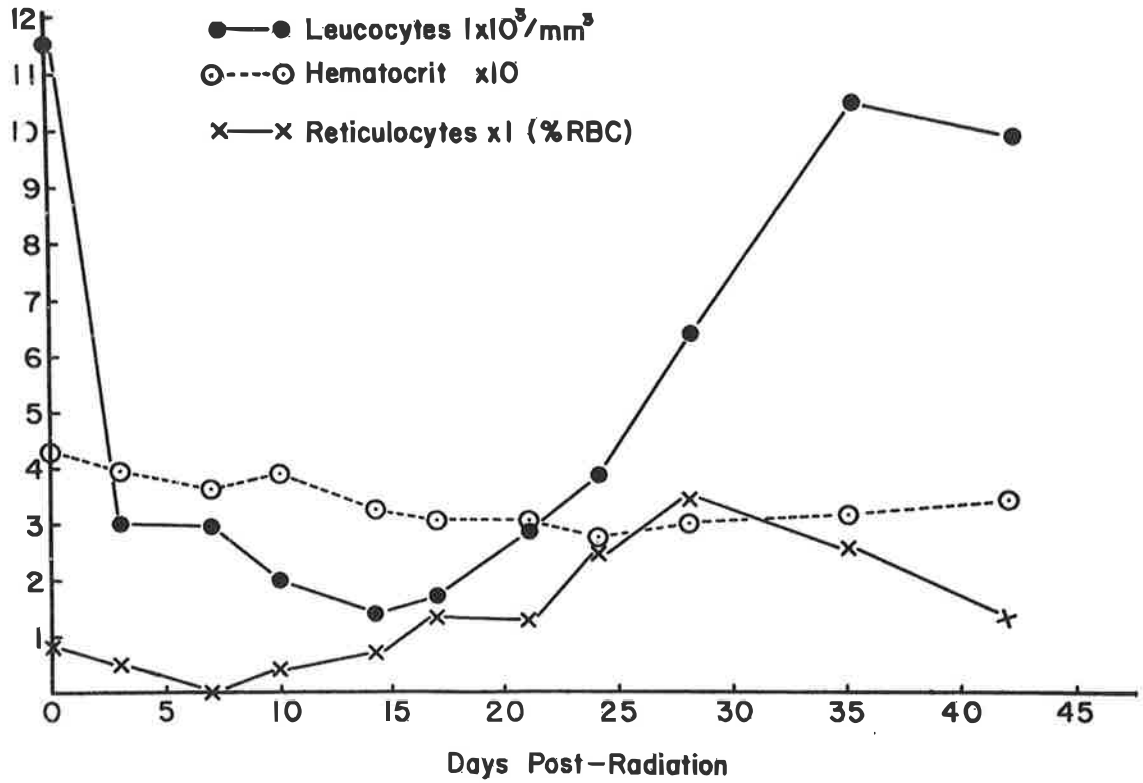

Figure 1: Peripheral blood values of five rhesus monkeys given AET and PAP prior to 700 r.

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DISCUSSION

C. M. AMBRUS I noticed that 3. 5 milligrams per kilo body weight of orally administered PAPP produced 70 % methemoglobinemia at about 2 hours. For what period of time was this maintained?

NEWSOME This, of course, varied from animal to animal, but in general this level was maintained for 3 or 4 hours. In the cases mentioned in the dog, the PAPP was given in gelatin capsules. The situation was different when PAPP was administered intravenously, in which case the methemoglobin levels increased much more rapidly.

OVERMAN The 3. 5 milligrams per kilo was in the dog. The monkey requires some 15 milligrams per kilo to produce approximately the same degree of methemoglobinemia.

C. M. AMBRUS And how long could this be maintained?

OVERMAN This depends entirely upon the route of administration.

C. M. AMBRUS Suppose it is administered in an enteric coated capsule?

OVERMAN Enteric coated sustained release capsules would maintain these levels for 4 or 5 hours, but in some cases these levels were maintained for as long as 9 hours.

AMBRUS I should like to ask whether you have noticed with these monkeys which had methemoglobin levels of 50 % or more, any impairment in their functional ability in the way they got around or tried to run away from people. I understand that quite some time ago some experiments were done with human volunteers and it was concluded that working ability was impaired at levels above 30 to 40 % methemoglobin.

F. E. NEWSOME I had to sample these monkeys with levels of about 40 to 50 % methemoglobinemia for several hours and they acted a little tired.

However, they still put up a good fight and I do not think they were too debilitated.

AMBRUS Have you information on the maximal dose of AET which can be given to a monkey without producing hypotension?

CROUCH A dose above approximately 75 milligrams per kilogram will certainly produce some degree of hypotension, but the hypotensive effect depends upon the route of administration of the AET. In any case, the hypotension is transient at this level.

AMBRUS Have you any information on the differences between different batches of AET? Some producers of the compound claim that on the basis of chromatography and other evidence their AET is "highly purified" and produces very few side effects.

OVERMAN We have seen some variation in batches of AET both from the same producer and from different producers. Therefore, we now check the melting point of all batches of AET before use and discard all batches which do not have the proper melting point.

CHEMICAL PROTECTION IN IRRADIATED MONKEYS [1,2]

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Chemical protection against the biological damage resulting from exposure to ionizing radiations has been achieved using sulfhydryl-containing compounds administered to several species of mammals prior to irradiation (Doherty 1955; Shapira 1957; Pihl 1958; Melville 1960). S, beta-aminoethylisothiuronium dibromide (AET) and cysteine have proved to be two of the better drugs utilized. Sedatives, tranquilizers, antibiotics, fluid therapy, and symptomatic medical treatment have also shown to confer some radioprotection and to improve postirradiation clinical condition.

The purpose of our studies has been to confirm or reject the working hypothesis--previously demonstrated in preliminary researches in our laboratories in rats and in many cases reported in the literature (Pihl 1958) using mice--that various protective effects can be combined to obtain an additive or synergistic result in an irradiated subhuman primate. This paper presents the results of combining two drugs which act by a similar mechanism, combining two drugs which act by different mechanisms, the same drug given by two routes of administration, and finally a preirradiation drug system combined with postirradiation medical treatment.

It is also proposed to comment briefly on a new entirely oral treatment which would appear to hold promise and to comment upon a novel, elegant, and simple method to select a better protective treatment in subhuman primates.

The monkeys used in these studies were of the Macaca mulatta strain of Rhesus monkey ranging in weight from 4 to 8 pounds and are pre-pubertal. The animals were screened for normal hematologic values and the absence of pathogenic organisms by parasitologic and bacteriologic examinations. No distinction was made as to the sex of the animals in their selection for the studies so that males and females are randomly distributed. The monkeys were caged individually in air conditioned quarters and baselined for experimental parameters for at least two weeks.

Whole-body x-irradiation of the animals was accomplished with a Picker X-ray machine operated at 250 KVP, 18 ma. with 1 mm. Al and 0.25 mm. Cu added filtration. At a target to object distance of 110 cm., the dose rate was 18 - 20 r/min. The exposure cage was rotated in the vertical plane at 15 r.p.m. (see table I).

TABLE I

X-Ray Dosimetry

Doses: 700r (588 rad), 800r (672 rad)

KVP: 250 + - 10

MA: 17.5 + - 0.5

Filtration: 1 mm Al and 0.25 mm Cu (added)

Dose Rate: 18 r/min + - 2

Cage Rotation: 15 rev/min

Target to Object Distance: 110 cm

S, beta-aminoethylisothiuronium dibromide (AET) was prepared by the method of Shapira (1957). Cysteine hydrochloride monohydrate was purchased from Nutritional Biochemicals Corporation, pentobarbital sodium

(nembutal^{R}) was purchased from Abbott Laboratories, methacholine bromide (mecholyl^{R}) from Merck Sharp and Dohme, thiethylperazine maleate (torecan^{R}) from Sandoz, and 5-hydroxytryptamine (serotonin) creatine sulfate complex from California Corporation for Biochemical Research.

Intravenous doses of AET and cysteine were 100 mg/Kg. body weight of AET and 750 mg/Kg. body weight of cysteine. The chemicals were weighed into a common vessel, neutralized to a pH of 7.2 - 7.4 with 2.5 N NaOH and diluted to 9 ml. with distilled water. 1 ml. of 2% procaine hydrochloride solution was added in order to reduce the trauma of injection. The final mixture was administered 60 minutes prior to irradiation.

Oral doses of AET and cysteine were from 205 to 325 mg/Kg. body weight and 800-2050 mg/Kg. body weight of cysteine neutralized as above, diluted to a final volume of 10 to 25 ml. with 0.5 M phosphate buffer at a pH of 7.2 to 7.4 and gavaged 20 minutes prior to irradiation.

Nembutal doses of 10 mg/lb. body weight were administered intravenously 15 minutes prior to irradiation. A minimum dose was used to effect anesthesia, ascertained by checking the eyelid reflex, so that many times the entire calculated dose was not used.

Mecholyl was administered orally at 182 mg/Kg. body weight using 200 mg. tablets.

Serotonin was administered intraperitoneally in 5 ml. of distilled water at a dose of 20 mg/Kg. body weight.

A combination of penicillin (100,000 units) and streptomycin (40 mg.) in any of several commercial preparations such as combiotic^{R} was administered I.M. daily for five consecutive days beginning on day 4 postirradiation.

Hematology was performed on venous blood withdrawn by femoral puncture. The methods for counting and other determinations were as specified by Wintrobe (1956).

Animals were weighed in tared individual cages; temperatures were obtained using a standard clinical rectal thermometer.

Protection against mortality from doses of x-rays has been accomplished in monkeys using multiple doses of AET given on successive days prior to irradiation (Crouch 1957). Preliminary work in our laboratory in primates indicated that single doses of AET given intravenously or orally confer protection (Melville 1961). This protection is, however, not above 50% survival at 700r and large doses of material are required. Cysteine did not offer effective protection in our animals.

In the first study, as described in figure 1, all protected animals received a single injection of AET-cysteine 60 minutes prior to irradiation. Five of the six treated animals were alive at 30 days; four of six at 120 days; two of six at approximately one year; these two animals are still alive at 450 and 730 days postirradiation. It is pertinent that the dose of AET for intravenous protection has, in this case, been reduced from 140 mg. to 100 mg., or almost 30%. No epilation, vomiting, bloody diarrhea, or febrile responses were seen in the treated irradiated animals. (see figure 1).

Oral radioprotection for many possible utilizations is more feasible but has proved to be more difficult and to require larger drug doses for comparable effects. Our early oral work was accomplished at 525 rad (625r) of x-rays and is shown in table II. This radiation dose killed 75% of the controls in 30 days, comparing favorably with Henshke and Morton (1957).

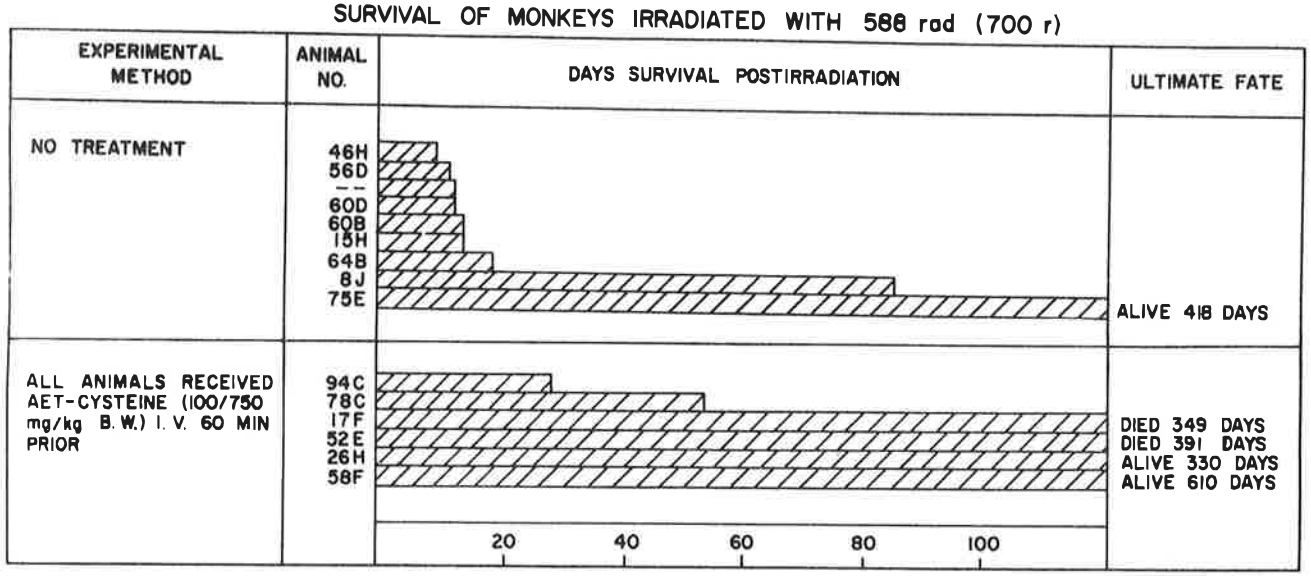

SURVIVAL OF MONKEYS IRRADIATED WITH 588 rad (700 r)
EXPERIMENTAL METHOD
ANIMAL NO.
DAYS SURVIVAL POSTIRRADIATION
ULTIMATE FATE
NO TREATMENT
46H
56D
--
60D
60B
15H
64B
8J
75E
ALIVE 418 DAYS
ALL ANIMALS RECEIVED AET-CYSTEINE (100/750 mg/kg B.W.) I.V. 60 MIN PRIOR
94C
78C
17F
52E
26H
58F
DIED 349 DAYS
DIED 391 DAYS
ALIVE 330 DAYS
ALIVE 610 DAYS
20
40
60
80
100

Figure 1

TABLE II

PROTECTION STUDIES AT 525 RAD (600 r) X-RAYS

Drug	Dose (mg/Kg.)	Route Admin.	Time Prior	Day of Death	Number of Animals
None	-	-	-	12,14,18,69	4
NEG	300	Oral (ST)	15	33,69	2
Cysteine	750	Oral (ST)	15	15	1
Nembutal	To Effect	IV	15	40	1
MEG + Nembutal	300 To Effect	Oral IV	15 15	16,16	2
Cysteine + Nembutal	750 To Effect	Oral (ST) IV	15 15	21	1
MEG-Cysteine	300-750	Oral (ST)	15	16,49	2
MEG-Cysteine + Nembutal	300-750 To Effect	Oral (ST) IV	15 15	300*, 700	2

*Sacrificed

30-day Survival: Controls 1/4
Treated 6/11

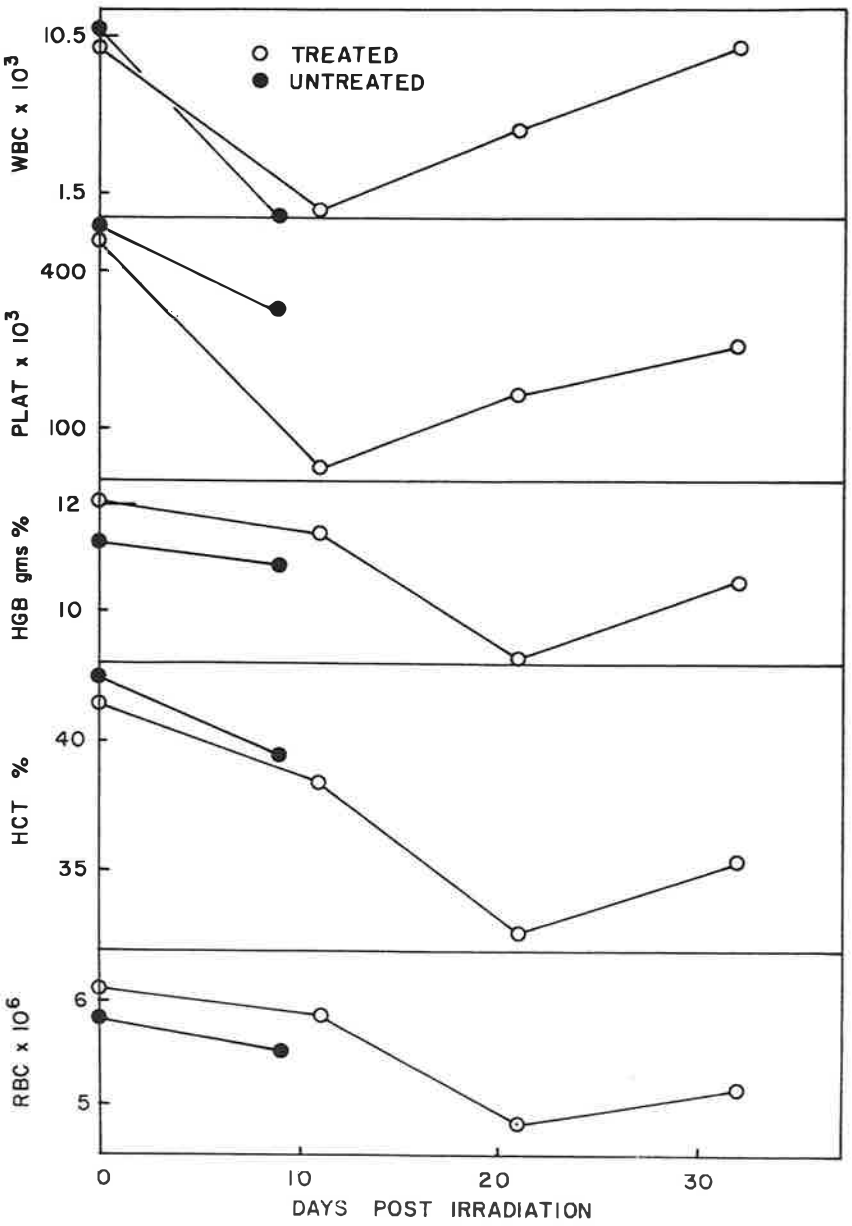


Figure 2: Hemogram for Monkeys Irradiated to 525 r (625 r) of X-rays

Of the treatments here, the combinations of mercaptoethylguanidine (MEG) --which is the form of AET at pH 7.3--and cysteine or MEG-cysteine plus nembutal are most suggestive of protection. Note that the weight ratio of cysteine to MEG has been reduced from 7.5 to 1 to 2.5 to 1; this was done since it was felt that the monkeys could not accept such large doses of cysteine or of total material. It will be seen as we proceed that this assumption is incorrect and the ratio is critical. Six of these animals survived 30 days or more. Figure 2 shows the hematology of these monkeys compared to the three controls who died at less than 30 days; there is no protection but recovery is almost complete by 30 days.

The implication of these studies have been evaluated and confirmed in rodents; particularly the fact that nembutal potentiates the protective effect of oral AET (Melville and Leffingwell 1961). Figure 3 describes the results of combining oral AET-cysteine with intravenous nembutal and combiotic postirradiation. It can be seen that only the total treatment offers truly extended survival time--five of seven animals were alive at 30 days and 120 days and all of these five monkeys are alive now at times ranging from 597 to 723 days postirradiation. The clinical condition of these animals is excellent. The weight and temperature curves in figure 4 show the maintenance of normal values when compared to irradiated controls which spontaneously survive the same dose of radiation.

Figure 5 is a hemogram showing the white blood cell counts, platelets, hemoglobin, and reticulocytes in the peripheral blood for the group just described. Again hematologic protection is not conferred by this treatment and recovery is somewhat slower than at 625r of x-rays. The onset of de-

SURVIVAL OF MONKEYS IRRADIATED WITH 588 rad (700 r)

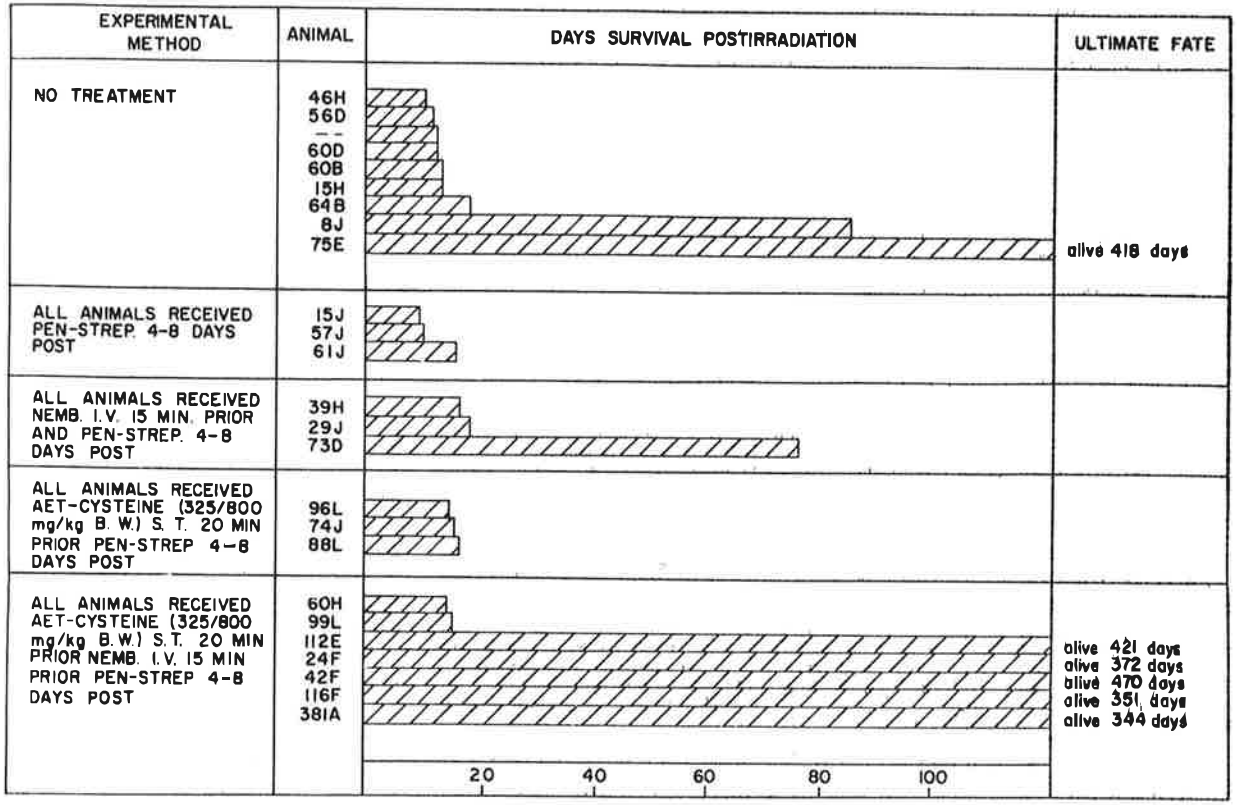


Figure 3

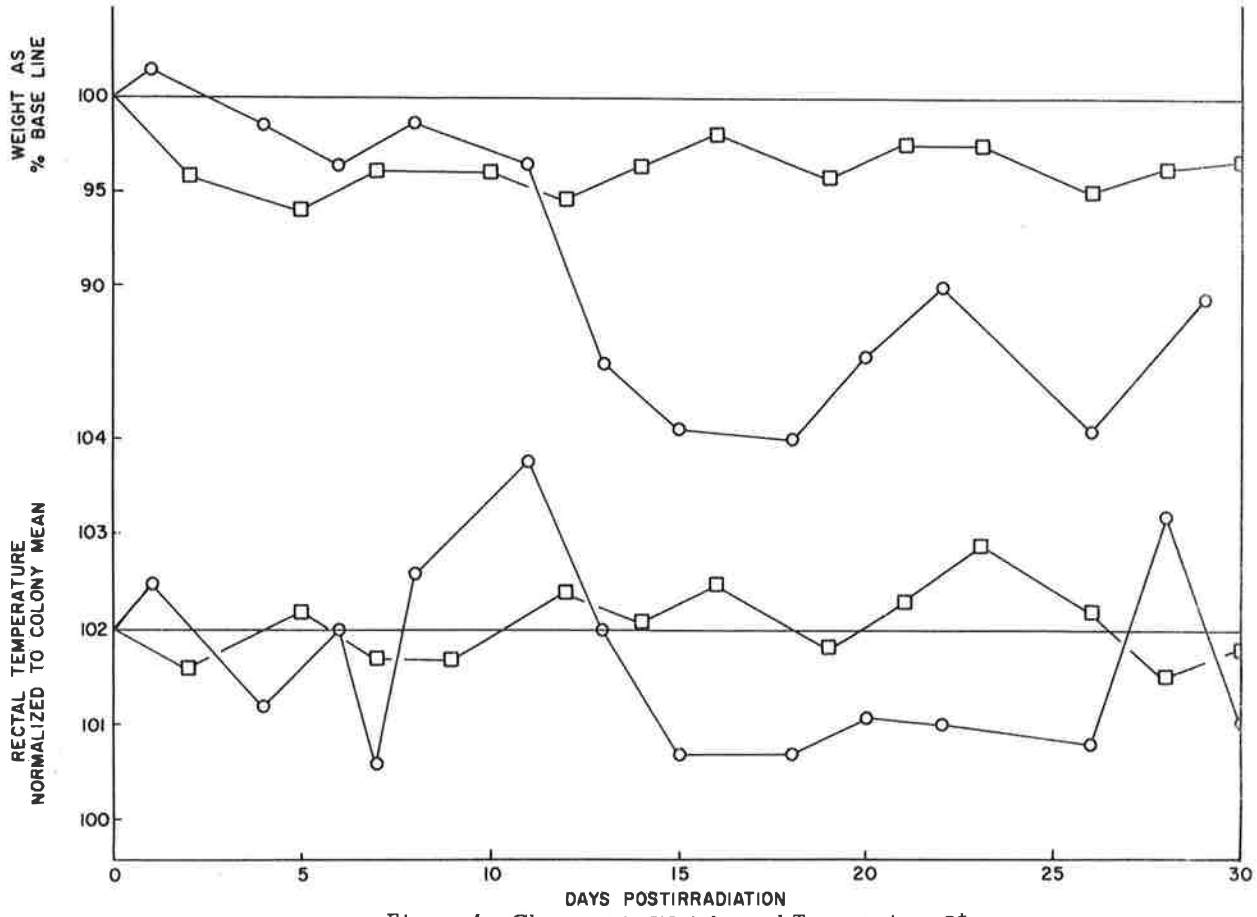


Figure 4: Changes in Weight and Temperature at 588 rad (700 r): Controls o, Treated

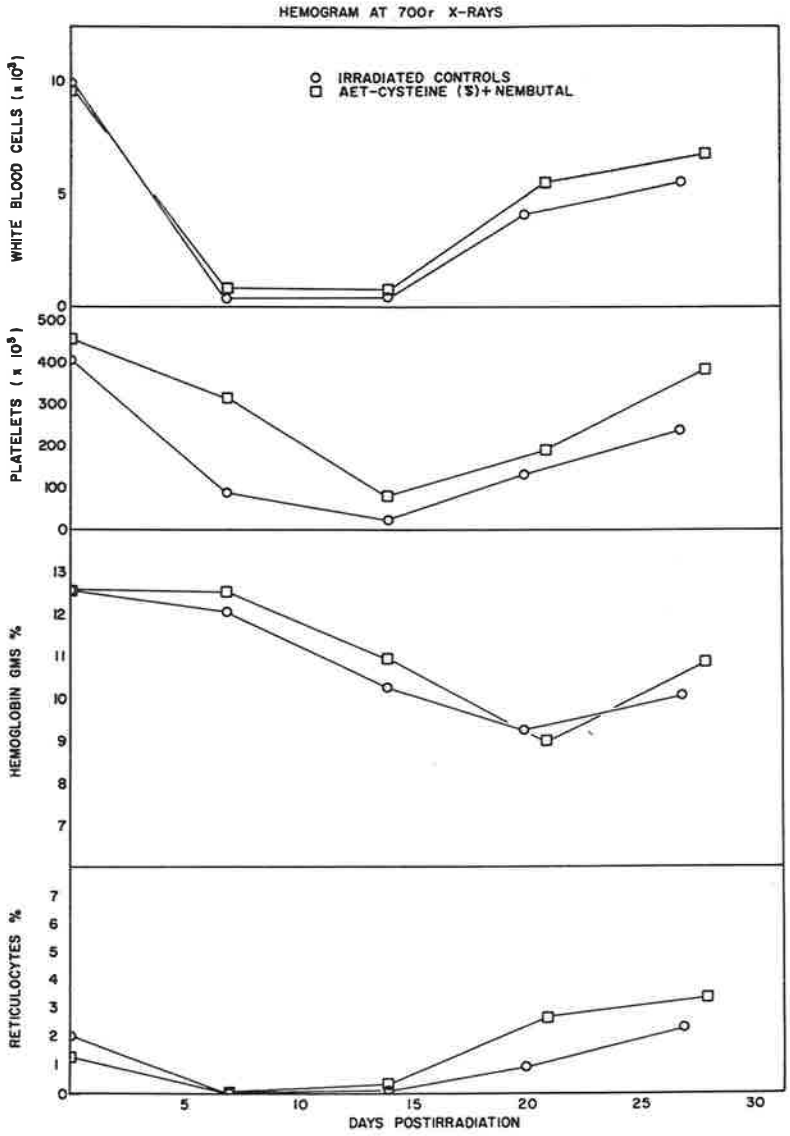

HEMOGRAM AT 700r X-RAYS
IRRADIATED CONTROLS
AET-CYSTEINE (S)+NEMBUTAL
WHITE BLOOD CELLS (x 10³)
PLATELETS (x 10⁵)
HEMOGLOBIN GMS %
RETICULOCYTES %
DAYS POSTIRRADIATION

Figure 5

pression of the peripheral platelet counts is delayed about 6 days. At 45 to 50 days, all these blood parameters are again in the normal range found for the Macaca mulatta under our conditions.

Since 700r (588 rad) is not 100% lethal and protection against higher total doses is desirable, the intravenous treatments with and without antibiotics were accomplished at 800r (672 rads) and are described in figure 6. To achieve significant protection, the penicillin-streptomycin appears to be necessary. Of the three animals alive at 30 days, two are still alive--at 115 and 262 days respectively. Figure 7 shows body weight and rectal temperature curves for the third group of monkeys--those receiving the antibiotic. Note that protection against weight loss is not achieved. However, there is no marked febrile response in the treated animals. It has been our finding that there is often an abortive febrile response in our animals during the period of 20 - 25 days; such monkeys usually recover and live well past 30 days. In figure 8, it can be seen that some blood parameters have not even reached their lowest point by 21 days--two values, however, show trends toward the normal ranges; white blood cells are normal for practical purposes by the 28th day and there is a definite reticulocytosis by the 21st day. These animals experienced some diarrhea during the first 15 days but otherwise did not demonstrate the other gross symptoms of the radiation syndrome (Eldred and Trowbridge 1954).

Despite the fact that these animals, from practical considerations, were run sequentially, the mortality data you have just seen were analyzed using non-parametric statistical methods (Siegel 1956), testing the irradiated controls versus the treated-protected groups. Fisher's Exact Test has been

SURVIVAL OF MONKEYS IRRADIATED WITH 672 rad (800 r)

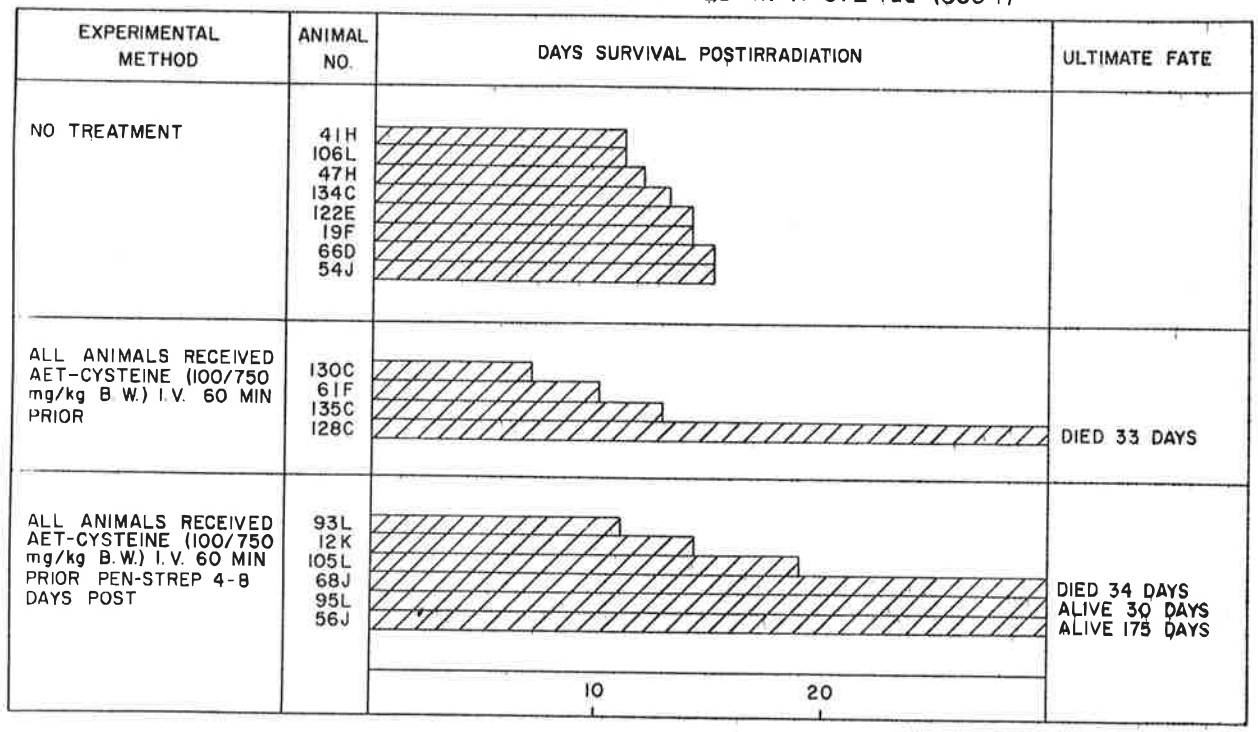


Figure 6

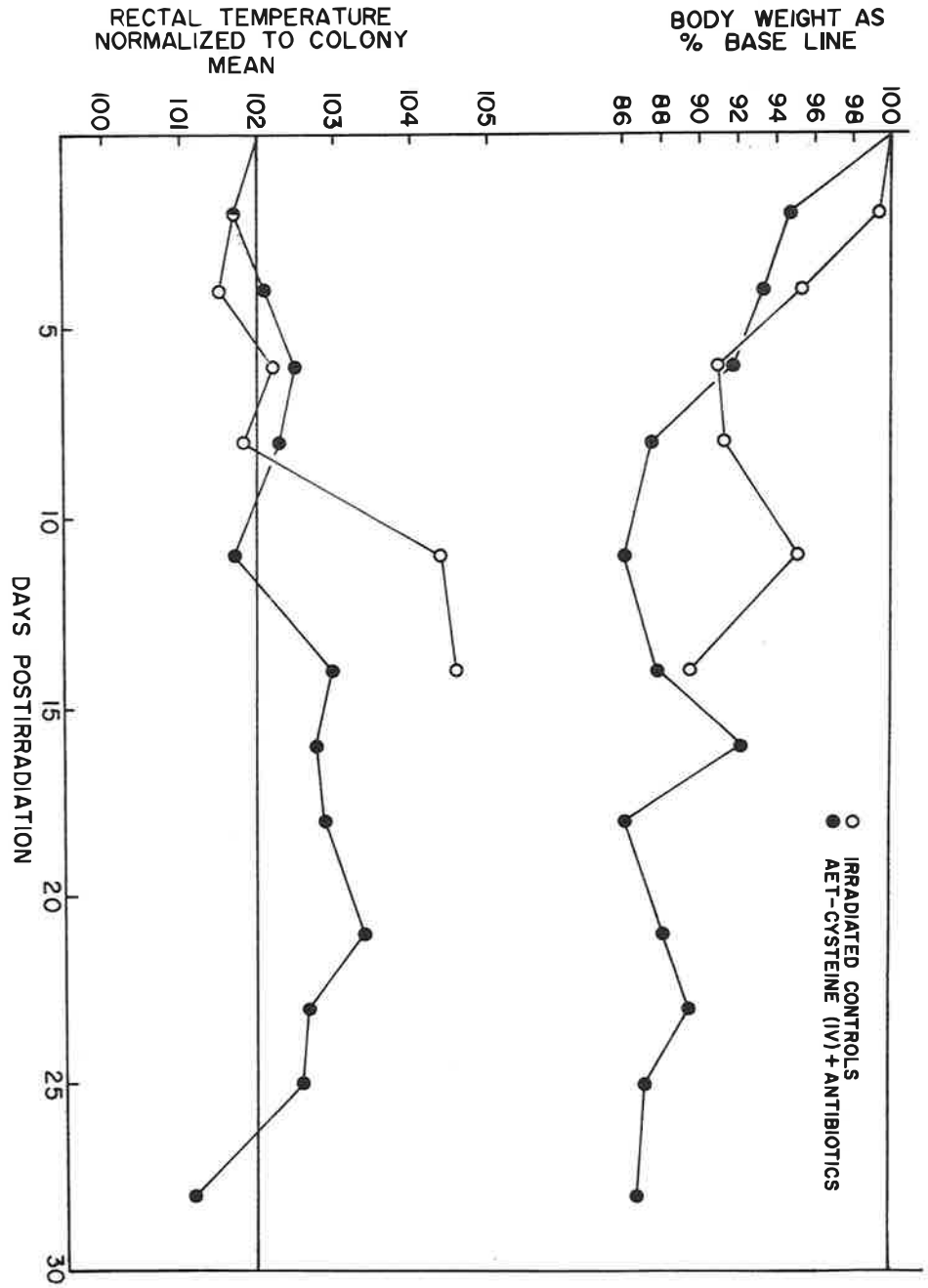


Figure 7: Changes in Weight and Temperature at 672 rad (800 r) of X-rays

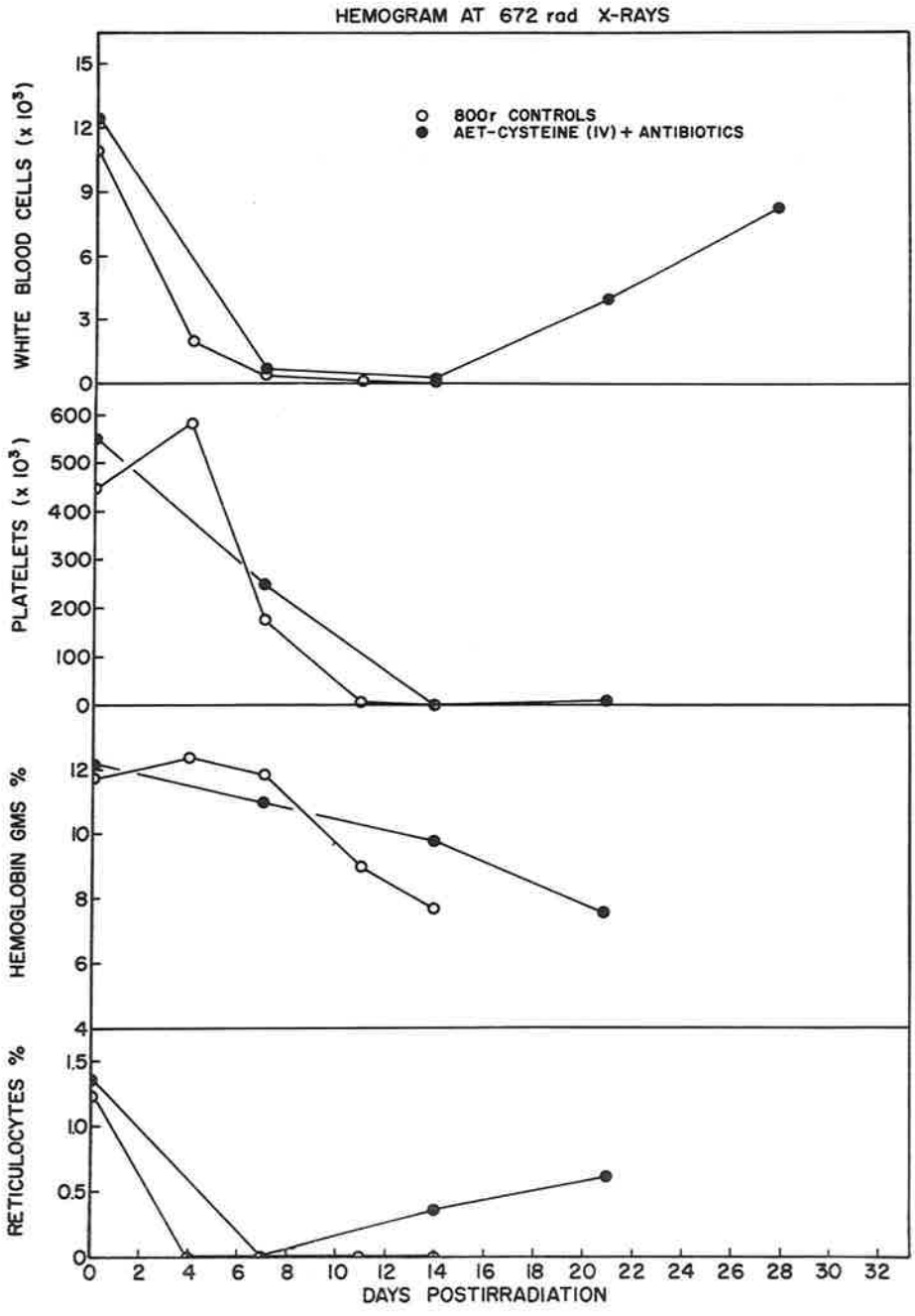

HEMOGRAM AT 672 rad X-RAYS
800r CONTROLS
AET-CYSTEINE (IV) + ANTIBIOTICS
WHITE BLOOD CELLS (x 10³)
PLATELETS (x 10³)
HEMOGLOBIN GMS %
RETICULOCYTES %
DAYS POSTIRRADIATION

Figure 8

used for mortality; the Rank-Sums Test and the Mann-Whitney "U" test for survival time expressed in days--these two tests give comparable answers.

Table III shows the probabilities which were calculated; three treatments are significant, the intravenous AET-cysteine at 700r (calculated at 120 days), oral AET-cysteine plus nembutal plus combiotic^{R} at 700r (calculated at 120 days), and intravenous AET-cysteine at 800r (calculated at 30 days).

TABLE III

STATISTICAL SUMMARY

	Probability	
	Fishers Exact Test	Ranks-Sum Test
AET-Cysteine (IV) vs Controls (700 r)	.044	.02
AET-Cysteine (oral) + Nembutal (IV) vs Controls (700 r)	.023	.04
AET-Cysteine (IV) + Combiotic vs Control	.055	.03

Our work in rodents indicated an oral treatment which did not require sedation to achieve protection at high radiation doses was entirely feasible. The findings indicated that AET-cysteine could be used in this way but the proper ratio of cysteine to AET lay in the range of 6 or 8 to 1. This, of course, encompasses the ratio of the successful intravenous treatments in the monkeys. Table IV shows some of our latest data for the use of oral AET-cysteine at a ratio of 6 to 1 in favor of cysteine. Five animals have been done to date--of these, one animal died on day 1 from drug reaction; the four others all lived 15 days or longer--equivalent to our longest-lived control at 800r. Of these four, two have survived longer than 30 days

and one of these is still alive. It will be noted that two animals received very low doses of torecan; at this level we have detected no adverse clinical effects or contribution to the protection effect. This antiemetic drug does, however, prevent any tendency to vomit or gag excessively during

TABLE IV

SUMMARY OF ORAL TREATMENT AND SURVIVAL

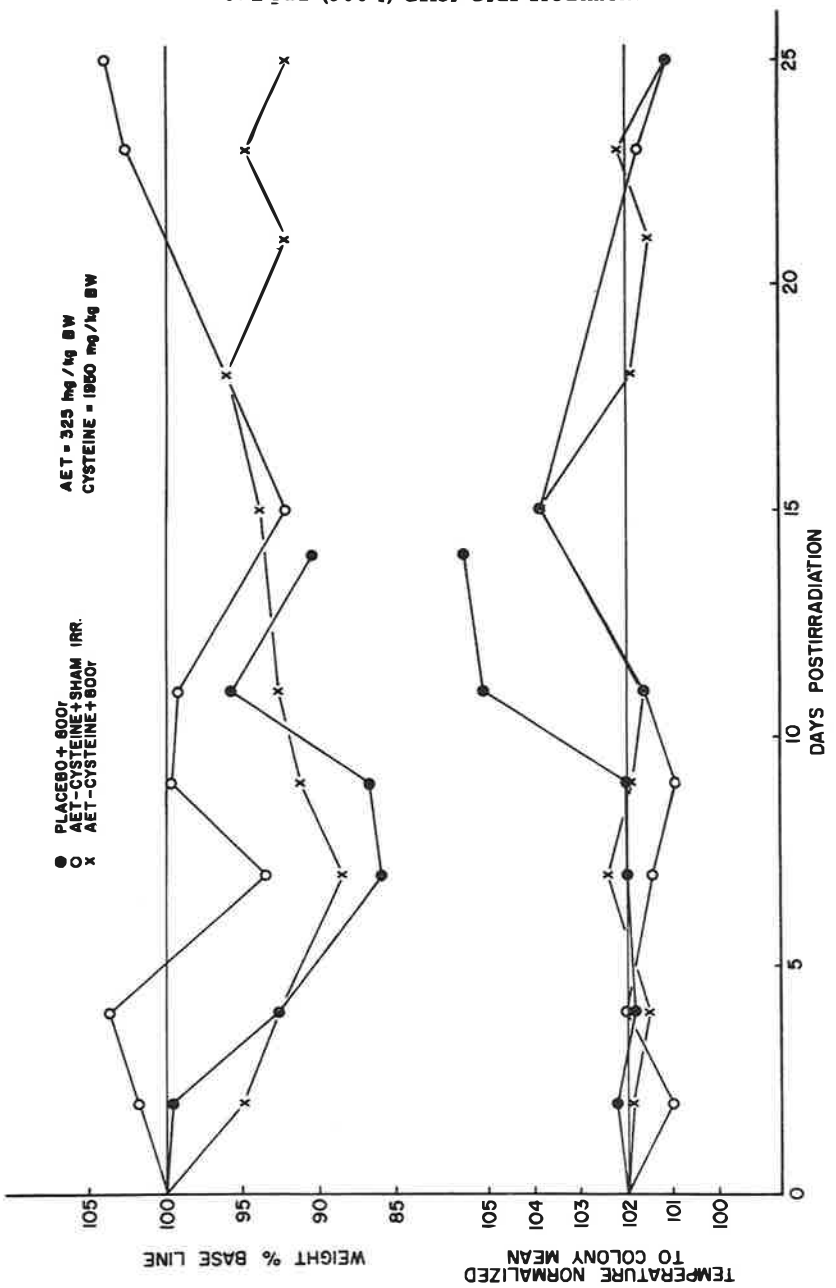
Animal Number	Treatment	S.T. (days)
106 L	Placebo + 800 r	11
54 J	Placebo + 800 r	15
98 L	AET 325 mg/Kg. B.W.; Cysteine 1950 mg/Kg. B.W.;	15
3 J	oral; 20 minutes prior	16
12 J		53
120 M	Torecan, 5 mg., IM, 60 minutes prior; AET, 325 mg/Kg.	1
112 M	B.W.; Cysteine, 1950 mg/Kg. B.W.; oral 20 minutes prior; 4 - 8 days Antibiotics	69 *

*Still alive

the administration of the oral dose--a problem encountered in monkeys which was not encountered in rats. Figure 9 describes the changes in weight and rectal temperatures seen in the animals treated at the 6/1 ratio of cysteine-AET--there is very little protection against weight loss, but recovery is evident during the third week; the febrile response is diminished in the treated animals. Figure 10 is the hemogram for these monkeys; the treatment does not prevent depressions but by the third week postirradiation the white blood cells have returned toward normal and evidences of recovery are beginning to be seen in platelet and reticulocyte counts.

One of the consistent effects of whole-body radiation in the Rhesus primate is an increased responsiveness to self stimuli which is manifest in

Figure 9: Changes in Weight and Temperature at 672 rad (800 r) after Oral Treatment



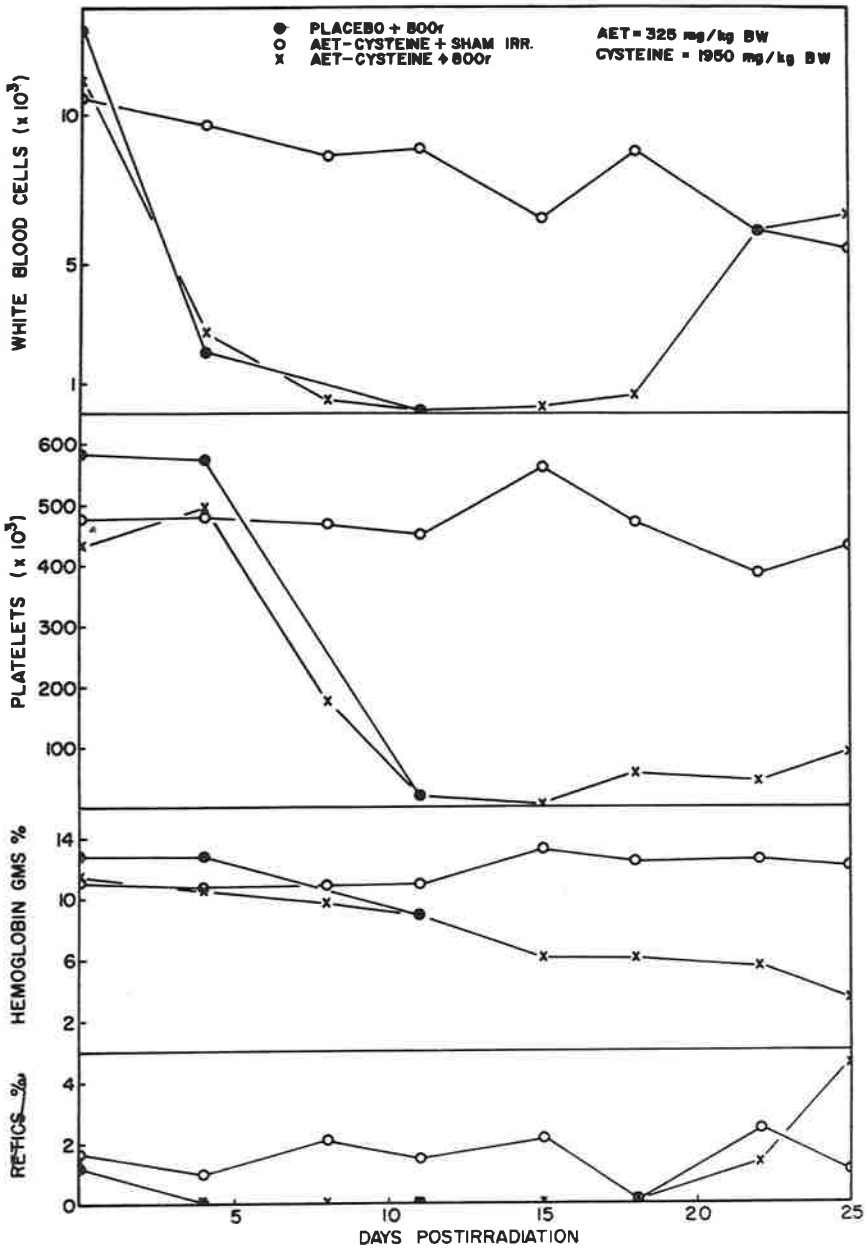


Figure 10: Hemogram for Orally-Treated Monkeys after 672 rad (800 r)

scratching and grooming of body parts and in sleeping during a time of day when normal monkeys are continuously alert and active (McDowell and Brown 1958). Reid et al. (1955) has demonstrated that such behavioral changes occur during irradiation; Hammack (1956) has found that similiar effects follow nitrogen mustard poisoning. It is our finding that these changes persist during the first 60 days of the postirradiation period. The method of obtaining data has been described by McDowell et al. (1956) and in essentials, involves only the observation of the animal at timed intervals in a 3 x 3 x 3 foot cage. The method involves no sampling of the animal or chemical procedures and very nearly everyone can learn to make the observations in a very few days. A classical "double-blind" experiment using appropriate shams, controls, and treated irradiates has shown that knowledge of the animal's history does not account for the results.

The utility of such a variable for the assay of radioprotectants appears obvious; the radiation dosage at which such protectants are tested is so high as to invariably produce the anticipated behavioral changes. This affords the opportunity to select for concentrated scrutiny those protectants which reduce the behavioral effect to the greatest degree although they may not at the present time, protect against mortality. Figure 11 shows the mean self responses for 15 days of three groups of two monkeys each receiving the oral AET-cysteine treatment. One of these treated-irradiated primates lived for 53 days and showed no sleeping and less than 10 self responses until three days prior to exitus when the values rose once again. Figure 12 shows mean self-responses for four groups of animals run at different ratios. The data at the higher ratios is confounded by the fact that the AET dose

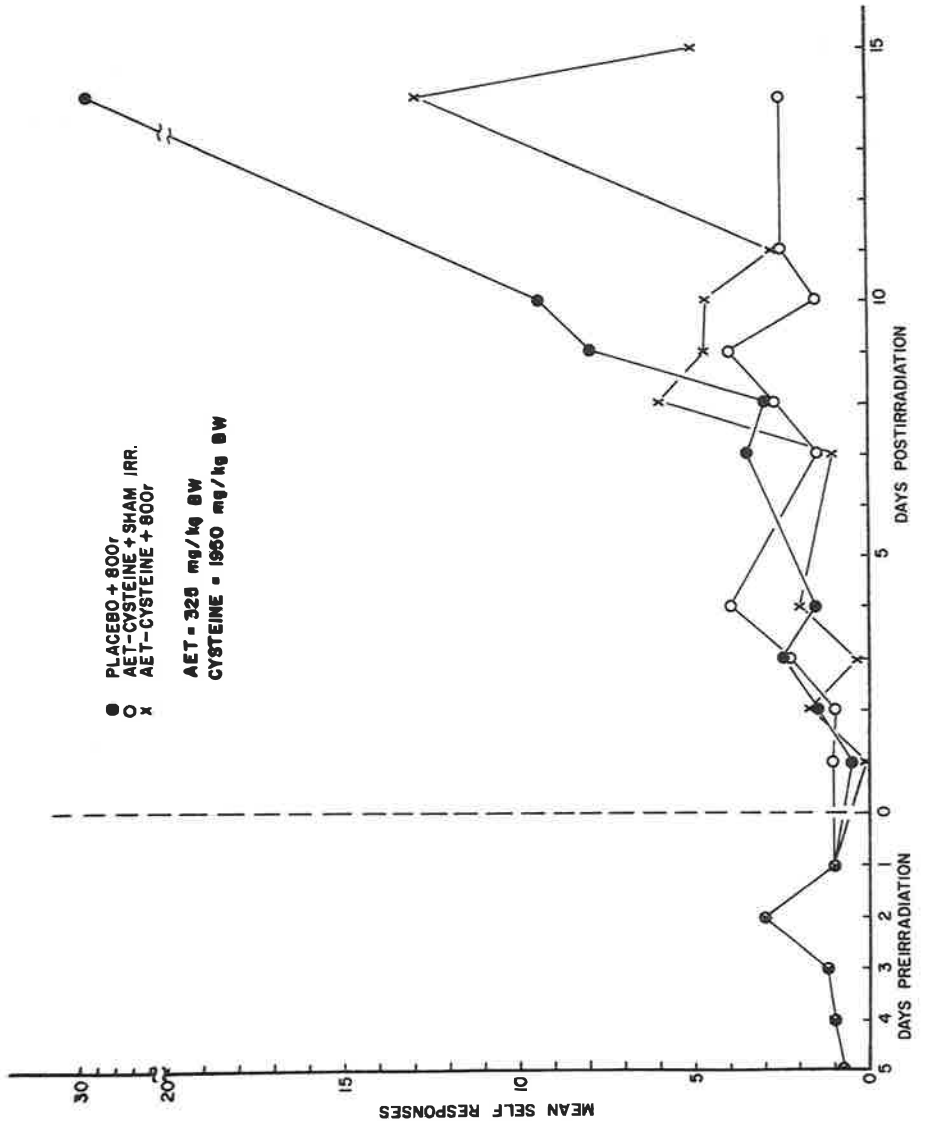


Figure 11: Mean Self-Responses in Orally-Treated Monkeys after 672 rad (800 r)

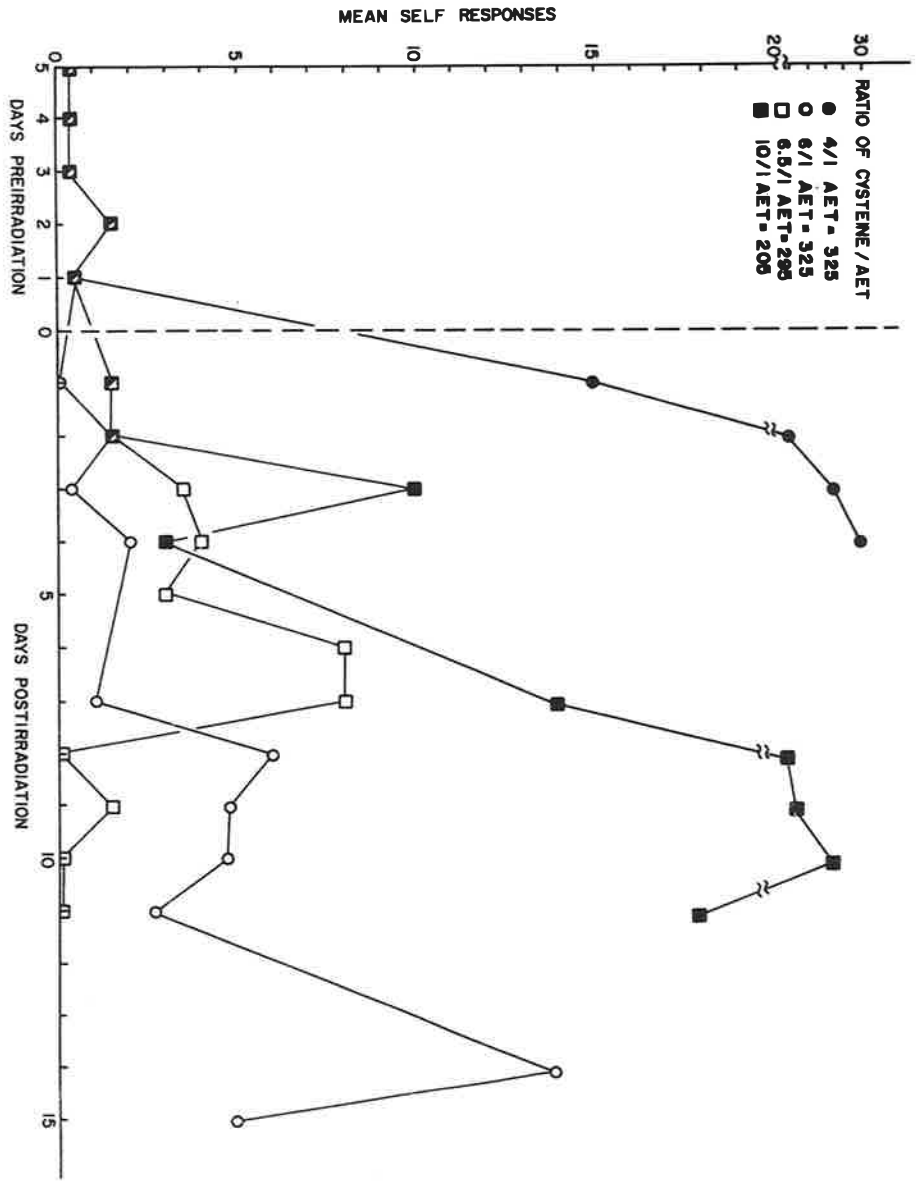


Figure 12: Effects of "ratio" Changes Upon Mean Self-Responses

must be reduced to prevent toxic and lethal effects from the drugs. It is quite clear, however, which treatments confer behavioral protection and our preliminary experiments indicate that our usual parameters will tell the same story. Statistics indicate that the extensions of survival time obtained with oral AET-cysteine (1/6) at 800r are significant at the 10% level.

Table V shows the mortality data for the first experiment accomplished

TABLE V

SURVIVAL OF MONKEYS IRRADIATED WITH 900r OF X-RAYS

	Animal Number	Experimental Treatment	S.T. (days)
Group I	115 C	None	9
	134 F		9
	74 E		9
	13 H		12
Group II	92 E	All animals received postirradiation	11
	93 E	therapy as indicated by the observed	11
	71 E	individual clinical symptoms.	12
	84 E		13
	78 E		15
	114 E		82
Group III	66 E	All animals received AET-DiBr (75 mg/kg	14
	98 E	B.W.) + Cysteine-HCl (225 mg/kg B.W.)	14
	80 E	I.V. 60 minutes prior AET-DiBr (150 mg/kg	19
	555 E	B.W.) + Cysteine HCl (150 mg/kg B.W.)	22
	83 E	oral 15 minutes prior. Nembutal (8-10	60
	125 E	mg/lb B.W.) I.V. 15 minutes prior and	81
	99 E	postirradiation therapy as indicated by	250
	48 E	observed individual clinical symptoms	618

at our laboratory in which radioprotective prophylaxis and medical therapy were utilized in the same group of primates. Mean survival time for Irradiated Controls: 10 days; Irradiated-Therapy-Controls: 24 days--one animal lived 82 days; Protected-Irradiated-Therapy animals: 135 days, 50% of the animals survived 50 days, three of these four over 60 days, and of these

one lived 250 days, the other 618 days postirradiation. The basis for the medical therapy was the treatment of the symptoms noted. The chemical treatment used is more extensive than any of our previous treatments. The approach has been to combine different mechanisms of protection, routes of administration, and sites and rates of absorption: 1) the intravenous dose affords a high blood level of a good radical sump--AET or a homologue--and an additional compound--cysteine--which may be both a radical sump, participate in normal oxidative metabolism, and is relatively nontoxic, 2) an oral dose of the same compounds which provides direct physical contact and presumably absorption in the gastrointestinal tract, and allows the total concentration of drugs to be increased without noticeably increasing the toxicity, and 3) nembutal introduces a drug which decreases respiration (in terms of relative O_2 concentrations), lowers the overall metabolism rates, creates some at least tissue hypoxia, and potentiates the protective effect of AET (Melville and Leffingwell 1961). Figure 13 shows the calorie and fluid intake expressed as the group mean percent of the amount offered. This data would indicate that failure to utilize available fluid is extremely important and the protectants do not alter this stiuation. Postirradiation anorexia is seen in both groups but is less severe and postponed some seven days in the therapy-protection group. Table VI summarizes the pathological findings in this experiment; these findings are typical. The most consistent findings which are not corrected by the chemicals utilized in these studies are the evidence of hemorrhage in many parts of the body and the generalized and severe atrophy of lymphoid tissue even to the almost complete absence of germinal centers. In the course of making pathologic

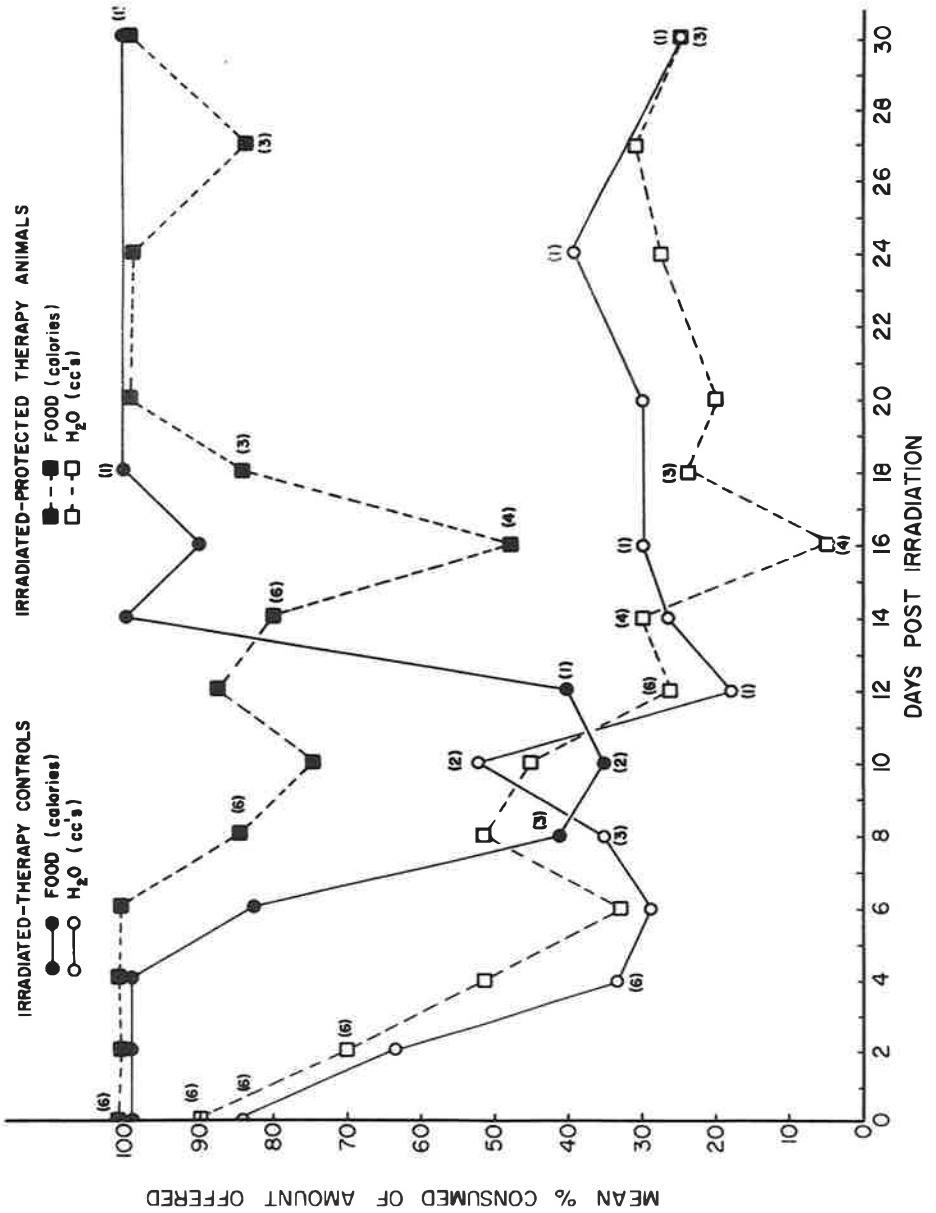


Figure 13: Changes in H_2O and Food Consumption in Treated Monkeys at 756 rad (900 r)

examination of all the decedents in our protection program, a significant number of animals were seen to have survived 30 days, appeared clinically

TABLE VI

PATHOLOGICAL SUMMARY

	Group I Controls	Group II Therapy	Group III Protected Therapy
Hemorrhage	3/4	4/6	6/8
Petechiae	3/4	5/6	3/8
Colitis	3/4	4/6	3/8
Bacteremia	2/4	5/6	4/8
Atrophic lymphoid tissue	4/4	6/6	8/8
Atrophic bone marrow	4/4	5/6	3/8
Parasitism	0/4	1/6	1/8

normal but then became ill and died at times as late as four months after irradiation. A complete discussion has been published (Pitcock and Melville 1962). Table VII summarizes the clinical symptoms in the animals dying subacute death Lymphocytopenia and diarrhea are the two

TABLE VII

CLINICAL FINDINGS IN PROTECTED ANIMALS - DYING SUBACUTE DEATH

Diarrhea	12/16
Inactivity	6/15
Poor Appetite	3/15
Eosinophilia	6/15
Lymphocytopenia	14/15

most consistent findings. Figure 14 shows the time distribution of deaths; within the 30-day period most deaths occur between 10 and 20 days, in the subacute region between 50 and 70 days. A variety of lesions was found at autopsy and the incidence is shown in Table VIII. In general the distribution of lesions is the same in acute death whether the animals were protected or not, but a different distribution was observed in protected animals

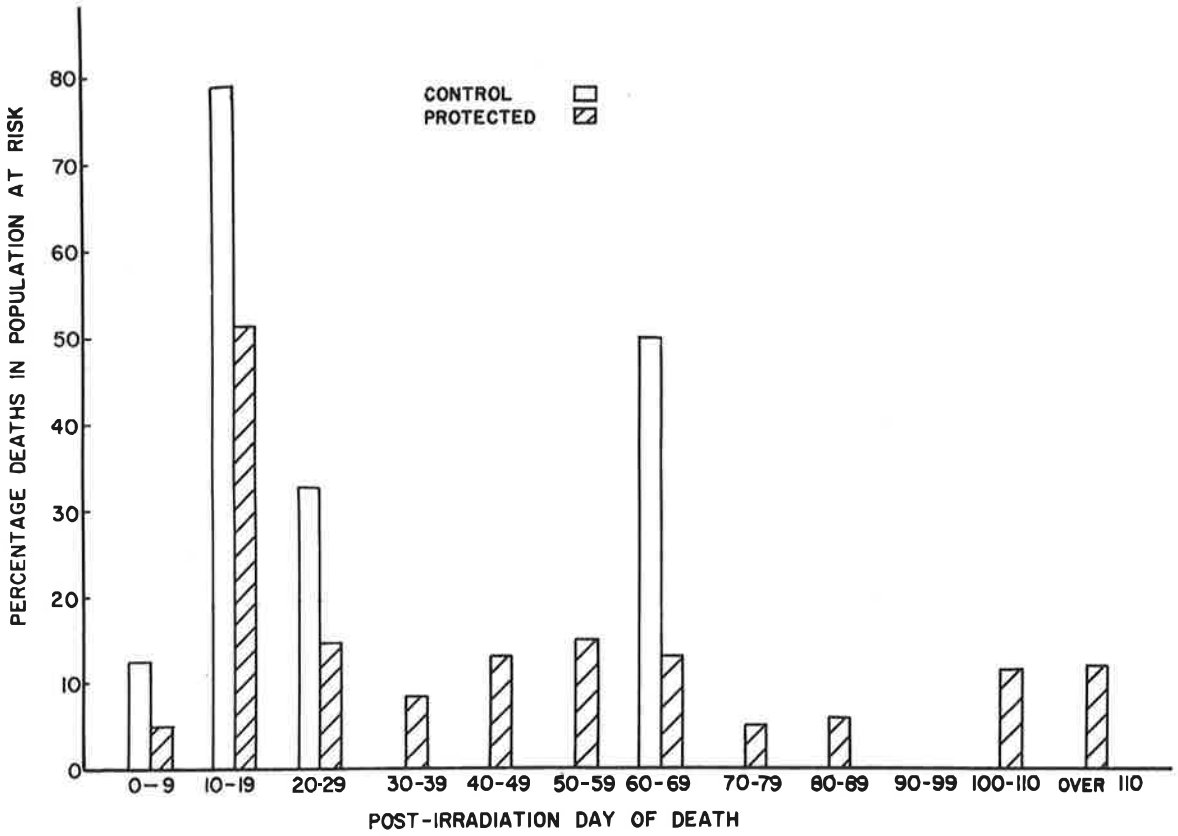


Figure 14: Distribution of Deaths in Irradiated Monkeys

Incidence of Lesions at Autopsy

Lesion	Controls, Acute Death (12)		Protected, Acute Death (47)		Protected, Subacute Death (15)	
Bone Marrow Hypocellularity						
Severe	84%		79%		7%	
Mild	8%		13%		40%	
None	8%		8%		53%	
Lymphoid Atrophy	100%		100%		67%	
Hemorrhagic Phenomena	100%		85%		20%	
Meningitis	8%		2%		40%	
Colitis						
Severe	33%)	75%	45%)	60%	20%)	53%
Slight	42%)		15%)		33%)	
Inflammation and/or Ulceration of Superficial Tissues	25%		30%		0	
Morphologic Lesion of Bacteremia	33%		23%		0	
Abscesses (large)	0		0		13%	
Gastric Lesions	8%		15%		20%	
Miscellaneous Acute Lesions	0		15%		47%	

TABLE 8

dying a subacute death. Meningitis and ependymitis were seen in subacute deaths but only rarely in acute deaths; inflammatory lesions are commonly seen in acute deaths, but not subacute; lesions of bacteremia are again seen in acute but not subacute deaths; lymphoid tissues are, in general, atrophic in both types of death. The bone marrow in animals dying acutely was severely atrophic with some regeneration by 20 days; in those dying subacutely, bone marrow was normal or only mildly atrophic. Bone marrow cellularity as a function of time after death is presented in figure 15.

In our rodents, a drug system using acetylcholine and serotonin creatinine sulfate has proved to be extremely successful; affording dose reduction factors as high as 1.6. The drugs can be administered either separately to the same animal or in a homogeneous mixture. This drug mixture can be combined synergistically with AET-cysteine to achieve protection in rodents at doses as high as 2000r. After some toxicity studies, a special combination study has been worked out in the monkey and the results of this treatment in six animals are described in Table IX. This is a complicated treat-

TABLE IX

SUMMARY OF SPECIAL TREATMENT

Animal Number	Treatment	S.T. (days)
106 L	800r	11
54 J	800r	15
	Mean for all other controls	13
114 M	All animals received 165 mg/Kg. B.W. Mecholyl 90	15*#
38 K	minutes prior orally; AET-Cysteine, 100 mg/Kg. and	18*
26 K	750 mg/Kg. IV, 60 minutes prior; Serotonin 20 mg/Kg.	18+
2 Y	B.W. IP, 30 minutes prior; Cysteine, oral days 1 - 3	20*
5 K	500 mg/Kg. B.W. + Antibiotics postirradiation	58*#
121 M		58*#

+Terramycin *Penicillin-Streptomycin #Still Alive

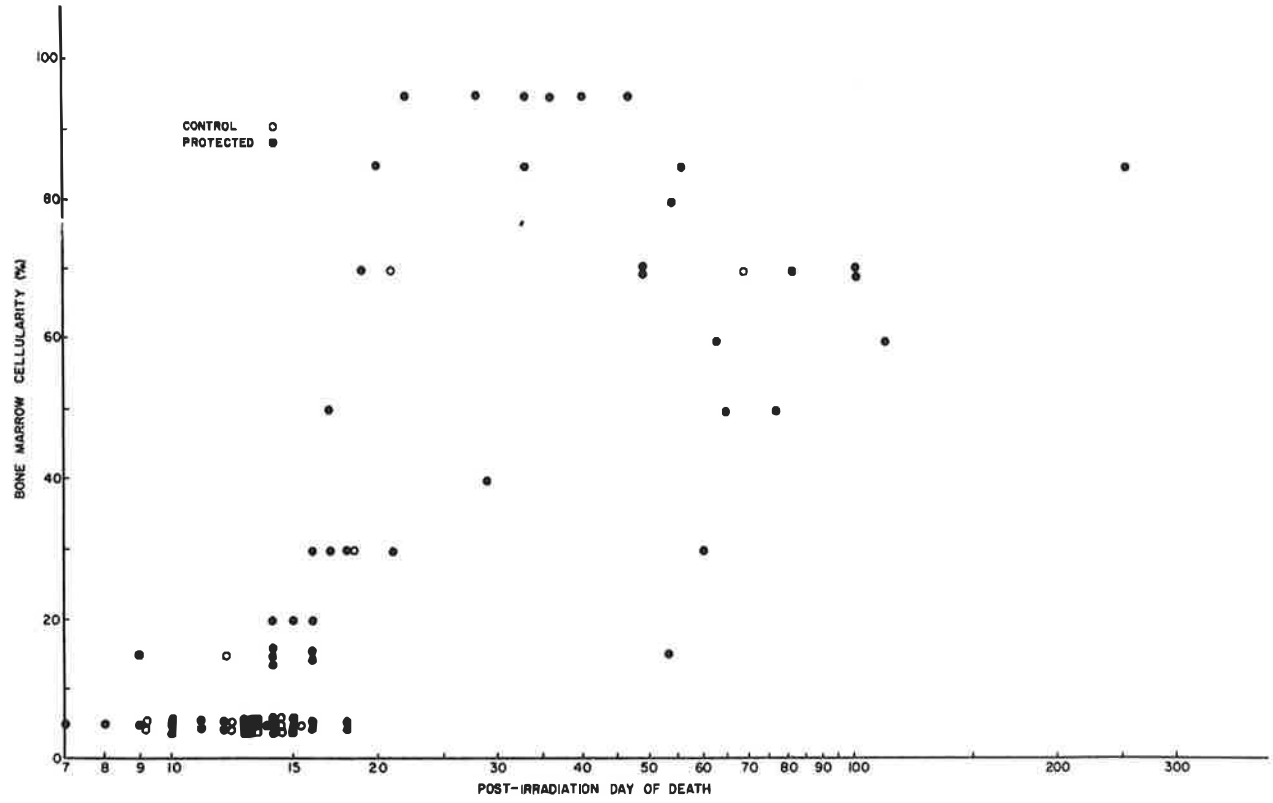


Figure 15: Bone Marrow Cellularity in Irradiated Monkeys

ment involving oral mecholyl--a Methyl analog of acetylcholine, AET-cysteine (IV), serotonin (IP), and cysteine and antibiotics given postirradiation. Four of the five animals are alive and all outlived the 800r controls. The clinical condition of these animals is excellent and there is little evidence of irradiation unless one notes the depressions in peripheral white cells and platelets. It would appear that this treatment will be amenable to use of oral AET-cysteine.

Table X summarizes the present status of our colony of long term survivors. It will be noted that most of the deaths occurred in the subacute

TABLE X

LONG-TERM SURVIVORS

Survival Time Range	X-ray Dose 625r	700r	800r	900r
30 - 45	2	2	2	0
45 - 110	5	5	1	2
110 - 365	3	4	1	1
365 - 730	0	1	0	1
Total Dead	10	12	4	4
Alive	1	9	9	0
Population	1	21	13	4
%	9	43	70	0

death region described a few moments ago. The apparent success at 800r is probably due only to the fact that by the time we began work at this dose, more effective treatments had been developed. There are 20 protection survivors presently alive--19 protected-irradiates and one 700r control-irradiated. In so far as we can ascertain from physical examination, peripheral hematology, clinical chemistries, and cataract examination, these animals are essentially normal.

In summation then, it would seem feasible to combine drugs of similar mechanisms, drugs of different mechanisms, drugs by different routes of administration, drugs and various types of postirradiation supportive treatment. AET-cysteine is a drug system which can be used either intravenously or orally and permits up to 40% reductions in the more toxic material. This basic drug can be used with either a sedative or a vasoconstrictor to achieve protection at lower drug doses or higher radiation doses. The classical depressions of the peripheral blood cell counts and of the cellularity of the bone marrow are not prevented. However, clinical condition is usually improved and survival time is extended even when 30-day mortality statistics are not improved. In effect, the total damage to these primates from these particular radiation doses has been reduced and the normal repair processes are then capable of sustaining life in a majority of the animals. Two new problem areas have been suggested: the problem of both protecting against and treating the subacute radiation death and the problem of protecting lymphoid tissue and function.

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[1] Investigations involving laboratory animals are conducted according to the Principals of Laboratory Animal Care as promulgated by the National Society for Medical Research. (Ref. DOD Instruction 3216.1).

[2] This investigation was supported in part by Air Force Contract AF 41 (657)-382.

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DISCUSSION

DE VRIES In view of the observations by Melville of late intestinal symptoms in chemically protected monkeys which may be related to a subacute form of death it seems appropriate to define briefly the three forms of intestinal lesions we have described in animals treated with bone marrow.

Following radiation doses above 1000 r the acute intestinal syndrome may develop during the first week, independent of the kind of treatment that is given. In the early form of secondary disease in monkeys irradiated with lower doses and treated with homologous bone marrow we have seen wide spread intestinal denudation without much inflammatory reaction and these lesions do not occur in autologous bone marrow treated monkeys which received the same dose of radiation.

In late secondary disease, which is most characteristically seen in mice treated with foreign bone marrow, inflammatory lesions in the colon are predominant and these may be called chronic collitis.

This late chronic colitis occurs also - though less constantly - in mice following repeated irradiation with sublethal doses as reported by Mole and it was described by van Bekkum in lethally irradiated mice treated with autologous bone marrow.

Probably van Bekkum will comment further on this problem.

VAN BEKKUM In order to avoid confusion secondary disease following foreign bone marrow transplantation should be carefully distinguished from any secondary complications that are being observed following irradiation and chemical protection. It seems advisable to reserve the term secondary disease for the bone marrow situation and to use the designation late disease or late symptoms for all other situations, since the picture in the latter cases is not at all clear at present. It is even impossible to conclude whether the intestinal symptoms described by Dr. Melville are comparable to those late symptoms that have been observed

in mice following very large doses of total body irradiation and restoration by isologous bone marrow.

We have maintained since 1959 (van Bekkum et al., J. Natl. Cancer Inst., 23, 75, 1959) that one of the factors that determines the incidence of secondary mortality as well as the severity of secondary intestinal symptoms following bone marrow transplantation is the initial dose of radiation. In other words, any late intestinal radiation damage will be aggravated by the graft versus host reaction, but in the absence of any graft versus host reaction (as for instance in animals treated with isologous bone marrow) late intestinal symptoms may occur during the second month when the dose of irradiation has been large enough. Melville's results are entirely compatible with this concept and it seems similarly possible that the condition in his cases could have been aggravated by the development of lymphoid atrophy which would promote infections in the damaged wall of the gut. The combination of late radiation damage of the gut and lymphoid atrophy can be encountered in a number of situations including isologous bone marrow treatment especially with minimal cell numbers and apparently also following chemical protection. Weight loss (or wasting) is a rather non-specific reaction of the organism to diarrhea and anorexia, whatever the cause of these symptoms be. In cases of chemical protection it should be kept in mind that some protective compounds might provide relatively less protection to the intestinal tract than to the bone marrow and thus promote the development of late intestinal lesions.

MELVILLE tended to agree with van Bekkum on the possibility that the initial amount of damage to the intestinal tract is actually the determining factor in the development of late intestinal symptoms.

PITCOCK also wished to avoid the use of the term secondary disease, mainly because the lesions of the colon in the monkeys reported on by Melville have

no resemblance to the lesions observed following foreign bone marrow transfusions. He found that there was no evidence bearing on the relative importance of lymphoid atrophy to the pathogenesis of late complications. Lymphoid atrophy does seem the most consistent finding and the lesions of the colon are seen less frequently. He had seen a variety of other lesions in these late deaths, which he preferred to call subacute deaths. These lesions include meningitis and ependymitis, which are only rarely seen in rhesus monkeys that die spontaneously.

KURNICK Could somebody comment on the causes of lymphoid atrophy in these animals: whether it is primary or secondary to possibly inanition, intestinal injury or stress in general.

PITCOCK This question cannot yet be answered conclusively but I prefer to think that the lymphoid atrophy comes not secondary to inanition etc. because the lymphocytopenia is present all along, also before wasting begins.

VAN BEKKUM I know of no instance when lymphoid atrophy in itself has been proved to be the cause of colitis, diarrhea, anorexia, wasting and finally subacute death. In all cases where this syndrome was observed the intestinal tract had received a large dose of radiation several weeks before the onset of the symptoms. It is not unlikely that both late radiation damage to the gut and lymphoid atrophy are required to bring about the full syndrome and that either one alone cannot provoke significant symptoms at all.

DE VRIES thought that the thymectomized mice in Miller's experiments developed late symptoms including diarrhea and colitis and this would be an instance of intestinal lesions in mice suffering from lymphoid atrophy without previous irradiation.

VAN BEKKUM As far as I know Miller has not provided any detailed pathological description of the intestinal lesions in his animals and since diarrhea

is not a very dependable symptom in rodents we will have to wait for further information on the thymectomized mice.

Chemical Protection of Rhesus Monkeys against Lethal Doses of X-Radiation*

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Many classes of chemical compounds have been considered as possible protective agents against radiation damage in a variety of laboratory animals. Of the compounds tested as possible radio-protective agents, one of the most effective appears to be aminoethylisothiouronium (AET) which is known to permit survival of rodents for 30 days following exposure to lethal doses of X-radiation. (1,2)

The protective effect of AET varies among species. In dogs levels of AET sufficient for protection are toxic. If, however, cysteine and AET are administered simultaneously, protection against a lethal exposure to X-radiation is obtained. (3) The protective effect of AET in primates has received only modest consideration. Further studies delineating the effect of radio-protective agents in primates exposed to large single doses of X-radiation are of interest for several reasons. Positive protective effects of the sulfhydryl containing compounds administered to monkeys would establish a continuity of the mechanism of action in the vertebrate kingdom. Moreover the phylogenetic relationship of monkey and man would suggest that knowledge of the active dose, toxicity of the drug, as well as the biological effect in monkeys could be more reasonably extrapolated to man. The present report is concerned with hematological, pathological and biochemical alterations which occur in both protected and nonprotected rhesus

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monkeys exposed to a single total body dosage of X-radiation.

Rhesus monkeys, weighing between 4 and 5 kg were exposed to 800 r total body X-radiation, under conditions described previously (4).

Two combinations of chemical compounds were administered, namely: AET and cysteine, and beta mercaptoethylamine (MEA) and cysteine. Cysteine was administered at a dose of 500 mg per kg of body weight, while AET and MEA were administered at a dosage of 100 and 50 mg per kg of body weight respectively. The dry compounds were weighed, solubilized, rapidly neutralized with 3 normal KOH, and the final 10 to 20 ml volume of solution injected intravenously 2 to 5 minutes prior to X-radiation.

A post-mortem examination, including the brain, was performed on animals sacrificed while still moribund, or within 2 hours after death. Samples of tissues were processed for histological examination. A group of animals, both unprotected and protected with AET and cysteine was sacrificed at 15, 30 and 60 days after X-radiation. All animals were killed by an intracardiac injection of 20 ml of air. The spleen was immediately excised, weighed and chilled in cold sucrose. A known amount of spleen was chopped and homogenized in the Potter-Elvejhem homogenizer while the remaining portion of the tissue was fixed in formalin for histological preparation. Bone marrow was obtained for biochemical purposes, by sawing both femoral diaphyses. The marrow was extruded from the bone cavity with the aid of a stirring rod into a tared beaker containing approximately 15 ml of sucrose. The marrow was weighed, transferred into cellulose tubes, and resuspended with a spherical glass homogenizer. This procedure suspended the marrow cells homogeneously without leading to marked cellular destruction. Both homogenates were incubated at 37° for 30 minutes in the presence of C-14 labelled thymidine, under conditions described in detail previously (5). Following incubation, the reaction was stopped with cold 10% trichloroacetic acid (TCA); the precipitate washed with 0.6M perchloric acid, 95% ethanol and Bloor solution;

and the nucleic acids extracted with 5% hot TCA. The specific radioactivity of the hot TCA extract was measured in a Packard scintillation spectrometer. DNA and nitrogen were quantitatively determined according to previously described methods (6).

RESULTS

Survival

The survival of rhesus monkeys receiving 800 r whole body radiation alone or together with either AET or MEA and cysteine is illustrated in Figure 1. Fourteen animals received a single total body exposure of 800 r. Eleven of these animals died within 10 to 18 days following radiation, while 3 animals survived for 30 days. All the surviving animals were exposed to a second dose of X-radiation; within 16 days all animals died. In contrast, 9 of the 11 monkeys pretreated with AET and cysteine not only survived the 30 day period following exposure to 800 r, but were alive and in apparent good health 100 days later, at which time three survivors were injected a second time with AET and cysteine, and re-exposed to X-radiation. These animals died on days 13, 17, and 25 after re-exposure. Two of the animals surviving the single 800 r exposure received no further treatment and are still alive approximately 1 year later. The four remaining protected animals were sacrificed for biochemical studies. MEA and cysteine was only moderately effective in permitting survival of irradiated monkeys for 30 days. Two of five treated animals are still alive approximately 8 months following exposure.

Since the use of monkeys for radiation protection experimentation involves considerable expense, the survival of mice protected against radiation administered under identical conditions was investigated. Figure 2 demonstrates that a combination of the 2 drugs at a concentration identical on a weight basis to that used in primates does not protect mice against lethal doses of X-radiation.

Hematology

A marked depression in total leucocyte count occurred in all animals, both protected and nonprotected, immediately following radiation (Fig.3,4). The white blood cell counts were maximally depressed by day 14, thereafter gradually increasing to reach pre-exposure levels approximately 7 weeks following radiation. The indicated values for week 3 and following for both the nonprotected and MEA and cysteine treated monkeys must be considered with reservation since at these times, only 3 of the nonprotected and two of the MEA and cysteine treated animals were alive.

The hematocrits reached minimal levels the third week following radiation, but returned to control values by the 7th week. (Fig.5,6)

Blood Proteins

The changes in serum gamma globulin concentrations of 2 animals sacrificed 60 days after X-radiation is illustrated in figure 7. The general shape of both curves is similar. A depression occurred at 2 weeks followed by an increase above normal between the 2nd and 4th week after X-radiation, which in one case is followed by an increase to values greater than normal, and in another, by a progressive decrease to slightly below normal values. Since in both cases, bone marrow and spleen regeneration were comparable histologically and biochemically no correlation between regeneration of the hematopoietic tissue and restoration of gamma globulin was apparent.

Pathology

Complete autopsies were performed on 10 nonprotected animals. Two other animals were sacrificed on day 14 after radiation. Considerable reduction in spleen size and a generalized hemorrhagic diatheses were the major findings on gross examination. All degrees of hemorrhage from petechia to massive were observed. Petechia were frequent on the face, particularly around the cheeks and mouth, under the gastric epithelium, the pericardium and endocardium. Small hemorrhagic foci were observed in

almost every organ, routinely in the intestinal mucosa, the myocardium and the lungs, occasionally in the adrenals, the testes and under the dura or the leptomeninges. Massive hemorrhage involved the lungs or intestinal cavity. Histologically, characteristic changes were observed in the hematopoietic system and gonads. Cellular depopulation of the splenic white pulp was marked, with only a few layers of immature lymphocytes, around the central artery of the malpighian bodies. The red pulp was congested and contained small foci of hemorrhage, and of hemosiderin accumulations. The main cell types found in the red pulp were fibroblasts, with occasional polymorphonuclear plasmocytes and lymphocytes; however, no elements of the erythropoietic or granulopoietic lineage were present. The cellular population of lymph nodes or thymus was much denser than that of the spleen, suggesting that either damage to these organs was less marked, or that regeneration developed earlier.

In the testes cells of the spermatic lineage were eliminated except for a few remaining spermatogonia. In the ovaries, oocytes and primordial follicles were rarely found but in most instances a persistent corpus luteum was present.

In the intestine no severe epithelial injuries were observed suggesting that no damage occurred after the administration of 800 r or that regeneration is complete 15 days after irradiation.

The importance of the hemorrhagic foci stems from the damage caused to the surrounding tissue. In the intestine large zones of hemorrhage were observed between the mucosa and the submucosal layers, leading to separation of the layers, and epithelial necrosis. In the myocardium, hemorrhagic zones were surrounded by degenerated myocardial fibers. Similar injuries were observed in the brain. Massive hemorrhage involving the entire lung or a single lobe were found. Vast hemorrhagic zones were often surrounded by foci of either atelectasis or emphysema.

Post-mortems were performed on 7 animals injected with AET and cysteine prior to the administration of 800 r. Two animals each were sacrificed at 15, 30 and 60 days after radiation. One animal died on the 29th day after exposure from a massive bilateral bronchial pneumonia. The sternal and femoral marrow of this animal presented a great cellular density with numerous immature elements. The degree of regeneration of the spleen was similar to that observed in animals sacrificed at the same interval after irradiation. The pneumonia was not of the type usually observed in animals or humans dying of exposure to lethal doses of X-radiation. In contrast to these cases, an abundant polymorphonuclear and lymphocytic exudate was present.

The post-mortem changes observed in protected animals sacrificed at 2 weeks closely resembled those found in unprotected animals except for the absence of hemmorrhage. Thirty days following exposure, however a conspicuous regeneration of both spleen and bone marrow occurred. In the spleen there was a diffuse lymphocytic proliferation, regeneration of the malpighian centers and developing foci of erythropoietic activity. The sternal marrow was highly cellular and contained numerous immature elements mainly of the granulopoietic lineage. The femoral diaphysis of one animal was entirely filled with highly cellular marrow, in another, only the proximal and distal ends of the diaphysis contained active marrow. Sixty days after irradiation, the regeneration of the lymphocytic tissue of the spleen was complete and active vicarious erythropoiesis present. The bone marrow was normal with respect to cellular density and composition.

In 2 animals injected with cysteine and MEA before administration of 800 r, the gross and histological changes were similar to those described in the nonprotected animals.

Three animals which received a second injection of AET and cysteine prior to a re-exposure to 800 r, presented injuries similar to those described in the controls except for an increased tendency to hemorrhage,

particularly in the lungs and at the surface of the heart. In the lungs, marked fibrosis was observed, resulting from sclerotic development in hemorrhagic areas which arose from the first exposure to radiation.

Biochemistry

Although preliminary in nature, the biochemical data are in agreement with the morphological findings. The DNA content of spleen and bone marrow, was identical in both protected and non-protected animals at day 15; but was increased in protected animals at 30 days and was decreased by the 60th day (Fig. 8) Such findings suggest that the rate of DNA synthesis must be elevated between the 15th and the 60th day after X-radiation. This, in fact, was observed when the incorporation of thymidylic acid C-14 into the spleen DNA was determined. Thus at 15 and 30 days the amount of precursor incorporated was proportional to the concentration of spleen homogenate used in the incubation. No differences between preparations obtained from animals sacrificed at days 15 and 30 were observed. In contrast, at day 60 the rate of incorporation, although linear for low concentrations, reaches a plateau at higher concentrations of homogenate. This suggests that by two months after exposure an inhibitory factor has developed in the spleen which interfers with thymidine C-14 incorporation into DNA at high concentrations of homogenate but is diluted out at low concentrations. (Fig. 9)

CONCLUSIONS

From these data the following conclusions appear valid:

1. AET and cysteine are effective in protecting rhesus monkeys against lethal effects of a single 800 r exposure to X-radiation.

2. A marked cellular regeneration of spleen and bone marrow is evident in protected monkeys within 30 days following irradiation.

3. Results obtained in rodents, although of great importance in screening radio-protective agents, cannot a priori be extrapolated to primates.

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ACKNOWLEDGMENTS

The authors acknowledge with gratitude the contributions of Drs. Nelson Fausto and Larry Gottleib to certain of the studies reported in this paper.

TREATMENT	NO. OF ANIMALS	DEATHS		NO. SURVIVING 30 DAYS	% SURVIVAL
		NO. OF ANIMALS	DAYS AFTER EXPOSURE		
800 r	14	1	10	3	21.4
		1	13		
		3	14		
		1	15		
		2	16		
		3	18		
800 r + AET + CYSTEINE	11	1	13	9	81.9
		1	29		
800 r + MEA + CYSTEINE	5	1	14	2	40
		1	16		
		1	18		

FIGURE 1

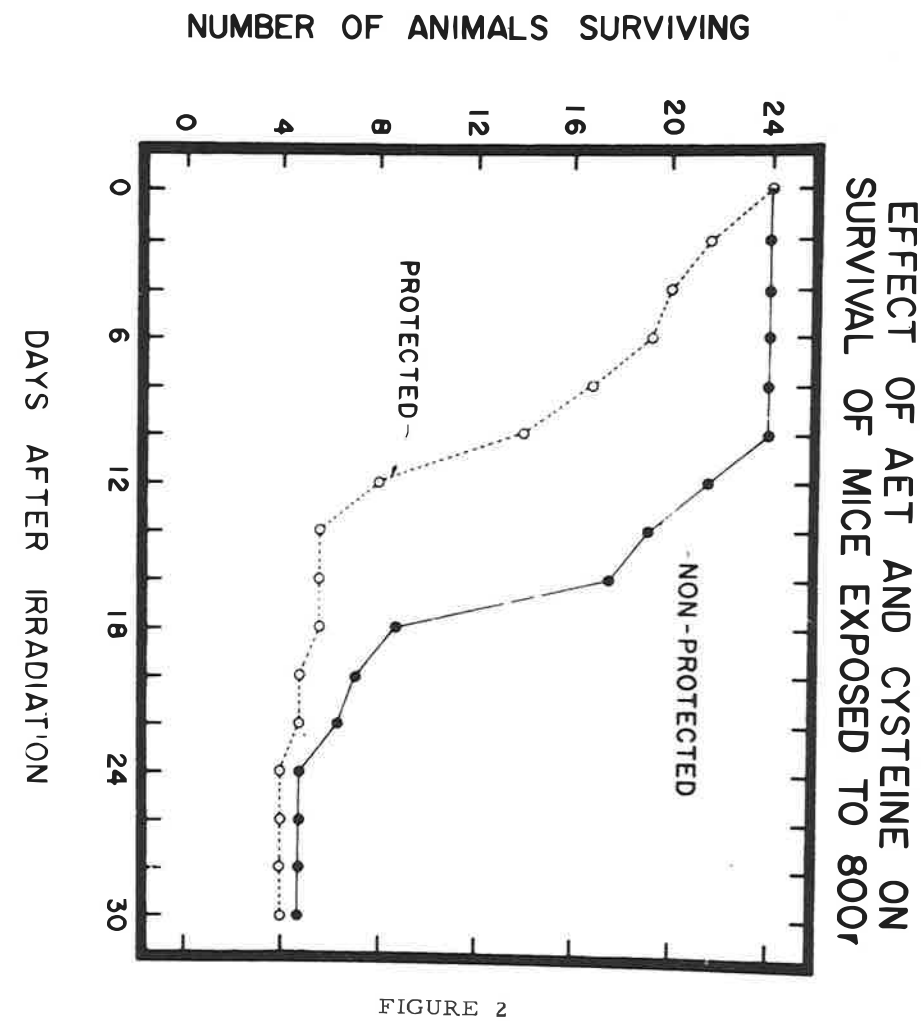

EFFECT OF AET AND CYSTEINE ON
SURVIVAL OF MICE EXPOSED TO 800r
NUMBER OF ANIMALS SURVIVING
0
4
8
12
16
20
24
PROTECTED
NON-PROTECTED
0
6
12
18
24
30
DAYS AFTER IRRADIATION

FIGURE 2

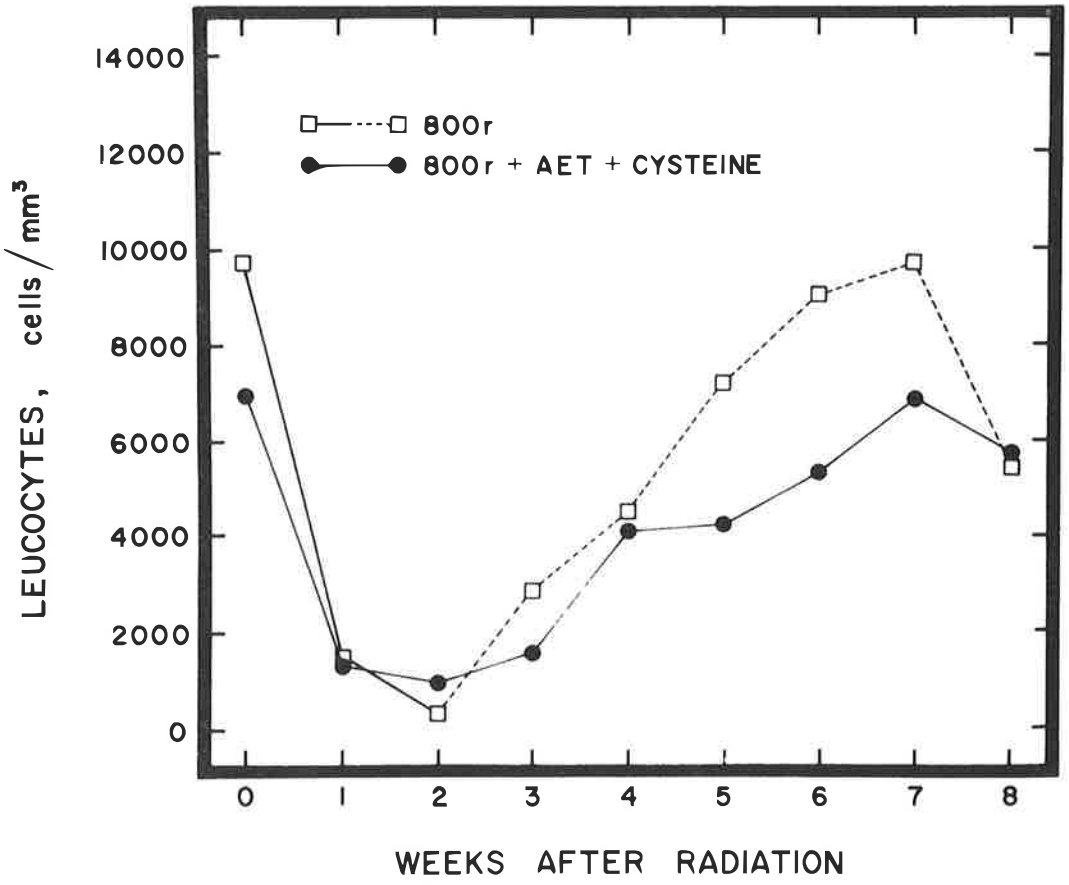

14000
12000
10000
8000
6000
4000
2000
0
LEUCOCYTES, cells / mm³
800r
800r + AET + CYSTEINE
0
1
2
3
4
5
6
7
8
WEEKS AFTER RADIATION

FIGURE 3

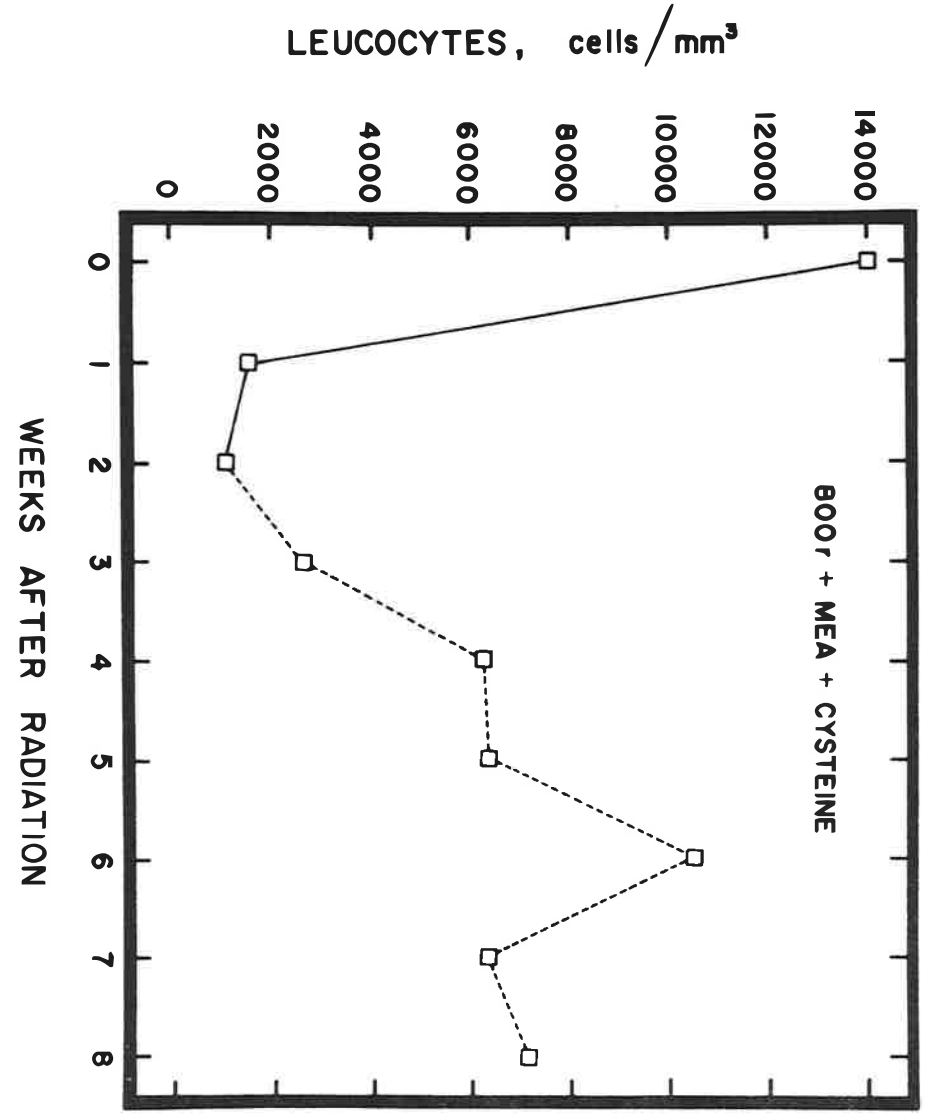

LEUCOCYTES, cells / mm³
0
2000
4000
6000
8000
10000
12000
14000
WEEKS AFTER RADIATION
0
1
2
3
4
5
6
7
8
800r + MEA + CYSTEINE

FIGURE 4

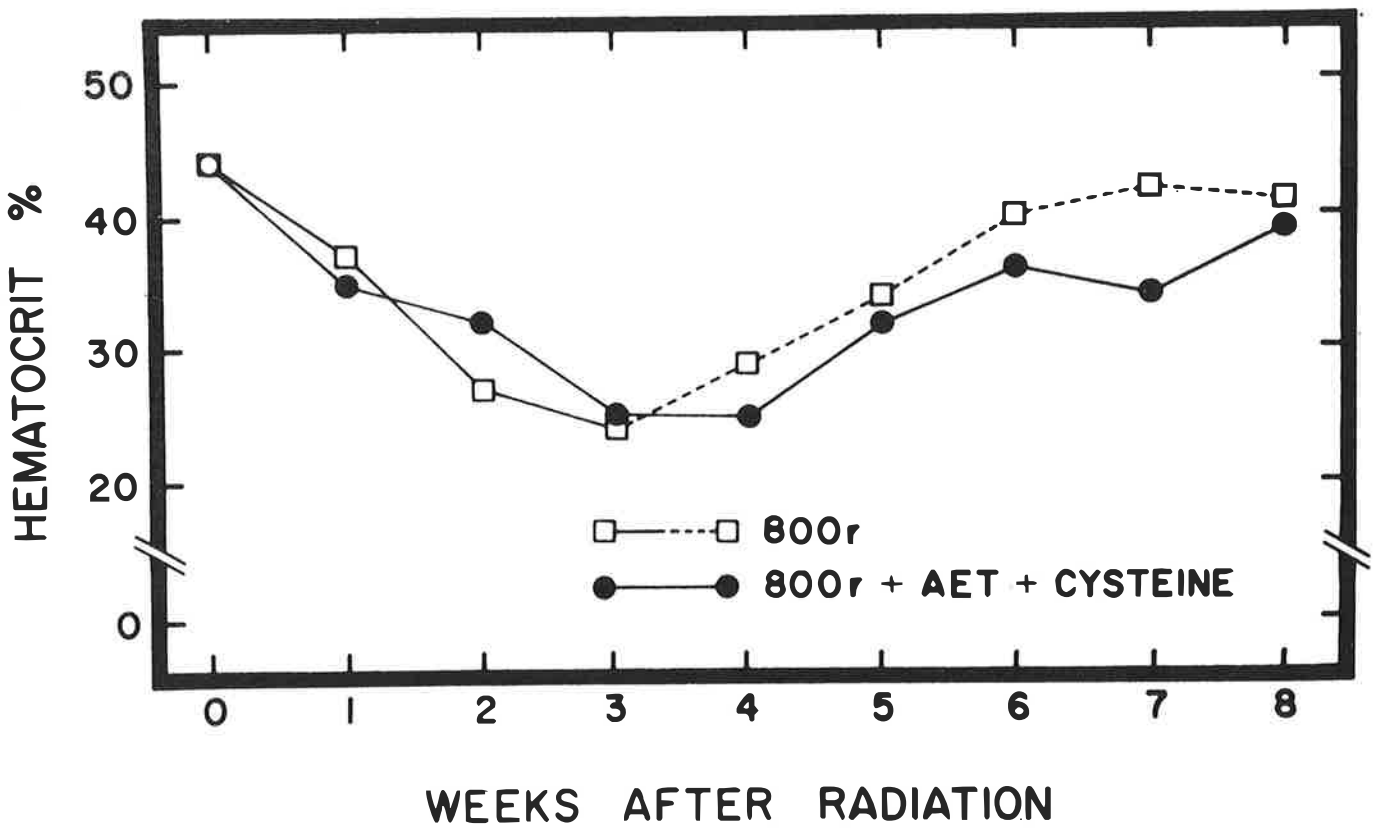

HEMATOCRIT %
50
40
30
20
0
0
1
2
3
4
5
6
7
8
800r
800r + AET + CYSTEINE
WEEKS AFTER RADIATION

FIGURE 5

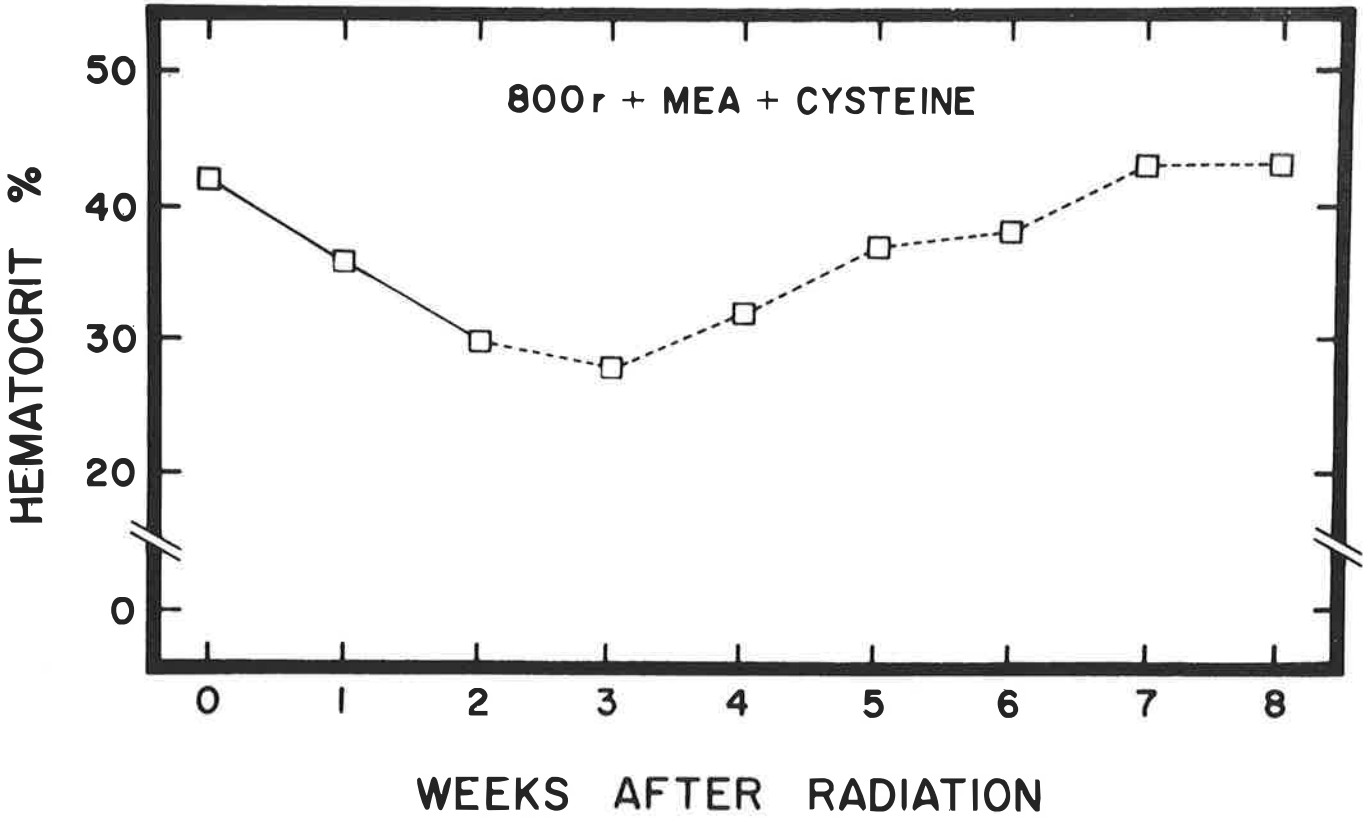

800r + MEA + CYSTEINE
HEMATOCRIT %
50
40
30
20
0
0
1
2
3
4
5
6
7
8
WEEKS AFTER RADIATION

FIGURE 6

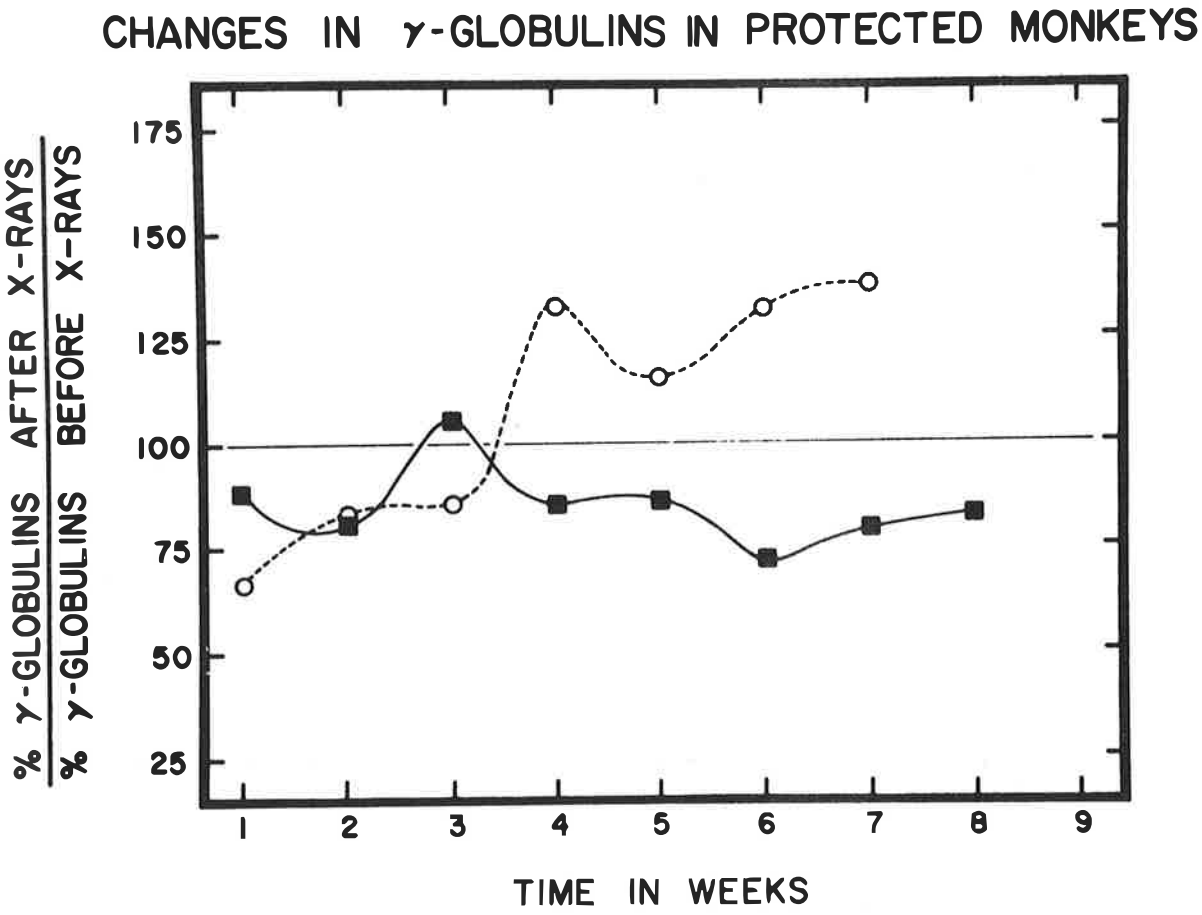

CHANGES IN γ-GLOBULINS IN PROTECTED MONKEYS
% γ-GLOBULINS AFTER X-RAYS / % γ-GLOBULINS BEFORE X-RAYS
175
150
125
100
75
50
25
1
2
3
4
5
6
7
8
9
TIME IN WEEKS

FIGURE 7

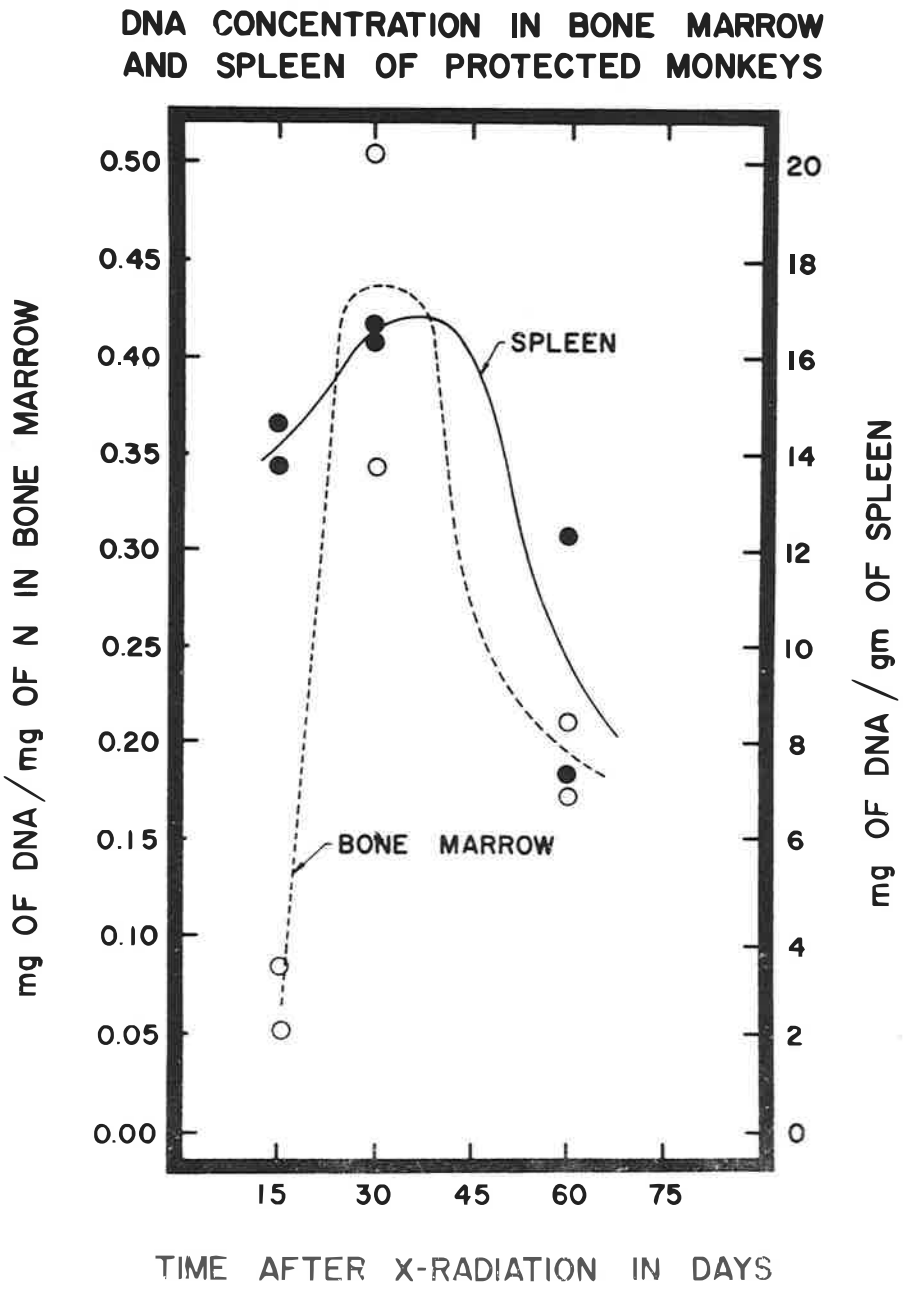

DNA CONCENTRATION IN BONE MARROW
AND SPLEEN OF PROTECTED MONKEYS
mg OF DNA / mg OF N IN BONE MARROW
mg OF DNA / gm OF SPLEEN
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
20
18
16
14
12
10
8
6
4
2
0
SPLEEN
BONE MARROW
15
30
45
60
75
TIME AFTER X-RADIATION IN DAYS

FIGURE 8

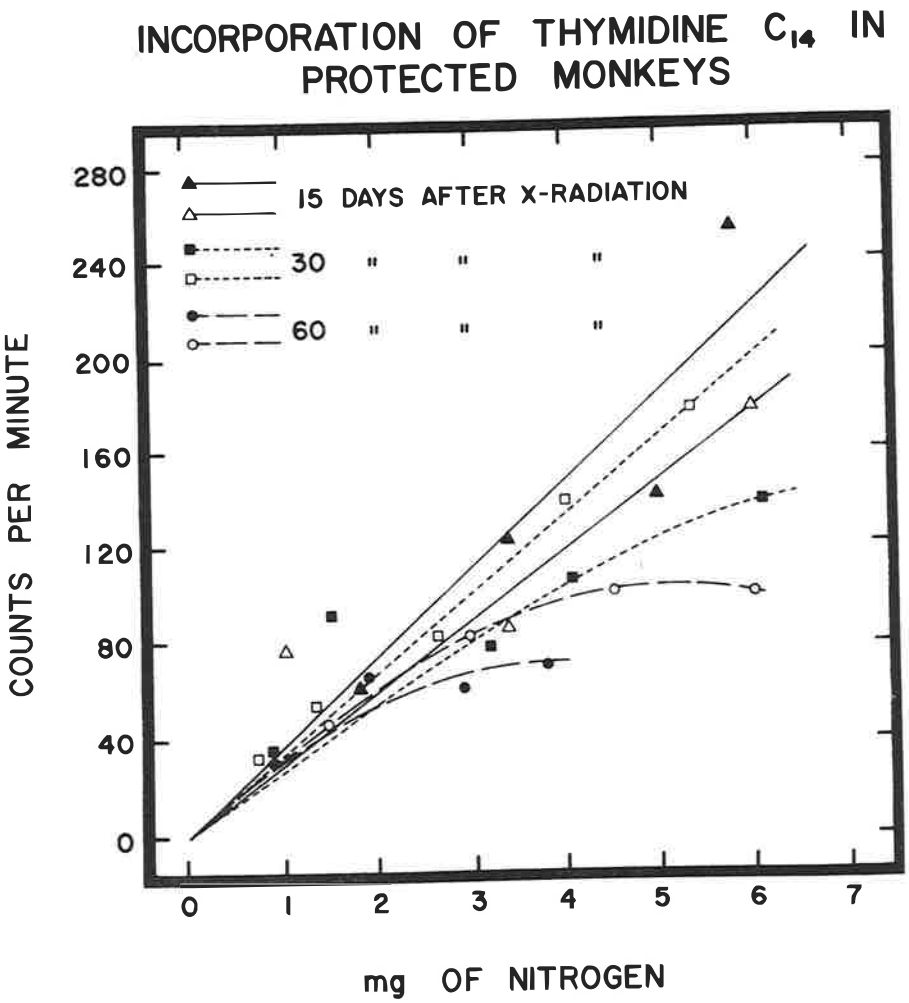

INCORPORATION OF THYMIDINE C_{14} IN PROTECTED MONKEYS
15 DAYS AFTER X-RADIATION
30 " " "
60 " " "
COUNTS PER MINUTE
280
240
200
160
120
80
40
0
0
1
2
3
4
5
6
7
mg OF NITROGEN

FIGURE 9

DISCUSSION

MELVILLE I'd like to inquire again about the doses of AET and MEA that were used and whether or not you tried any different ratios of AET and cysteine?

VAN LANCKER The dose of AET was 100 mg per kg body weight and the MEA-dose was 50 mg per kg. We had no trouble with any of our animals using these doses. We have never killed an animal using AET and cysteine, but I should add that we routinely inject epinephrine while giving AET and cysteine intravenously. We originally tried 100 mg per kg MEA and, unfortunately, loss that animal. Since that experience we have reduced the MEA-dose to 50 mg per kg and have given atropine along with it.

KROHN Might I inquire whether there were any signs of protection of the gonads of the treated animals?

VAN LANCKER I think it is too early for us to answer that. Dr. Wolfe in our laboratory has taken testicular biopsies and we plan to observe some of these animals more extensively.

OVERMAN We have protected both male and female dogs against 650 r whole body irradiation using AET alone and got long term survival of both animals. These were later bred and subsequently a normal litter of 6 pups (3 male, 3 female) was produced.

KROHN I am rather surprized that such a radiation dose did n't destroy all the oocytes in the ovary. I can well believe that spermatogenia would have remained to repopulate the testis, but my understanding is that the ovary is extremely radiosensitive and that unless you breed the animals soon after radiation, the total oocyte population would be gone.

OVERMAN I thought this too at the outset, however my radiologist friends tell me that to sterilize a bitch with X-radiation it is necessary to deliver

at least 2000 r to the ovary. As for breeding time, we did wait beyond 30 days in order to be sure of 30 day survival and it is my recollection that it was several weeks after that before the animals were bred.

KROHN I think that this time interval would be insufficient to remove all the large oocytes but all the primordial oocytes would probably have gone. Certainly in the mouse it only requires 20 or 30 r to completely sterilize the ovary.

BENSON I think we should call attention to the fact that the mouse ovary is uniquely sensitive. We have some investigations under way in Colorado on dogs in which we are giving doses up to 20,000 r X-radiation locally to the exposed ovary and are not consistently producing permanent sterility in this species.

GENERAL DISCUSSION
CHEMICAL PROTECTION OF PRIMATES

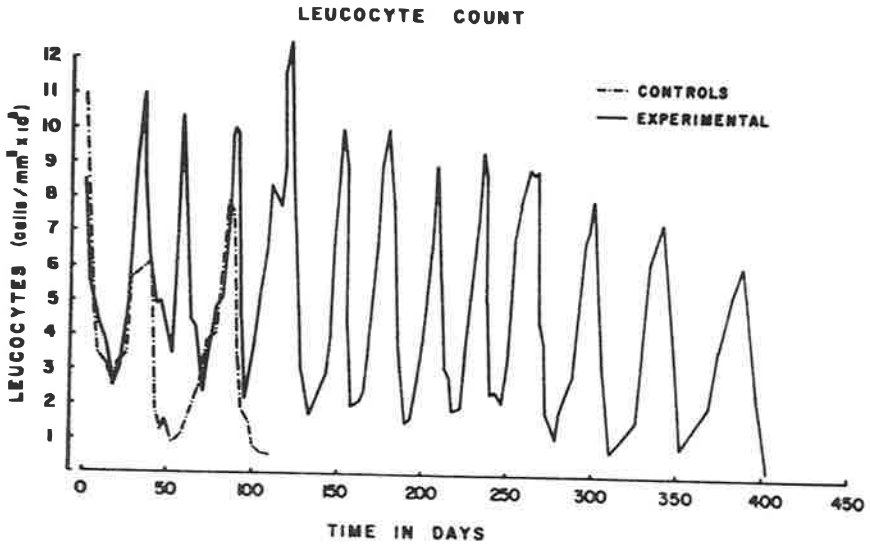
Chairman : R. R. Overman

OVERMAN I might open this general discussion by asking whether anyone here, other than Dr. van Lancker has tried protecting animals against repeated sublethal or lethal doses of irradiation?

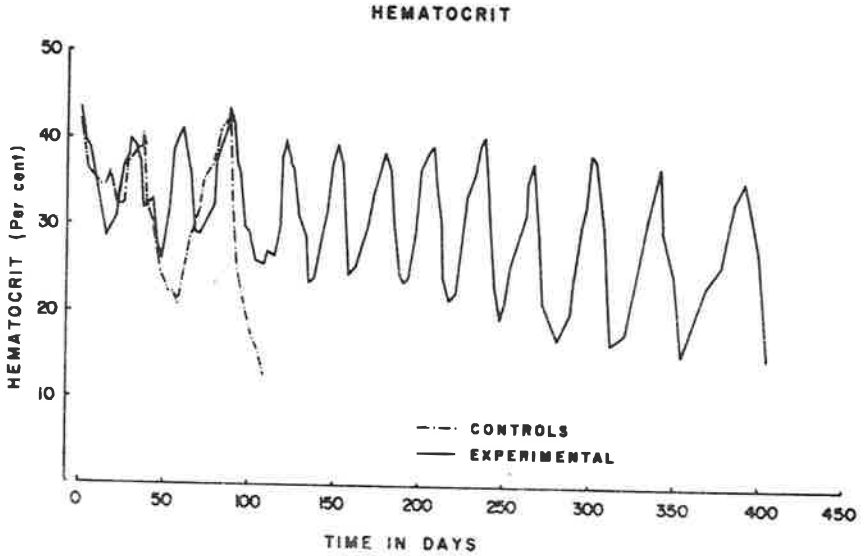
MELVILLE We did an experiment essentially similar to Dr. van Lancker's in which we retreated and reirradiated a chemically protected 800 r survivor. The second irradiation of 800 r was given 180 days after the first and the animal lived for exactly 8 days afterwards.

OVERMAN In preparation for studies to be made in primates, we have conducted experiments on the protection of dogs against repeated sublethal doses of irradiation. This work was done by repeatedly exposing dogs to 265 r at a dose rate of 13 r/min. using a 240 kV X-ray machine. The animals were divided into two groups, one receiving AET plus PAPP before each irradiation and the other receiving no treatment. Between each radiation exposure the animals were allowed to recover in so far as the peripheral blood elements were concerned as can be seen in the 1st slide.
This slide also points up the fact that none of the unprotected animals survived more than 3 exposures to radiation. In contrast, the protected dogs were able to survive 13 exposures and indeed a few survived as many as 15 such reirradiations. It can also be seen from the 1st slide that peak leucocyte counts progressively decline with successive exposures. Slide 2 indicates similar changes in hematocrit. The table in slide 3 illustrates in addition 3 other experiments using various lower radiation and drug doses, in which the same pattern of response was seen.

SLIDE 1



SLIDE 2



SLIDE 3

Irradiation per Exposure	Group	No. Dogs	Drug Dose (mg./kg.) AET	PAPP	Average Recovery Time (days)	Average Accumulated Irradiation (r)	Increase Over Controls %	Average % Non-recuperable Fraction Per Exposure
Series I	Controls	6	0	0	44.0	707	-	58.8
265r	AET	6	75	0	39.4	1413	100	20.7
	PAPP	6	0	2.5	35.6	1506	113	19.0
	AET+PAPP	6	75	2.5	31.8	3268	362	7.9
Series II	Controls	6	0	0	34.5	866	-	45.8
200r	AET	6	50	0	32.1	2000	131	16.8
	PAPP	6	0	2.0	31.5	2066	139	16.3
	AET+PAPP	6	50	2.0	29.9	3550	310	8.9
Series III	Controls	6	0	0	32.5	1066	-	38.3
	AET	6	50	0	31.5	2176	104	17.3
150r	PAPP	6[1]	0	2.0	31.7	2200[1]	106[1]	17.0
	AET+PAPP	3[1]	50	2.0	29.1	2700[1]	153[1]	-
Series IV	Controls	4[2]	0	0	31.2	1189	-	-
100r	AET+PAPP	4[1]	50	2.0	38.0	1300	-	-

[1] One animal still surviving.

[2] Two animals still surviving.

Although the drug combination used confers considerable protection, it is apparent that it is not 100 % effective in eliminating or protecting against non-recuperable injury to the stem cells of the bone marrow.

WHITCOMB Dr. M. D. Harris and I have irradiated the polycythemic mouse preparation of Gurney and Jacobson on 3 occasions at a dose of 200 r at weekly intervals and protected these with AET given 15 minutes beforehand. We then challenged these animals with phenylhydrazine anemic rabbit plasma, and while these are preliminary, the data thus far suggest that we find no difference between the non-irradiated animals and those that were protected, while the irradiated controls showed a decrement in their response. Another point I would like to raise is that some time ago Congdon and Cudcowitz suggested that perhaps AET might protect the immune mechanism less than other biological systems. I would like to say that in the primate, at least, our data suggest that the immune mechanism is indeed protected in animals treated with both AET and bone marrow.

OVERMAN Before bringing the scientific sessions of this symposium to a close, it seems germane to provide a brief summary of our accomplishments. This has been, by modern standards, a small working conference, at which there has been ample time, both here on the floor of the meeting and in numberless informal sessions and conversations, to review in a thorough manner the present state of our combined knowledge regarding the use of bone marrow and other tissues in the post-hoc treatment of radiation disease in the primates and the problems, apparent efficacy and perhaps future hope of the applications of the principles of ad hoc chemical protection against ionizing radiation in these species. A reasonably unique feature of our meeting has been the formal recognition of our problems relative to procurement and maintenance of experimental animals, a subject on which the greatest disparity has often obtained between its importance on

the one hand and its public recognition on the other. Our membership is representative of a number of countries, a variety of disciplines and a broad spectrum of experience. We have come together in a spirit of friendship, offering to share both our successes, which have often been few, and perhaps more importantly our failures which have been legio. In a relatively short time we have passed from the 200 gm Tamarin to 200 lb man, and from the intricacies of immunology, to the necessities of cage cleaning.

We have discovered areas of agreement and often been able, at least to recognize and define our differences. We have reviewed the past, attempted to judge the work of the present, and with the hope, which is a sine qua non to all who profess and call themselves scientists, we have suggested the future. In providing the support and the environment in which these things have been accomplished our Dutch colleagues have presented us with a demonstration of what such a conference can be, have given us a prototype of organisation and service which we might all do well to emulate. I suggest that strong personal and scientific friendships have arisen here, and that our future intercommunication has been assured.

I declare the scientific session of this symposium closed.

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