RAT KIDNEY TRANSPLANTATION

AN EVALUATION OF IMMUNOSUPPRESSION

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CHAPTER I

INTRODUCTION

General

To allow the successful transplantation of tissues and organs – be it for experimental or for therapeutical purposes, a number of problems has to be solved. These problems may be roughly divided into two distinct groups.

The first category includes problems concerned with the process of transplantation itself. They are essentially of a technical nature, such as the procurement, preparation, temporary storage and finally the relocation of the graft in a living organism. In the case of organ transplants the latter has to be accomplished by surgical techniques, e.g. by methods of vessel and duct anastomoses which will result in the grafts' continued viability and the conservation of normal structure and function.

The second category of problems encountered in transplantation is much more complex and originates from the incompatibility of allografts: grafts exchanged between genetically dissimilar individuals of the same species. After a transient period of functional integrity that rarely exceeds one to two weeks, allografts are normally rejected while autografts of the same tissue or organ continue to perform normally. It is this latent unrelenting state of intolerance that has appeared to be the main barrier to successful allotransplantation in man as well as in virtually all other vertebrates.

So far the majority of investigations on the transplantation of intact vascularized organs has been performed with the dog kidney. The reason for this is a most practical one: a generally useful technical procedure for kidney transplantation in the dog had already been developed many years ago.

As early as 1905 Carrel and Guthrie succeeded in transplanting an allogeneic dog kidney to the abdominal cavity, after they had introduced a method of direct suture of anastomoses of blood vessels that has since been adopted for all types of vascular surgery. From then on this technique, after some improvements and modifications, has been universally applied and the dog has appeared to be an excellent experimental animal: the surgery is not difficult and the details of renal function are well established.

As the technical problems had thus been overcome, transplantation studies in the dog could concentrate on the mechanism and prevention of rejection of the allograft. Yet, after the first technical successes of kidney transplantation had been achieved, many years elapsed before effective measures of prolonging allograft survival were reported. This was due to the fact that for a long time the nature of the allograft reaction remained completely obscure. Not until

1944 systematic analysis of the phenomenon was initiated by Medawar. He demonstrated in rabbits that a second skin graft from the same donor has a much shorter survival period than the primary graft. This finding probably provided the most convincing evidence for the notion that the allograft rejection is based on immunological reactivity originating in the lymphatic system of the recipient.

It was only after this idea had been recognized and generally accepted, that the way was open to a rational approach to the control of the allograft reaction.

The first observations of this kind were published by Dempster et al. (1950) and Billingham and coworkers (1951a) who demonstrated that X-irradiation and treatment with cortisone acetate respectively, delayed the rejection of skin allografts in the rabbit. Subsequent studies have since led to the development of various methods of suppression of the immune response, the most important of which are outlined in table I. The effect of non-specific immunosuppression

Table I Major methods of immunosuppression

NON SPECIFIC		SPECIFIC
Chemical and biological agents	Surgical and physical methods	
Antimetabolites: 6-Mercaptopurine Azathioprine Amethopterine	Splenectomy Thymectomy Excision of lymphoid tissue	Acquired immunological tolerance Enhancement
Alkylating agents: Cyclophosphamide	Thoracic duct drainage	
Hormones: Corticosteroids	Irradiation: Total body Graft	
Antibiotics: Actinomycin C and D	Blood (extracorporeal)	
Antilymphocyte serum		

by chemical and biological agents is mainly due to a general lymphoid depletion which is usually accompanied by a bone marrow depression leading to a decreased resistance of the host against infectious micro-organisms. This undesirable side-effect is obviously avoided when the response to donor specific antigens is selectively suppressed, as is the case with immunological tolerance and enhancement. The latter phenomena are induced by means of specific antigen and specific antibody, respectively.

All of the non-specific methods have been tested in the experimental situation, both on skin and on kidney. When a significant effect was observed and

serious toxicity did not occur, these methods were extrapolated to the clinic, often before the basic mode of action had been sufficiently elucidated. At present, treatment with Imuran, prednisolone and antilymphocyte serum is universally applied in human transplantation, and although there is little doubt that matching of donor and recipient by leucocyte typing improves the prognosis (Van Rood, 1969), the i.s. agents so far remain essential in preventing transplant rejection.

Investigations on kidney graft rejection with the dog as test animal, however, have the inherent disadvantage of the inconsistency in the tempo of rejection. The reason for this is that so far most transplantations had to be performed among dogs obtained at random, differing widely in histocompatibility factors. Consequently, a considerable variation may be observed in results, which necessitates large series of experiments and requires the use of many animals.

Allografts between members of two different inbred and thus genetically uniform strains, however, display a more constant and reproducible rejection pattern as has been demonstrated in skin transplantation among rodents. Until recently, no laboratory animal of which inbred strains were readily available could be considered suitable for kidney transplantation, mainly because of the technical limitations imposed by the calibre of the vessels and ureter. When Fisher and Lee in 1965, after more than a decade of experience with microvascular surgical techniques, published a method of renal transplantation in the rat, a model was provided for investigations of kidney rejection under genetically controlled conditions. Since then several publications on this subject have appeared. These came from a relatively small number of research centres, indicating the fact that the challenge of the skill required for this technique has not yet appealed to many investigators in this field.

For years most transplantation biologists felt that study of the allograft problem with intact organs was a waste of time until adequate means of inducing acceptance of skin allografts had been devised. It was argued that skin was far more easy to transplant and that there was no convincing evidence of any difference between the rejection mechanism of skin and organ grafts. Surgeons have finally disproved this premise, and we are now liberated of what Medawar (1965) once termed "the doctrinal tyranny of skin grafts".

Several studies have indicated that the skin and the kidney may not be compared in all respects. Moseley and coworkers (1966) found that a regimen of immunosuppressive chemotherapy that permitted long-term survival of kidney allografts in dogs failed to permit a similarly prolonged survival of skin allografts. This difference in graft survival has been related by some to the fact that the kidney is an important route of secretion of the drugs used (Elion et al., 1961) and may be subject to more intense local drug action. When rejection begins and the excretary function starts to deteriorate, blood levels of the drug would be expected to rise, and this would lead to a decrease in

severity of the rejection response to the kidney. Also, the additional factor of the recipient's uraemia which has been described as nature's own immunosuppressive device (Wilson et al., 1965) favours acceptance of the renal transplant. The additional presence of a well functioning kidney, however, does not significantly alter the fate of a kidney allograft under immunosuppressive therapy, which seems to exclude the mechanism described above.

In any event, it should not be surprising that although the immunological principles may be quite similar, the detailed reactions observed in skin allografts do not always apply to those of the kidney. The larger mass of the renal allograft, the antigens of which drain directly into the venous circulation contrasts with the skin graft which is much smaller and dependant for survival on capillaries only. In addition, the renal glomeruli by nature of their plasma clearance function are apt to be exposed to high concentrations of circulating macromolecules, among which are antibodies.

If, as it appears to be (Kountz et al., 1963), the immunological damage of the vascular bed plays an important role in graft rejection, it should be realized that the greatest part of the ultimate vascular network of the skin graft is genetically not a part of the graft but is instead derived from the host. The transplanted kidney, however, as all other vascularized organs retains its own endothelium, all of which including the large vessels may thus be subject to immunological attack. Moreover, the experiments of Barker and Billingham (1967) demonstrated that prolonged acceptance of skin allografts in guinea pigs could be obtained when they were transplanted onto a full thickness vascularized skin pedicle in which the lymphatics had been interrupted. This emphasizes the importance of the lymphatic system in the induction of skin allograft rejection. In contrast, the role of lymphatics in the afferent pathway of kidney rejection has been proved to be of little significance, since Hume and Egdahl (1955) and later Vetto and Lawson (1967) showed that rejection was unmodified in dog kidney grafts permanently devoid of lymphatic connections.

Recently, some more interesting differences in behaviour of skin and kidney allografts without immunosuppressive therapy were reported. White and coworkers (1969) and Mahabir et al., (1969) obtained a greatly prolonged, if not permanent, survival of kidney allografts in unmodified inbred rats when the donors and recipients were matched at the AgB (R_tH-1) or major rat histocompatibility locus. This was in spite of the fact that the strains involved differed at numerous minor histocompatibility loci and vigorously rejected each others' skin allografts. Previous findings of Linder (1961) did show that when skin and ovarian allografts were transplanted across a weak histocompatibility barrier in mice, the skin grafts were rejected promptly whereas the ovarian grafts were accepted for a prolonged period. This investigator had demonstrated earlier that skin rejection does not necessarily predict the survival of other tissue allografts.

It has thus been sufficiently recognized that the rejection processes in skin and kidney are quite distinct phenomena and the question remains whether the kidney may be considered representative for all vascularized intact organs.

Calne and associates (1967) reported on liver transplants in the pig, which survived consistently for long periods of time without immunosuppression in immunologically mature animals, even when the donor and recipient were from widely disparate genetic sources. The behaviour of the liver was in marked contrast to that of kidney and skin transplants, which were rapidly rejected in similar pigs. Comparable results, however, have never been obtained with the dog, indicating that species specific factors also play a role in organ transplantation. There is increasing evidence that the survival times of grafts vary with different organs in different species.

In our laboratory it was recently demonstrated (Van Bekkum et al., 1969) that a longer survival of the rat kidney as compared with that of the heart allograft could be achieved under identical genetical and immunosuppressive conditions. A similar difference between kidney and heart allograft rejection was observed by Freeman and Steinmuller (1969) with unmodified rats in a weaker histo-incompatibility system. Consequently, it may be stated that each organ has its own qualities in transplantation, part of them possibly influenced by specific differences in tissue antigenicity (Gittes et al., 1964).

The kidney is an ideal organ to transplant because it is paired, its function can be easily assessed and its vascular supply is uncomplicated compared with many other organs. Consequently, clinical transplantation of the kidney is far more advanced than that of any other organ. This may also be due to the fact that failure of the graft will not be fatal, since in that case the possibility remains of regular haemodialysis treatment.

However, human kidney transplantation is still in the stage of development and although the results have improved considerably with time, the optimal immunosuppression available at present has not yet led to a high proportion of indefinite acceptance of a kidney allograft as with the identical twin situation. In order to gain better results in kidney transplantation, the need for investigations exploring new and more effective immunosuppressive agents is obvious and the kidney itself should be the object of such studies, considering its quite distinct organ specific qualities.

Purpose of the present study

The primary purpose of the present experiments is the screening of some immunosuppressive agents on the survival of kidney allografts under genetically controlled conditions. As stated before this has not been possible until recently, since larger laboratory animals than the rat did not provide a proper model for this purpose.

The use of the rat in this type of study, besides avoiding the problem of variable histocompatibility, offers additional advantages over other animals currently used in allografting. The costs of a rat and consequently of each experiment are relatively low. In addition, the housing of great numbers of rats for long periods of observation is possible in a limited space. The room needed for the housing and care of an equal number of dogs, supposing that they could be handled, would be more than a hundred times as large and more personnel would be needed. Moreover, it was known that surgery on rats does not require strict sterile conditions and that operations on the rat take less time and can be performed by a single operator. Finally, another important advantage is the fact that by the use of specific pathogen free rats, one may reduce the incidence of infectious complications that occur frequently during immunosuppressive treatment with conventional animals. Thus the mere evaluation of the rat kidney model for its routine use, allowing the production of relatively large series of transplantations is in itself worthwhile and represents another objective of the present study.

After the technique had been sufficiently controlled by means of isogeneic and allogeneic transplantations, the effects of immunosuppressive agents were studied, most of which with an already established positive value, both in experimental and in clinical kidney grafting. These methods include the application of Imuran (azathioprine), prednisolone, local irradiation of the graft, splenectomy and thymectomy.

When the predictive value of the model had been demonstrated with conventional immunosuppressive agents it was subsequently employed for a more extensive investigation into the effects of antilymphocyte serum, including its ability to inhibit second set rejection. In the course of the latter experiments involving pre-immunization of the host with donor antigens it was found that the intravenous administration of allogeneic donor type whole blood resulted in an unexpected prolongation of survival time of allografts.

The final experiments of the present study are dealing with this way of conditioning of the recipient prior to a subsequent kidney allograft.

CHAPTER II

PRELIMINARY HISTORICAL AND TECHNICAL NOTES

Microvascular surgery

The development of the technique of kidney transplantation in a laboratory animal as small as the rat would not have been possible without the advances made by a number of investigators in microvascular surgery.

Although Carrel was able to reconstruct vessels like the femoral artery of the cat as early as 1908, the first results with suturing of vessels with a diameter less than 4 mm were uniformly poor.

Using conventional sutures, frequent thrombosis and stenosis seemed to be unavoidable. Shumacker and Lowenberg (1948) and Hurwitt et al. (1953) for instance recorded 40–50% failures of end to end anastomoses of small abdominal vessels in the dog. These discouraging results caused a great deal of pessimism as to the feasability of such microvascular surgery with the use of needle and thread. Consequently, a variety of approaches involving new techniques and devices have been introduced to improve the results.

In order to reduce vascular spasm which frequently adds to the difficulties encountered in this kind of surgery, Buncke and Schulz (1965) applied chlorpromazine (0.25 mgm per ml) locally, while Nakayama and coworkers (1964) advised the use of a 2% solution of xylocaine, both of which drugs appeared to be beneficial. Preliminary sympathectomy was found by Casten et al. (1962) to almost double the patency rate in dog femoral artery anastomoses, and Hedberg (1962) successfully employed hydrostatic pressure dilatation in the repair of the same vessel. Engler and his associates (1962) have reported that the induction of a negative electrostatic field around the site of anastomosis aids in maintaining patency.

Several investigators have used an intraluminal splint to temporary hold the walls of small arteries in a distended position during suturing (Man and Kohn, 1962; Mozes et al., 1963). By means of a conventional straight needle, which was withdrawn through the wall after the anastomosis was completed, Mozes obtained 70% patency in vessels less than 2 mm in diameter such as the superior mesenteric or thyroid artery of the dog.

Others have experimented with a technique to widen the vessel at the site of suture by employing an arterial or venous patch (Crawford et al., 1959; de Leon et al., 1961; Lindstrom and de Takats, 1963; Stahl and Katsumura, 1964).

Yasargil (1965) combined a patch with a bypassing T tube and after successful experiments on the femoral artery of the rabbit, he introduced this method

in neurosurgical reconstruction by performing anastomoses between the superficial temporal artery and the median cerebral artery of the dog.

Many different non-suture methods utilizing metal or plastic rings and clips have been described. Some investigators (Carter and Roth, 1958; Goetz et al., 1961; Rohman et al., 1960; Urschel and Roth, 1961) have resorted to cuffing the vessel over the ring so as to accomplish a rapid intima to intima approximation and one of them (Rohman, 1960) reports a lasting success of coronary-internal mammary artery anastomosis in 13 out of 15 dogs. Others (Weiss and Lam, 1950) have used tantalum or polyethylene tubes lined with vein grafts or have effected anastomoses with individual clips (Gonzalez and Nathan, 1963) or pins (Holt and Lewis, 1960). These methods, however, tend to narrow the vascular lumen at the site of juncture and to leave behind a considerable amount of foreign material.

Since 1956 (Androsov) a number of instruments for stapling blood vessels have been designed, to avoid the problem of suturing. Its use in joining vessels 1 to 4 mm in diameter has been evaluated (Williams and Takaro, 1963; Zwaveling, 1963) and found to have certain major disadvantages e.g. those related to loading the bushings with staples, the requirement of a long cuff on the vessel ends and the removal of the instrument after the anastomosis.

One of the most recent developments in the field of non-suture anastomosis has been the introduction of tissue adhesives, which may also be used as a reinforcement or adjuvant to conventional repairs. The experimental use of methyl 2-cyanoacrylate monomer in the junction of small vessels has mostly been disappointing (Hafner et al., 1963; Hosbein and Blumenstock, 1964). Thrombosis readily occurs when even minute amounts of adhesive gain access to the luminal surface of the vessel. Moreover, extensive necrosis has been found after its application, followed by the formation of a sterile abscess, which is subsequently replaced by fibrous tissue, leading to narrowing of the lumen (Weissberg and Goetz, 1964). Recently however, more promising results were reported when n-butyl cyanoacrylate monomer was used, which appeared to be less toxic (Matsumoto et al., 1968).

It was Jacobson (1960) who emphasized the importance of the dissecting microscope for small vascular anastomosis. His efforts have led to the constitution of what has been termed "*micro*-vascular surgery" with the annotation that special and different methods and techniques from those used in vascular surgery in general are necessary. However, after a long period of experience in this field, Fisher and Lee (1965) proved that the general principles of large blood vessel surgery are applicable and that basically the only differences are the most meticulous technique and skill required in the case of smaller vessels.

It is remarkable that in spite of the invention of so many aids and techniques as were previously mentioned, none of them have had any significant influence on the results of microsurgery involving vessels with a diameter of 1 mm or less.

With these very small vessels the perfection and refinement of the instruments used for conventional suturing with needle and thread has so far yielded the most encouraging results.

Buncke and Schulz (1965) were the pioneers in this respect. They succeeded in the reimplantation of rabbit ears and even of the human finger using a diploscope. Before that time investigations on tissue transplantation in continuity with its small vessels had been scarce and of limited success (Goldwyn et al., 1963; Seidenberg et al., 1959). Yet it should be mentioned that Krizek and associates (1965) transferred free composite grafts of skin and subcutaneous tissue from the abdominal wall of dogs to the neck. The arteries on which this transplantation was based varied in diameter from 0.6 to 1.2 mm while the corresponding veins were in the range of 2 to 4 mm. Success was reported in 19 out of 20 dogs. In their procedure however, the arterial end to end anastomosis was converted to an end to side anastomosis, changing the 1 to 2 mm repair into a 3 mm repair.

A similar principle was used by Fisher and Lee (1965) for the transplantation of organs in rats, allowing the use of relative conventional suture material, without the need of a diploscope. The development of this technique for rat kidney transplantation by these investigators has only been achieved after extensive vascular surgical experiments in this test animal, which finally led to such successful operations as a porto-caval shunt (Lee and Fisher, 1961), arterialization of the rat liver (Fisher et al., 1961) and an arterio-venous fistula (Lee et al., 1962). A year later Lee (1966) reported his successful liver transplantation in the rat.

Rat kidney transplantation

Natural vascularization of the kidney has appeared to be essential for ensuring viability of the entire organ after transplantation. Evidence that this condition is not obligatory for small amounts of kidney tissue, however, was provided by Kamrin and Kamrin (1955). These investigators transplanted the lateral one third of the kidney, after it was cut off sharply from the donor kidney during temporary occlusion of the hilar vessels. This piece of tissue was carefully placed onto the excision site of a similarly prepared recipient kidney. Digital pressure was applied for about one minute to assure adhearance to the host organ before circulation was restored. It was shown that such a graft was not always doomed to complete necrosis and a histological success of 25 to 30% was reported with isologous as well as with allogeneic transplants. Later Koldovský (1961) obtained comparable results with this method, and Wheeler and associates (1966) recently applied the technique with equal success in mice. The limited value of such a partial transplant with regard to the evaluation of its function is obvious, yet its use in studies on rejection and its suppression

Schematic illustration of various technical methods used in rat kidney transplantation.

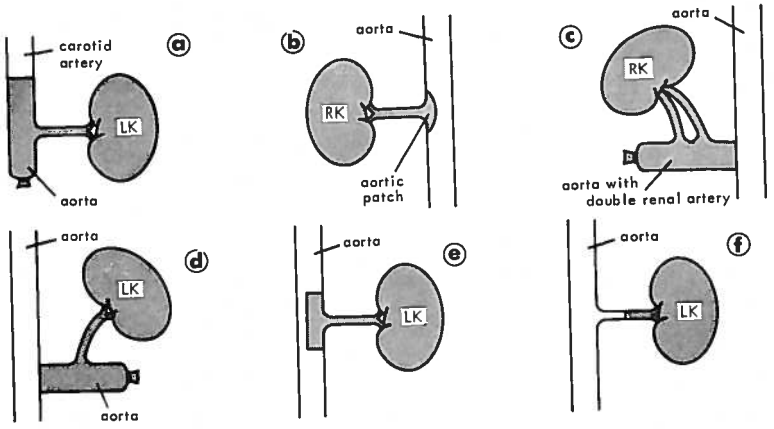

Fig. 1
Arterial reconstruction

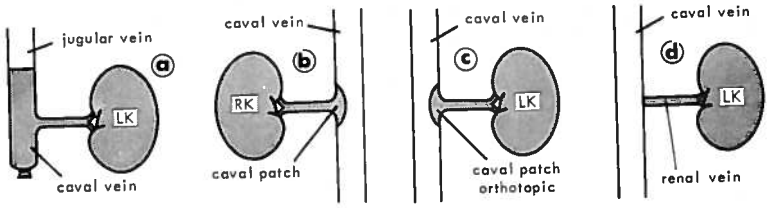

Fig. 2
Venous reconstruction

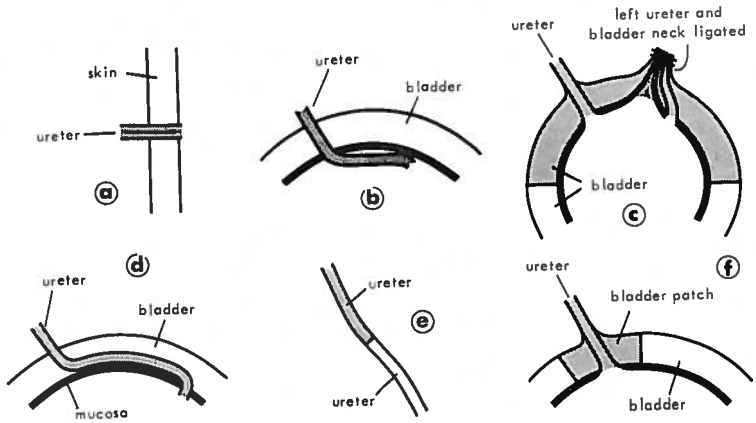

Fig. 3
Urinary tract reconstruction

LK = left kidney
donor origin

RK = right kidney
host origin

could be very valuable. Unfortunately the method appeared to be irreproducible in the course of our own experiments.

The first to describe a method for transplantation of the intact rat kidney were Gonzalez and associates (1962) after they had developed the procedure successfully in the rabbit (Nathan et al., 1961). With this technique (fig. 1a, 2a, 3a) the left kidney of the donor together with the main part of the abdominal aorta and caval vein, of which all branches except the left renal vessels are ligated, is transplanted subcutaneously to the neck of the recipient. The proximal ends of the big donor vessels are anastomosed end to end with the right common carotid artery and jugular vein of the host, after which their distal parts are ligated. Temporary intraluminal plastic tubes are used to facilitate vascular anastomoses, which are performed with a continuous running suture, combined with silver clips. The discrepancy between the vessels to be connected was avoided by using rats of different sizes. The total period of ischaemia of the graft, which was not cooled, often exceeded one hour. The main obstacle to long term survival was the tendency of the rat skin in the neck to close over the ureter of the transplant, thereby blocking the flow of urine. This caused hydronephrosis, infection and destruction of the graft. Consequently, it was necessary to reconstruct the ureteral orifice every 7 to 10 days. Nevertheless these authors reported one survival of 71 days after bilateral nephrectomy in the isologous situation.

In 1965 Lee (Fisher and Lee, 1965) introduced his famous technique of rat kidney transplantation, whereby cuffs left on the donor kidney vessels are anastomosed end to side with the large abdominal vessels of the recipient (fig. 1b, 2b, 3b). The ureter is connected to the bladder providing a more natural drainage of urine. A detailed description of this technical procedure will be given in chapter III.

More recently Lee (1967) has reported a variation of this technique (fig. 1c, 2b, 3c). In the Lewis strain of rats, used by this investigator, the incidence of double renal arteries to the right kidney had appeared to be 53 percent. To preserve both vessels, transplantation of the corresponding aortic segment is recommended. This is performed by joining it end to side with the aorta of the host. Simultaneously, a modification of the reconstruction of the urinary tract was described by performing an end to end bladder to bladder anastomosis, following transection of both host's and donor's bladder at their middle portion. Several other technical variations have been reported lately. Stuart et al. (1968) and Cortesini et al. (1969) prefer the use of the left donor kidney for transplantation (fig. 1d). Taguchi and associates (1968) place the renal vein orthotopically in the caval vein and employ a rectangular aortic patch in coherence with the renal artery (fig. 1e, 2c). Moreover they succeeded in burying a short length of distal ureter under a submucosal tunnel of the receiving bladder (fig. 3d).

By utilizing a dissecting microscope and the ultrafine instruments of Buncke and Schultz, a quite different technique was recently developed by Daniller and his associates (1968). These investigators managed to perform an end to end anastomosis between the renal arteries of the donor and the recipient, providing a method of orthotopic transplantation (fig. 1f). Likewise their ureter to ureter connection is quite unique (fig. 3e), a silicone tube which is used for a stent in this procedure is left in situ after the anastomosis is completed. The renal vein is sutured end to side into the caval vein without a patch (fig. 2d).

Although most recent reports dealing with rat kidney transplantation contain few exact data about the failures and difficulties encountered with reconstruction of the urinary outflow, the impression is gained that up to now such complications present the most important problem in these experiments, especially when studies have the object of long term survival. In the course of their respective investigations Lee has changed from ureter – bladder to bladder – bladder anastomosis, Daniller et al. (1968) switched from their ureter – bladder to ureter – ureter anastomosis, White et al. (1969) changed the bladder – bladder into a ureter – ureter connection, and Sakai et al. (1969) left the bladder – bladder technique of Lee for a procedure whereby a very small bladder patch around the donor ureter is sutured into the bladder of the host (fig. 3f). Owen (1970) has resorted lately to a proximal ureter – ureter connection, removing the temporary stent after the anastomosis is completed. These continuous changes of techniques with regard to the urinary tract reconstruction seem to underline the fact that it remains a major stumbling block in rat kidney transplantation.

Preliminary experiments

It goes without saying that in order to acquire a sufficiently large series of transplantations, the technical method requiring the least possible aids and manpower is to be preferred. At the beginning of the present study the tissue slice technique of Kamrin and Kamrin (1955) was tried out, to evaluate its possible use in pilot studies on immunosuppression. Although this method turned out to be easily and quickly accomplished, using the isologous and autologous model both in rats and in mice, in none of the 50 transplantations performed, a take, that is to say evidence of survival of the grafted tissue was obtained. Histological examination revealed complete necrosis of the graft after a few days in all cases. The reason for these disappointing results, compared with those reported by others, could not be determined. Interference of tissue contact by a blood clot barrier, for instance, was never found. More experience with this method could have improved the results, but the ultimate possible yield of the experiments still would have been poor so that this technique was soon abandoned. Some

experience was also gained initially with Conzalez' (1962) technique, since the utilization of stents during vascular suturing appealed to the beginner. Moreover it was at that time the only available technique. To avoid the difficulties occurring after uretero-cutaneostomy in the rat, an abdominal localisation of the graft would be preferable. An exploration of the possibilities of intra-abdominal transplantation seemed therefore worthwhile. It was found that after ligation of both aorta and inferior vena cava just proximal to their bifurcation, all of six rats thus treated survived with sufficient vascularization and permanent good function of the hind legs. Thus converting the technique of Gonzalez into an abdominal end to end aortic and a similar vena caval connection appeared to be feasible and subsequently was tried in isogeneic transplantations. However, following the dissection and mobilization of the distal 1 cm of the large abdominal vessels of the recipient, which is necessary to allow the vascular anastomoses, it was found that in the majority of cases the hind legs became necrotic and the animals died in a few days. Probably too many collaterals to the distal part of the body including the lumbar vessels were sacrificed in this way. Moreover the dissection of the transplant with its extensive vascular addendum took a relatively long time and required a great deal of training and much skill and accuracy, especially during ligation of the very tiny and vulnerable lumbar veins. To arrange the position of the transplant in such a way that venous obstruction did not eventually occur was another problem, caused by the length of the grafted venous traject which includes a right angle (see fig. 2a). All this was sufficient reason to leave this method and start concentrating on the technique of Lee which only just about that time had been introduced. Initially, this technique was practiced on the guinea pig because inbred strains of this species are available. It was found, however, that although the vessels appeared to be of a larger calibre, they were much more vulnerable than those of the rat, which made the latter the experimental animal of choice.

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CHAPTER III

MATERIALS AND METHODS USED IN RAT KIDNEY TRANSPLANTATION

Animals

Two inbred strains of rats, the WAG/Rij and the BN/Bi, were employed. In the first few experiments conventional rats were used but all other experiments were performed with rats bred under specific pathogen free conditions, that is free of pleuropneumonia like organisms or any other endemic infections. In the case of allografts, WAG male rats weighing between 250 and 350 g served as recipients, while male BN donors of 200–300 g were used. Both strains were employed in syngeneic grafting for the evaluation of the technique. The genetic homogeneity of both rat strains has been confirmed by the permanent survival of intra-strain skin grafts. BN skin grafts are invariably rejected within 12 days by WAG recipients. Recently (Štark and Křen, 1969), the two strains were typed for all antigens of the major histocompatibility locus of the rat (R_tH-1 or $H-1$). The BN strain was found to be $H-1^n$ and the WAG strain was $H-1^w$.

The rats were caged, never more than 4 of them together and fed commercial pellets and water at lib.

The weight of a rat kidney is about 1.2 gram. The outer diameter of the renal artery is consistently less than 1 mm; that of the renal vein is approximately 1 mm and the ureter measures 0.5 mm or less in diameter.

Anaesthesia and preparation for surgery

After anaesthesia is induced in an ether chamber the abdomen of the rat is carefully shaved. The animal is then fixed on its back by taping its extremities to a mobile operating board. Anaesthesia is further maintained by open drop ether, regularly applied to a small nose cone (fig. 4a). In early experiments nembutal, administered intra-peritoneally was used for the induction of anaesthesia and supplemented with ether if necessary. In our hands this method was rather troublesome. Moreover an advantage of ether is that it is excreted
[illegible]gs and that normal renal function is not essential for its elimi-
[illegible]scitate the occasional animal that becomes apnoeic because of
[illegible]naesthetic, short term artificial respiration by means of a short
[illegible] tubing large enough to cover the animals nose (fig. 4b), is
[illegible]. The rare case of cardiac arrest, can successfully be handled
[illegible]ac massage.

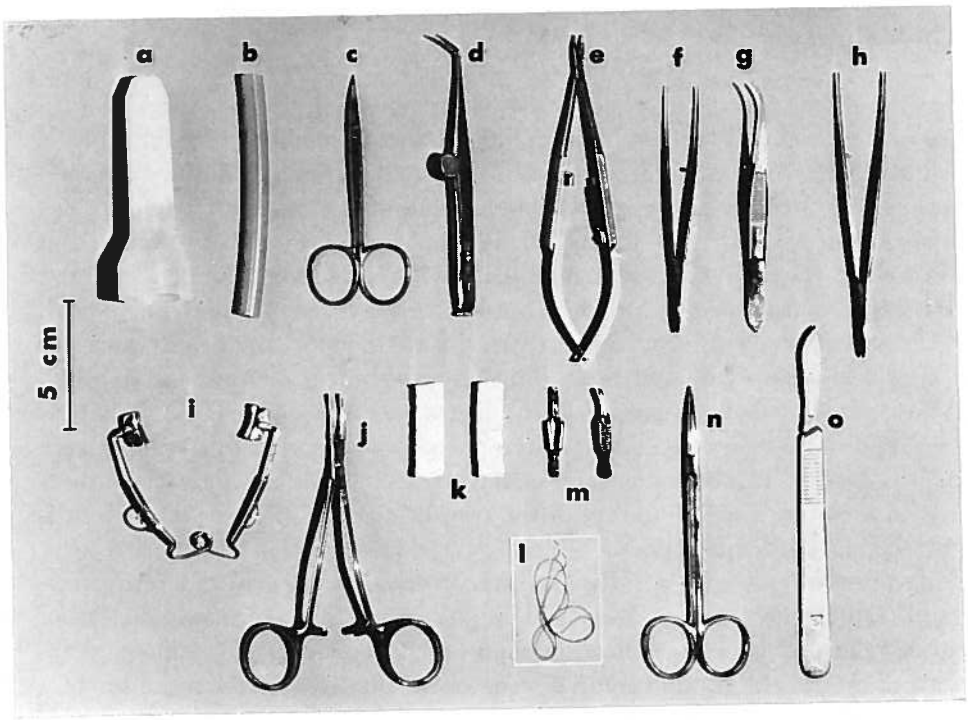

Fig. 4 Instruments used for rat kidney transplantation.

Instruments

It has become evident during practice that while ingenuity may be used in developing instruments suitable for microvascular techniques, the smallest commercially available instruments are usually adequate. Those used in eye surgery are ideal (fig. 4c–h). The small iris scissors, especially the one with the right angle (fig. 4d), eye needle holders, and mosquito forceps, together with a variety of small tissue forceps work well. Small anatomical forceps with rather flat jaws may also be used as needle holders. A self-retaining retractor is used for separation of the wound edges (fig. 4i). To temporarily clamp the aorta and vena cava a small haemostat is used, which has been curved to resemble a Satinsky blood vessel clamp (fig. 4j). To prevent damage of the vessel walls, both ends of the clamp are covered with a short piece of silastic rubber tubing. The cotton wool rolls commonly used in dental surgery (fig. 4k) have been found to be very convenient in obtaining haemostasis by pressure. All vascular anastomoses are performed with No. 7–0 black braided silk (Ethicon), armed with two 3/8 circle taper needles (fig. 4l).

Technical procedure

When the donor rat has been prepared as previously described, the abdomen is elaborately cleaned with alcohol, mainly to avoid loose hairs to get into the operation field. The abdominal cavity is entered via a long midline incision and the wound edges are separated by self-retaining retractors. After the intestines are moved laterally to the left side and have been loosely wrapped in a wet gauze, the right kidney is mobilized while preserving the perirenal fat. This is best accomplished by blunt dissection with a curved mosquito haemostat. The right renal, spermatic and adrenal arteries are then identified, and the latter two vessels are doubly ligated close to the aorta with 6–0 silk ties, and divided. It is advantageous to first dissect out and ligate a vein draining the right suprarenal gland. This vein consistently enters the superior border of the renal vein and, if it is torn, troublesome haemorrhage ensues. This tie may serve afterwards as a marker for orientation of the transected vein in order to prevent it from twisting. Subsequently, the kidney is retracted medially to the left after which the right renal artery is dissected free from the renal vein in a retrograde fashion. The kidney is then placed back into the original position and dissection is carried out to the aorta. Following complete dissection of the right renal artery from the renal vein and inferior vena cava, the left renal vein and artery are ligated and divided.

The ureter is then easily mobilized, but care must be taken not to strip it, otherwise ischaemia of its distal part will occur. Subsequently the ureter is transected near the bladder junction. Prior to removal of the right kidney, 1500 I.U. of heparin are injected into the vena cava. The renal artery and vein are severed with an attached elliptical cuff of aorta and vena cava, completely freeing the kidney, which is immediately placed in a cold saline solution (4 °C) and stored in the refrigerator until the recipient rat is ready.

After preparation of the host animal in the same way as the donor, its aorta and vena cava below the renal vessels are dissected free and are completely clamped together with the curved rubber-shod Satinsky-like clamp. To facilitate application of this clamp, the anterior wall of both aorta and vena cava are grasped and "tented" by a non-toothed thump forceps held in the left hand while the vascular clamp is applied with the right hand.

A small elliptical opening, about 4 mm in length, is made proximally in the clamped part of the aorta with one snip of the iris scissors and clots are washed out using a 2 ml syringe with a short 27 gauge needle attached. The donor kidney which is wrapped in wet gauze is then positioned in such a way that the anterior wall of its aortic cuff can be sutured to the left margin of the aortic incision with a continuous no. 7–0 silk suture (fig. 5a). This suturing is facilitated by first placing two stay sutures at each end of the incision, which are kept under slight tension by means of the weight of a bulldog clamp fixed at

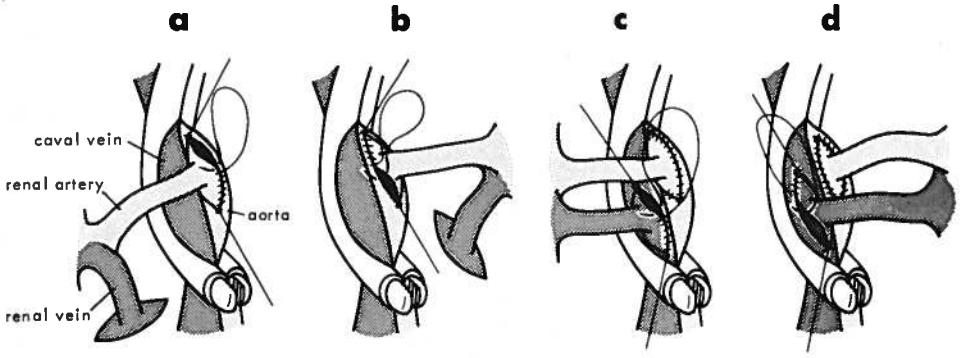

Fig. 5 Various stages in vascular reconstruction (see text for description).

their end. After completion of the left suture line, the kidney is flipped to the left side so that the posterior wall of the aortic cuff may be sutured (fig. 5b). The last two stitches are not drawn tight until the final stitch is placed in order to avoid catching the opposite wall. A similar elliptical opening is now made in the vena cava at a level just distal to where the artery is inserted into the aorta. As with the aortic anastomosis, the anterior wall of the venous patch is sutured to the vena cava (fig. 5c), following which the transplant is retracted to the left, which allows the posterior wall to be sutured (fig. 5d). Before removal of the clamp all suture lines are inspected and should there be a visible gap, additional interrupted sutures are placed. After release of the clamp, pressure is maintained on the anastomoses for a few minutes with a cotton wool roll. The total time of renal ischaemia does not need to exceed 30 minutes.

Just as in the dog and in man, peristalsis and bleeding of the cut end of the ureter are observed, following reestablishment of the blood flow. The transplant may now be fixed and protected by placing it under the mobilized right dorsal peritoneum, giving it a retroperitonal position.

A small stab wound is then made in the fundus of the bladder and a small curved mosquito clamp is inserted and pierced outwards through the posterior wall. The tip of the ureter is grasped and drawn into the bladder so that it fits comfortably without tension or torsion, and subsequently a No. 7–0 silk stitch is placed in the serosa at the site where the ureter passes into the bladder, thus securing it to the bladder wall (fig. 6a).

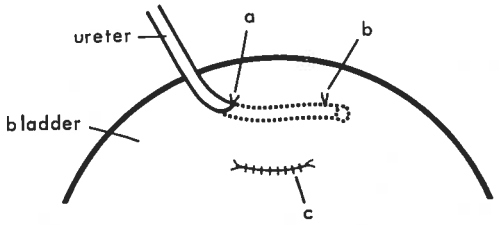

Fig. 6 Urinary tract reconstruction completed (see text for description).

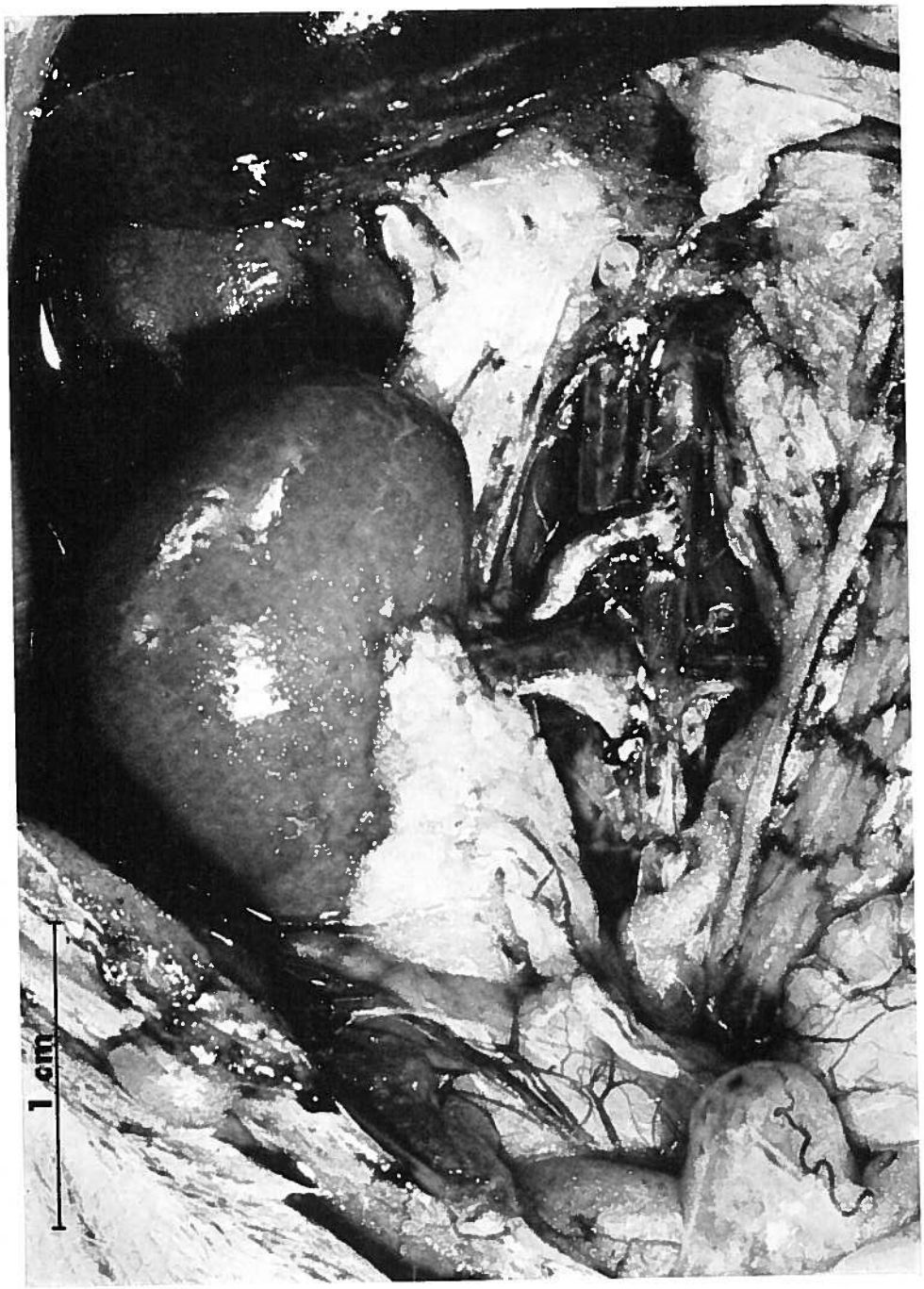

Fig. 7 The transplanted kidney in its abdominal position.

A short length of ureter, which is permitted to protude into the vesicle lumen, is anchored to the mucosa by passing both ends of a single stitch through the bladder wall and tying them on the outside (fig. 6b). The stab wound of the bladder is closed with a short continuous silk suture (fig. 6c).

Fig. 7 shows the freshly transplanted kidney in its abdominal position while in fig. 8 an angiogram of a rat with three kidneys is demonstrated.

Depending on the nature of the experiment, only the right kidney or both kidneys of the host are removed, whereby a 5–0 ligature is placed, tying artery, vein and ureter together close to the hilus, so as to preserve vascularization of the adrenal gland. Only after the surgeon has assured that optimal vascularization of the entire graft and complete haemostasis is achieved, the abdomen is closed. The peritoneum together with the fascia is approximated by means of a running 5–0 catgut suture. The skin is closed with metal clips or with a continuous linen suture.

In certain cases the left kidney of the recipient is removed after 4–7 days, by using a pararectal incision, which at the same time enables macroscopic evaluation of the graft. Moreover, a wedge-shaped biopsy of the transplant may be taken at that time by sharply cutting it longitudinally from its anterior side. Subsequent gentle pressure applied with a piece of gauze usually results in satisfactory haemostasis.

Antibiotic treatment

In the first series of experiments all animals were treated for one week with daily intramuscular injections of penicillin and streptomycin, 10,000 I.U. and 10 mg resp. (Retromypen S, Mycofarm, Delft, Holland). In the experiments dealing with antigen pretreatment of the host (chapter VIII) the antibiotic treatment was changed into a single injection of 60,000 I.U. of benzathine – penicillin (Penidural, Mycofarm) at the time of transplantation.

Technical annotations

As mentioned before, the operation is carried out employing clean but not sterile techniques, the instruments are soaked in alcohol or any suitable antiseptic solution prior to use, drapes are fashioned from gauze sponges and the operator is not wearing gloves but merely scrubs before starting the operation.

The whole procedure can be carried out by a single operator, although the preparation of the recipient rat by an assistant during the final stage of the removal of the donor kidney may shorten the time of cold ischaemia of the graft, but this is not at all obligatory.

While performing the anastomoses, experience has taught that even with trained assistance, simultaneous movement of both operator and assistant may

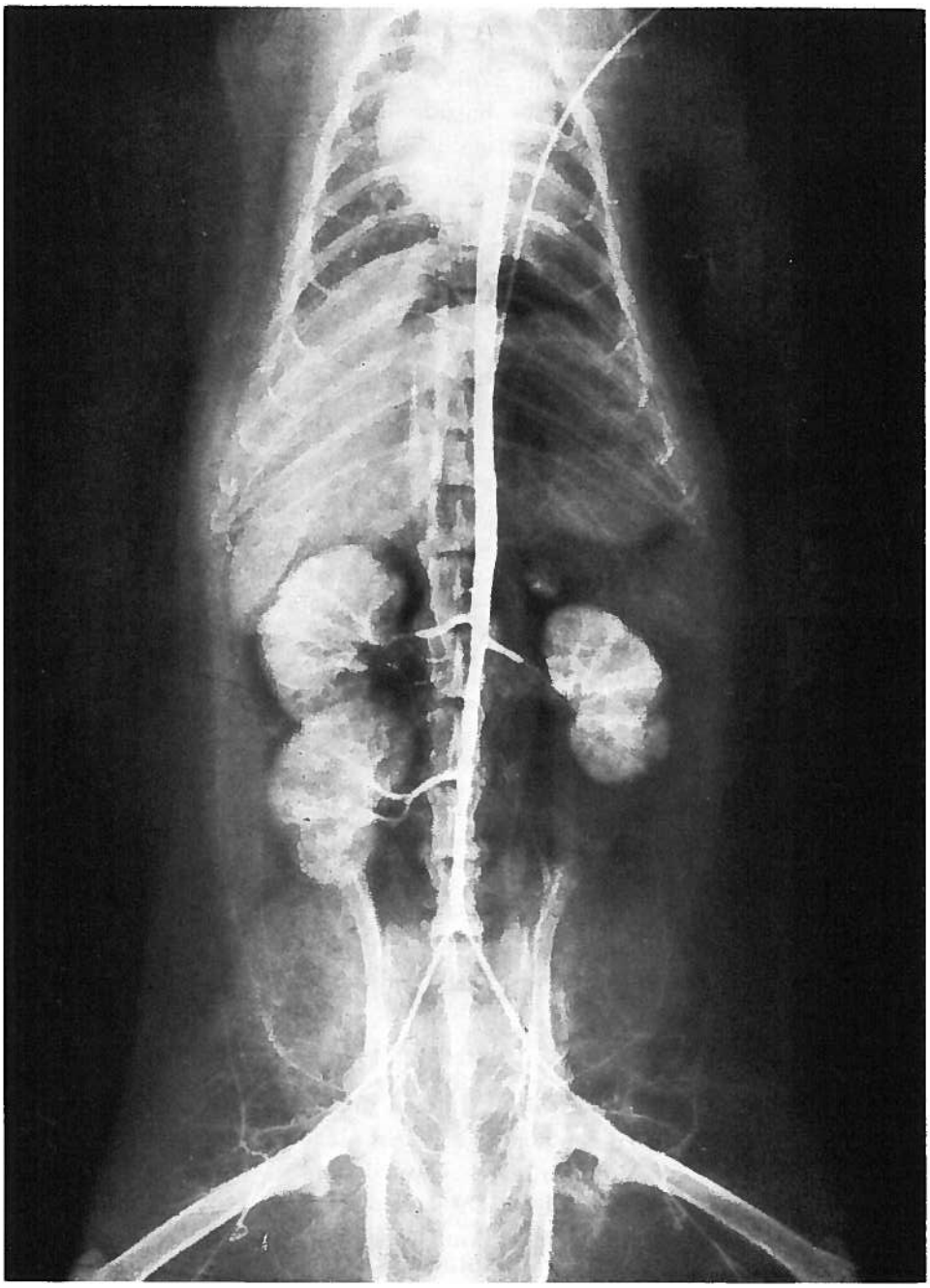

Fig. 8 Angiogram of a rat with three kidneys.

To this end 1 ml of Urografin 76% (Schering A. G., Berlin) was injected retrograde into the coeliac artery. The graft is the inferior one on the right side.

be hazardous if not well coordinated. Moreover only the highest accuracy can be obtained when the operator is supporting the ulnar sides of his hands and forearms on the operating table, thus eliminating movement of the elbow and shoulder. Under those conditions four hands in the small operating area are too much. One disadvantage of operating single-handed is the lack of attention for the anaesthesia, especially during concentration on the vessel anastomoses, with obvious frustating consequences in case irreversible apnoe or cardiac arrest develops.

The limitations of this micro-surgical procedure are mainly determined by the exact control over the movements executed and not so much by whether magnifying devices are used or not. The use of a binocular magnifier with a 2,25 diameter magnification and a 25 cm focal length worn by the operator may be satisfying, although one needs to get used to it and some of the operators engaged in the latter part of the present study obtained equally good results without the use of visual aids. Proper light with minimal thermal radiation to prevent desiccation is indispensable.

Using very large rats for recipients is not advantageous with regard to the calibre of the vessels. It only takes more time to prepare the larger vessels, because of excessive adipositas and fibrosis encountered in these older animals.

Bleeding should be kept to a minimum at all stages to enable these animals with their small blood volume of about 20 ml, to survive. During the period of training it often happens that lethal bleeding recommences after closure of the abdomen over dry suture lines of the vessels. This is probably caused by normalization of the blood pressure and an accompanying dilution of the blood. It remains possible that the small amount of heparine, present in the graft may also play a role in this respect.

Although administration of plasma or of its substitutes is feasable via the tail vein, it has been of little advantage in our hands, since it causes a decrease of the clotting capacity. Replenishment with isogeneic blood is preferable to other fluids though even then the small amount of citrate that has to be added seems to be hazardous.

Occasionally a haemorrhage occurs during the dissection of the venous tract of the donor kidney, especially while it is severed from the renal artery. Even when at that time the bleeding can be handled, it is advised not to use the kidney for grafting. If in such a case the bleeding stops spontaneously following pressure, it will certainly recommence after transplantation while a stitch applied at that time is likely to cause stenosis.

In his improved technique Lee (1967) describes perfusion of the graft before it is removed with 1 percent heparine in saline via the aorta. This procedure is followed by others (Daniller et al., 1968; Cortesini et al., 1969). It has however not been employed in the present experiments, since after minimal blood loss, recirculation rarely presented a problem.

In a minority of cases it took several minutes after release of the occluding clamp before recirculation of the entire kidney became established. In such a case reclamping with the object to apply an additional stitch at a bleeding point of the suture-line, should not be done before the graft has become fully "flushed". Otherwise, after the second removal of the clamp, frequently the whole kidney or one of its segments will never fully regain its proper circulation, due to intra-renal thrombosis.

It is recommended to place the vascular clamp proximally in the abdomen because at that level the large vessels run separate and have their greatest calibre. Prior removal of the host's own right kidney may facilitate this procedure. However, operating high in the abdomen may be troublesome because of the increased depth of the working site.

Some experience was gained with the use of the tissue adhesive methyl 2 – cyanoacrylate (Eastman 910 monomer) as an additive to the vascular suture. The results were disappointing. The hardened material prevented the collapsed vessel to expand, while it excluded the possibility of applying an additional stitch when needed.

When performing the venous anastomosis it may be hard to keep both suture lines separate from each other. This can be facilitated by leaving the graft in the dextroposition and by first suturing the posterior wall from the luminal side.

The bladder to bladder anastomosis as advocated by Lee (1967) proved to be disadvantageous in our experiments. In 9 out of 10 cases necrosis due to ischaemia or venous thrombosis of the grafted bladder section resulted. This complication has been observed by others (Ockner 1970). Lack of sufficient experience may well have been the cause, but on the other hand the difference between Lee's and our results may have been due to the different rat strains used. Moreover another hazard of the bladder to bladder procedure should be mentioned. Sakai and coworkers (1968) found that 6 months after transplantation, the contralateral kidney of the host, which was left in situ, showed hydronephrotic and pyelonephritic changes similar to those seen in the grafted kidney. They concluded that this technique obviously interferes with normal vesical function.

In surgery it is usually agreed that before starting to practice a certain new technique, the close observation of or careful assistance to an experienced operator, is much more profitable than the mere following of its description. This goes particularly for the present procedure since it took the author four months of full time practice before any success was obtained, while subsequently trained technical assistants usually reached their first successful transplantation within a few weeks.

CHAPTER IV

EVALUATION OF THE TECHNICAL RESULTS

General

With the transplantation procedure as it was finally developed, the total period of renal ischaemia ranged between 20 and 40 minutes, the average being 30 minutes. In general the whole operation takes $1^1/_2$–2 hours, although an experienced worker may occasionally perform it within one hour.

Operative mortality

An impression of the yield of technical success can be derived best from transplantations performed by a constant and experienced surgical team.

The experiments carried out in the period that methods to study the function of the graft were developed (De Bruin, 1970) meet these conditions and will be considered as being representative in this respect.

The incidence of death during operation was 73 out of 282 cases which means a mortality rate of 24%. The main cause of peroperative death was related to the occurrence of uncontrollable haemorrhage. In those instances either the bleeding itself was fatal or the subsequent correction of the leak by means of one or two extra stitches resulted in irreparable stenosis, in which case the animal was discarded. Far less frequently, death was due to irreversable apnoe or cardiac arrest caused by an excess of anaesthetic. An occasional animal was sacrificed because of deficient peripheral circulation of the graft due to irreversible intrarenal arterial spasm or thrombosis.

Of the 209 rats that recovered from anaesthesia, another 12 animals (5,7%) died within the next 24 hours. With our own experiments the incidence of death during this immediate postoperative period was quite similar (16 out of 311 cases = 5,1%). Although strictly this should not be classified as peroperative death, this complication was so closely linked to the operation itself that it is being discussed under this heading. In all these cases it appeared at autopsy that death could be attributed to recurrent haemorrhage or inadequately treated hypovolaemic shock.

Thus it may be stated that after 24 hours a total of 30% of the animals had died. All animals that died during the operation or within 24 hours thereafter are further excluded from our data.

Mortality during the first two weeks after transplantation

In the first series of the present experiments the left kidney of the recipient

was not removed until 4 to 7 days after transplantation as advised by Fisher and Lee (1965). This procedure (two stage nephrectomy) was used in isografting among WAG strain rats as well as in the experiments with allografts. In the latter situation the BN strain rats served as donor and the WAG rats as recipient. The two stage procedure was also used in most of the experiments dealt with in chapter V, VI and VII.

When measurements of the kidney grafts function were developed in our laboratory the procedure described above was modified in that bilateral nephrectomy was performed during transplantation (one stage nephrectomy). Consequently, the examination of the function of the graft became possible immediately following the operation. After it was found that the one stage procedure yielded good results it subsequently was used for all other experiments, such as those described in chapter VIII.

As will be shown in the next pages mortality during the first week could always be attributed to technical complications in both the isografted and the allografted rats. However, apart from late technical failures, death of allografted animals during the second week was mainly due to graft rejection.

The incidence of fatal technical complications during the first two weeks will therefore first be evaluated for rats bearing isografts, since the rejection phenomenon does not interfere in this situation (fig. 9). When WAG strain rats were

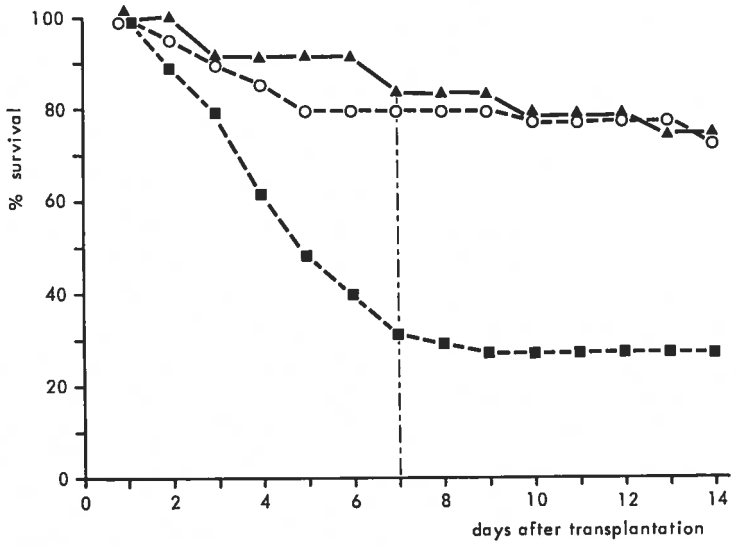

Fig. 9 Survival of rats bearing kidney isografts.

▲—▲ 23 WAG rats; two stage nephrectomy.
■--■ 62 WAG rats; one stage nephrectomy.
o--o 39 BN rats; one stage nephrectomy.
Animals that died during transplantation or within 24 hours thereafter are not included.

used, there was a marked difference in first week mortality between the one stage and two stage nephrectomy groups. In the latter group 83% of the animals survived the first week, while in the first group this was only 31%. However, when the one stage procedure was used with the BN strain rat, no such high first week mortality occurred. The BN group may not be considered as wholly comparable in this respect, because in half of these experiments isografts were performed from old to young rats (Hollander and De Leeuw-Israel, 1969). This factor, however, could only be expected to *increase* the mortality, if anything. The incidence of mortality during the second week was less pronounced and quite similar for all groups.

Rats can live for 4 to 5 days after bilateral nephrectomy. Consequently it could be expected that a comparable death rate as observed in the first week among the one stage WAG group would occur during the second week among the two stage WAG group. This, however, was not the case. Apparently the presence of a kidney of the recipient during the first days has a protective function on the WAG type graft, which seems to be much more vulnerable to the transplantation process than the BN kidney.

In fig. 10 the survival during the first two weeks is shown among untreated allografted rats. Both the one stage and the two stage group demonstrate a

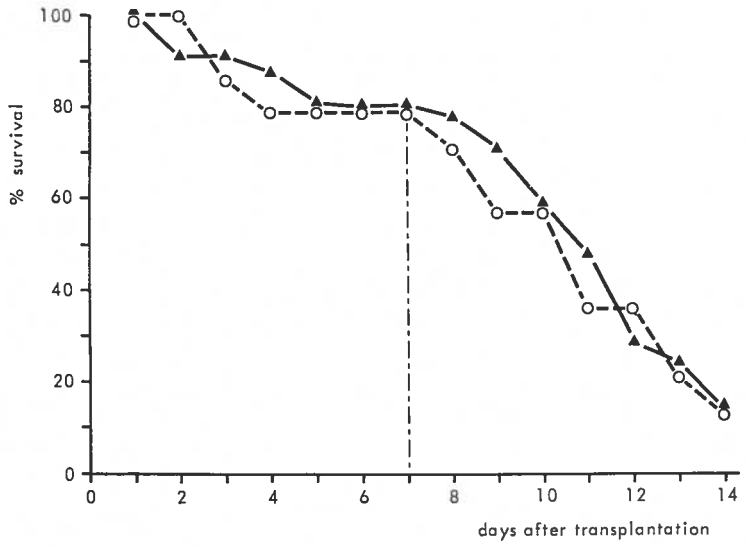

Fig. 10 Survival of untreated WAG rats bearing BN kidney allografts.

▲—▲ 58 rats; two stage nephrectomy.
o--o 14 rats; one stage nephrectomy.
Animals that died during transplantation or within 24 hours thereafter are not included.

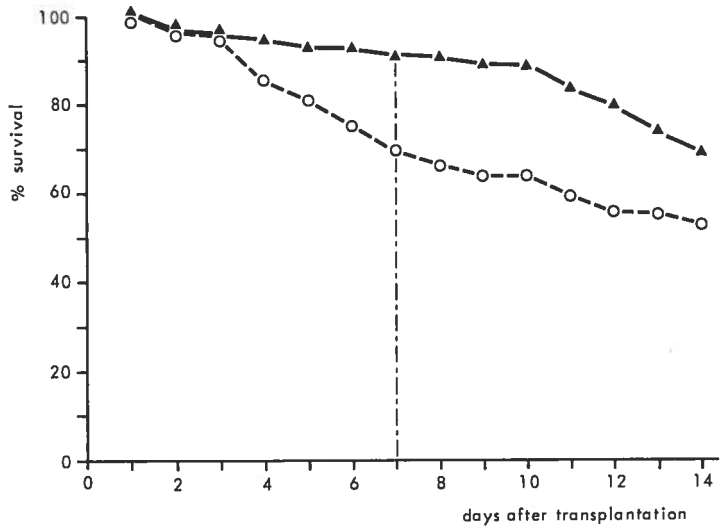

Fig. 11 Survival of treated WAG rats bearing BN kidney allografts.

▲—▲ 97 rats; two stage nephrectomy; non-specific immunosuppression.
o--o 118 rats; one stage nephrectomy; specific immunosuppression.
Animals that died during transplantation or within 24 hours thereafter are not included.

similar mortality pattern and the high incidence of death during the second week is mainly due to rejection of the graft.

When allografted rats receiving some kind of immunosuppressive treatment are considered (fig. 11), a 20% difference in first week survival can be noted between the one and two stage groups. To evaluate the possibility that this difference were due to the specific immunosuppressive treatment received by the one stage nephrectomy group, these data have been compared with those obtained by De Bruin (1970) in a study on non-specific immunosuppression, where the one stage procedure was also employed. The survival curve of the latter group did not differ significantly from that of our own one stage group. These results suggest that the BN allograft, in particular the ureter (see table II), becomes more vulnerable to the damage associated with transplantation during immunosuppressive treatment.

The causes of death during the first week after transplantation

In table II the nature of the technical failures leading to death during the first week is presented. The cause of death was deduced mainly from the macroscopic findings at autopsy. The formation of blood clots or of lumps of inspissated protein substance in the bladder was the cause of death in those cases listed as bladder neck obstruction. The cause of death was considered as being

Table II Cause of death during the first week.

	TWO STAGE NEPHRECTOMY				ONE STAGE NEPHRECTOMY					
CAUSE OF DEATH	Total	Iso WAG	Allo contr.	Allo treated*	Total	Iso BN	Iso WAG	Allo contr.	Allo treated**	Allo treated*
Total transplantations	178	23	58	97	381	39	62	14	118	148 [1])
Total deaths	25	4	12	9	137	8	43	3	35	48
Disconnection ureter	1	1			51	3	13	1	16	18
Obstruction ureter					10	1	4		2	3
Bladderneck obstruction	1		1		10		2		5	3
Anuria					15	2	10		1	2
Late haemorrhage	4		2	2	3		1	1	1	
Shock	2		2		3		1		2	
Anaesthesia 2nd oper.	3		2	1	3		1		1	1
Haemorrhage 2nd oper.	6	1	2	3	6		1		1	4
Infection	2	1	1		1					1
Thrombosis					16	1	4	1	3	7
Wound disruption	1	1			3		2			1
Accidents	2			2	4					4
Unidentified	3		2	1	12	1	4		3	4

* Non-specific immunosuppression.
** Specific immunosuppression.
[1]) Data of De Bruin (1970) for comparison.
Animals that died during transplantation or within 24 hours thereafter are not included.

due to anuria in those instances where diuresis had not been observed at all after transplantation and no obvious technical imperfection was found at autopsy. "Accidents" represent death from injection injury during intraperitoneal administration of drugs or from anaesthesia during local irradiation of the graft. The majority of deaths in the two stage groups was closely related to the first or second operation itself. In the one stage groups, however, it is obvious that the high mortality was mainly due to complications of a urological nature. Either a total obstruction was observed with a greatly distended pyelum and ureter, or disruption or disconnection of the distal section of the ureter had occurred. The decreased vitality of this part of the ureter caused by relative ischaemia possibly plays an additional role in those cases. There was a high incidence of anuria among the isogeneic WAG group (10 out of 62 cases). In the latter group disconnection or obstruction of the ureter was seen twice as often as in the BN donor situation (27% v.s. 14%), again indicating the high vulnerability of both kidney and ureter of the WAG rat. Obstruction of the vascular anastomosis caused by thrombosis was another notable complication, which obviously only led to death in the one stage groups. When the transplantations performed with BN type grafts are considered, which represents the most favourable situation, the overall death rate was 29% for the one stage, and only 14% for the two stage procedure. This means that after 30% of the

Table III Cause of death during the second week.

	TWO STAGE NEPHRECTOMY				ONE STAGE NEPHRECTOMY					
CAUSE OF DEATH	Total	Iso WAG	Allo contr.	Allo treated*	Total	Iso BN	Iso WAG	Allo contr.	Allo treated**	Allo treated*
Total transplantations	178	23	58	97	381	39	62	14	118	148 [1])
Total rats surv. 1 week	153	19	46	88	244	31	19	11	83	100
Total deaths	61	2	38	21	71	3	2	9	20	37
Disconnection ureter					7	1	1		2	3
Obstruction ureter	6	2	2	2	4	1			1	2
Bladderneck obstruction	1		1		4		1			3
Thrombosis	2		1	1	4	1			1	2
Infection	2			2	3				1	2
Accidents	1			1	2				1	1
Rejection	48		33	15	43			9	13	21
Unidentified	1		1		4				1	3

* Non-specific immunosuppression.
** Specific immunosuppression.
[1]) Data of De Bruin (1970) for comparison.

animals had died during the first 24 hours after transplantation, another 30% of the one day survivors died in the course of the first week when bilateral nephrectomy was performed at the time of the transplantation.

The causes of death during the second week after transplantation

Table III shows the causes of death within the second week after transplantation. Rejection was the dominant feature during this period. Only one out of ten animals that survived the first week, died in the isogeneic groups. A wide variation of complications can be seen and it may be noted that the incidence of fatal infection i.c. pyelonephritis and or septichaemia is low and was only found among those animals which received immunosuppressive treatment, as might be expected.

Mortality after the second week

When the animals have survived the first two weeks following transplantation, it may be expected that mortality due to complications occurring thereafter will be less influenced by the operation technique. Consequently, isografted rats have been evaluated, without regard to the applied technique (fig. 12). Of 23 isografted WAG rats alive after two weeks, 5 died before day 50. The cause of death, which could be established in 4 of the animals, was severe hydronephrosis due to obstruction of the ureter in three cases and to bladder neck obstruction in one. In one rat urinary infection had occurred as well.

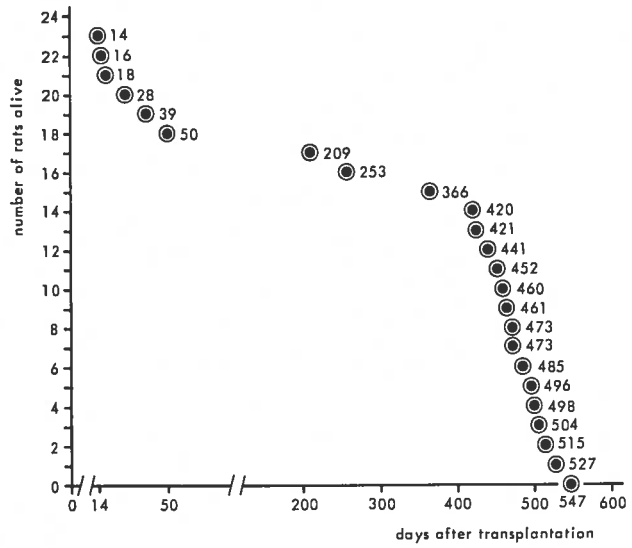

Fig. 12 Survival after two weeks of WAG rats bearing kidney isografts.

It appears that once the rats have escaped fatal technical complications during the first 6 to 7 weeks, they are apt to survive for a relatively long period of time. Most of the 18 rats still alive after 200 days, died between day 400 and 500. The longest survivor died 547 days after transplantation. In about half of the long term survivors (> 200 days) hydronephrosis was found at autopsy, with varying degrees of urine outflow obstruction. The latter complication again seemed to be the major cause of death of these animals.

However, most of the long term survivors were permitted to live out the greatest part of their normal life span, since a WAG rat rarely becomes older than three years and the majority of the recipients were more than one year old at the time of transplantation. No signs of rejection were found in these animals, which is in accordance with the high degree of homogeneity of the WAG strain.

Control allografts

It has already been shown that among all experimental groups, mortality during the first week could be attributed to technical complications. Furthermore, it was found that the kind of immunosuppression used, and whether it was employed at all did not affect the first week mortality to a notable degree. It therefore seemed justified to further exclude from our data all animals that died during the first week after transplantation.

Fig. 13 shows the mortality pattern of 57 control untreated allografted rats.

served among control allografted animals is in accordance with previous investigations by others. However, literature data on survival in the presence of incompatibility at the AgB or R_tH-1 locus, the major histocompatibility locus and the mediator of "strong" allograft immunity in the rat, are based on relatively small series of animals. Stuart and associates (1968) reported a mean survival time of 17.3 $\pm$2.7 days in 14 Lewis rats bearing BN allografts, the longest survivor living for 45 days. When allografts were performed from Buffalo to Lewis strain rats, Sakai (1969) obtained a mean survival time of 14.3 $\pm$1.7 days in 6 animals. Both investigators found a shorter and more constant survival of corresponding skin allografts.

All these results are quite similar to those obtained by some investigators with kidney transplantations performed among randomly selected mongrel dogs. Zukoski and associates (1965) for instance reported a survival time of 9–25 days, with an average of 15 days, while Shanfield and coworkers (1968) found a survival of 8–41 days (mean 13 days) in a series of 20 dogs.

The variation mentioned above implies that the expected advantage of a larger homogenicity has not been realized and that as with the dog experiments

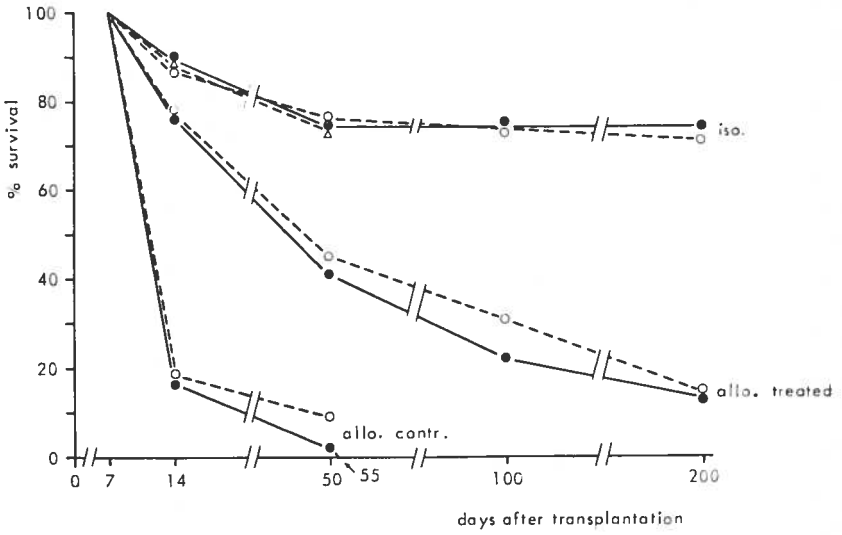

Fig. 14 Survey of survival in allografts.

Isografts:	●—●	19 WAG rats; two stage nephrectomy
	o--o	31 BN rats; one stage nephrectomy
	Δ--Δ	19 WAG rats; one stage nephrectomy
Treated allografts:	●—●	88 rats, two stage nephrectomy; non-specific immunosuppression
	o--o	83 rats; one stage nephrectomy; specific immunosuppression
Control allografts:	●—●	46 rats; two stage nephrectomy
	o--o	11 rats; one stage nephrectomy

it necessitates the use of large groups of animals. The latter, however, can be accomplished much more easily when rats are being employed.

Our results indicate that the rat kidney model provides sufficient possibility for studies on the effect of immunosuppressive agents, as is shown in fig. 14. It clearly demonstrates that the survival curves of the treated allografted rats are situated between those of the isografts and the control allografts, as might be expected. Moreover, it should be noted that whether or not one of the kidneys of the recipient is temporarily left in situ, the results are not significantly different.

Urinary tract complications were commonly found to various degrees among all groups of animals at autopsy and did not seem to interfere seriously with prolonged graft function and survival. These cases therefore were not excluded from our data, also because the simultaneous occurrence of graft rejection made it impossible to assess the actual cause of death with certainty. The same applies for the few rats that died of infection. The latter complication is usually observed more frequently in experiments dealing with dog kidney transplantation. Diethelm and coworkers (1968) reported that in a series of 33 dogs treated with drugs such as Imuran, azaserine and prednisolone, the causes of death were equally distributed between renal allograft rejection and drug induced infection. The low incidence of complicating infections in the present study on the other hand, seems to be an important advantage of the rat model.

In the subsequent chapters the different methods of immunosuppression will be evaluated separately, using the survival of the recipient as the main parameter. In addition to individual survival times, the mean survival time of each experimental group will be presented. In several groups a great variety of survival times was observed with occasionally an exceptional long term survivor. Of such groups the median survival time will also be given. In case of an even number of animals, the median survival time was fixed at that time falling half way the interval between the survival times of the two middle animals of the group.

CHAPTER V

THE EFFECT OF PREDNISOLONE AND AZATHIOPRINE (IMURAN)

Introduction

General

The first experiments were designed to examine the effect of well established immunosuppressive drugs such as prednisolone and Imuran on the BN→WAG kidney allograft model in order to evaluate its use in studies on immunosuppression compared with those performed on the dog and man. A preliminary report on these data has been published (Tinbergen, 1968).

Prednisolone

Prednisolone is a synthetic corticosteroid and has many similarities of action to cortisone, which belongs to the first drugs that appeared to have a suppressing influence on the homograft reaction. Early investigators showed a definite prolongation of skin graft survival in animals treated with cortisone acetate (Billingham et al., 1951a; Morgan, 1951). The first authors demonstrated in the rabbit a prolonged survival of skin homografts 3–4 times the normal surviving period of 8 days. They assumed that the delayed release of antigen due to the retarded vascularization of skin homografts treated with cortisone was the main factor of this prolongation. However, some other mechanisms may be involved, indicating a more central inhibitory action of the corticoids on the R.E.S. system by means of a combination of several factors.

Evidence for the inhibition of antigen uptake by immunosuppressive agents has so far been found only for cortisone, which prolongs skin graft survival even when applied to the graft locally (Billingham et al., 1951b). Moreover, cortisone has been shown to impair phagocyte function (Nicol et al., 1958). Dougherty et al. (1944) found that within three hours after a single dose of cortisone, lymphocytolysis occurs in the germinal follicles of the lymph nodes and the spleen.

In addition to destruction of small and medium sized lymphocytes, lymph nodes are depleted of plasma cells of animals treated with corticoisteroids. (Baker et al., 1951). Moreover, these drugs may cause a marked drop in the number of lymphocytes circulating in the blood as demonstrated by Reinhardt et al. (1944) in rats as well as in dogs.

Presumably, it is mainly by this mechanism that these hormones suppress the formation of antibodies. Cortisone administration to rats just prior to an-

tigen challenge with intravenously administered heterologous red blood cells prevents splenic macrophages from incorporating the antigen, and is associated with a decrease in antibody production. The inability of the splenic macrophages to capture the antigen, but not the depletion of lymphocytes was considered to have led to a decrease in antibody production (Craddock et al., 1967).

Cortisone has been shown to depress antibody formation to purified antigens (Woodruff, 1960), and Kaliss and Hoecker (1954) have reported that cortisone caused a marked depression of the haemagglutinins which developed in association with the injection of tumor tissue extracts. Such a depression of circulating antibodies may be beneficial to transplanted tissues.

The allograft rejection of skin was not the only phenomenon susceptible to corticosteroids. In 1965 Zukoski et al. reported on prolonged survival of kidney allografts in dogs treated with prednisolone.

Azathioprine

Azathioprine (Imuran) is an imidazol conjugate of 6-Mercaptopurine (6-MP). It is readily split to 6-MP *in vivo* and was synthesized as part of a program designed to present the thiopurine in a masked but metabolically active form with a slower degradation (Elion and Hitchings, 1959). Imuran is less toxic for the bone marrow than 6-MP (Calne and Murray, 1961) and although the biological activity of both drugs seems to be similar, there might be an additional independent action of the split imidazolyl group (Hitchings and Elion, 1963). 6-MP is an analogue of hypoxanthine and the purine base adenine which is an essential constituent of both ribose and desoxyribose nucleic acids, and also of other vital metabolites. It apparently acts as an antimetabolite, competing with hypoxanthine and adenin for their natural receptors and probably interfering with numerous complex biochemical reactions, among which is inhibition of nucleic acid synthesis (Elion et al., 1954). This may be the reason for the selective damage to rapidly dividing cells produced by the purine analogues. 6-Mercaptopurine was originally developed as an antileucaemic agent; its influence on lymphoid cells during their intensive proliferation observed in response to specific antigenic stimulation (Scothorne and McGregor, 1955) may likewise explain its immunosuppressive action.

The potential usefulness of antimetabolites in the field of transplantation was first demonstrated by the now classic report of Schwartz, Eisner and Dameshek of 1959. These investigators showed that the administration of large doses of 6-MP daily for 14 days would entirely prevent the antibody response of rabbits to the initial injection of a foreign protein. Subsequently André et al. (1962) found that 6-MP blocked the appearance of pyroninophylic blast cells (immunoblasts) in rabbits challenged with a skin allograft. In such animals the grafts had a prolonged survival. Calne (1960) and Zukoski et al. (1960)

were the first to investigate the effect of 6-MP on renal allografts in dogs. Both authors reported a significant prolongation of survival, while slightly better results were produced with Imuran (Calne and Murray, 1961).

Methods of application

Prednisolone sodium succinate (Organon Oss Holland) was used as a 2 mg/ml solution in aqua dest. and dissolved just prior to use. It was administered subcutaneously starting on the day of operation in a daily dose of 4 mg/kg bodyweight. The treatment was continued until death of the animal.

Imuran [1]) was used as a solution of 4 mg/ml, prepared from a suspension in 0.9% NaCl by adding NaOH until pH 8 was reached. The solution was never kept longer than 1 week before use and was administered intra-peritoneally starting on the day of transplantation. One group of rats received a daily dose of 4 mg/kg and a second group was given 8 mg/kg.

All rats were treated indefinitely, unless stated otherwise.

Results

Table IV shows the individual survival times of all rats treated with either one of the drugs, or with a combination. With all treatment schedules a definite prolongation of survival was obtained. Prednisolone as sole i.s. agent was the

Table IV The effect of Imuran and prednisolone on the survival of WAG rats bearing BN kidney allografts.

Nr. of rats	Treatment	Mean surv. time	Median surv. time	Survival times (days)
57	None	14	12	see fig. 13
15	prednisolone 4 mg/kg	32	17	11 13 13 13 13 16 17 17 19 19 20 39 45 48 170
16	Imuran 8 mg/kg	99	95	11 13 14 28 37 41 42 92 98 102 117 125 173* 190* 235* 273*
10	Imuran 4 mg/kg	41	40	9 11 20 32 37 43 51 58 71 76
11	Imuran 4 mg/kg+ prednisolone 4 mg/kg	91	34	12 14 14 20 34 34 90 125* 208* 220* 238*

All treatment was started on the day of transplantation and continued daily thereafter. Prednisolone was administered s.c. and Imuran i.p. Animals that died during transplantation or within the first week thereafter are not included.

* Treatment discontinued, see text.

[1]) Imuran was made available by Burroughs Wellcome and Company, London, through the courtesy of Dr. D. A. Long.

least effective and produced a relatively long survival in one case only. The prolongation achieved with Imuran 8 mg/kg was more pronounced than with 4 mg/kg. However, in the 8 mg/kg group most of the animals sooner or later developed signs of drug toxicity such as wasting, anaemia and diarrhoea, which were aggravated by the state of chronic uraemia. The toxicity was less obvious in the 4 mg/kg group. In the last four cases of the animals receiving 8 mg/kg daily, the administration of Imuran was discontinued after 138, 139, 160 and 161 days. These rats subsequently survived for another 135, 34, 30 and 74 days respectively without any further treatment.

Combination of both drugs added to the immunosuppressive effect of each single drug given at the same dose, as might be expected. As with some Imuran treated animals, 4 of the animals receiving the combined treatment with Imuran and prednisolone were permitted to survive following withdrawal of all therapy after 100 days. In these cases an additional survival of 25, 108, 120 and 138 days was observed.

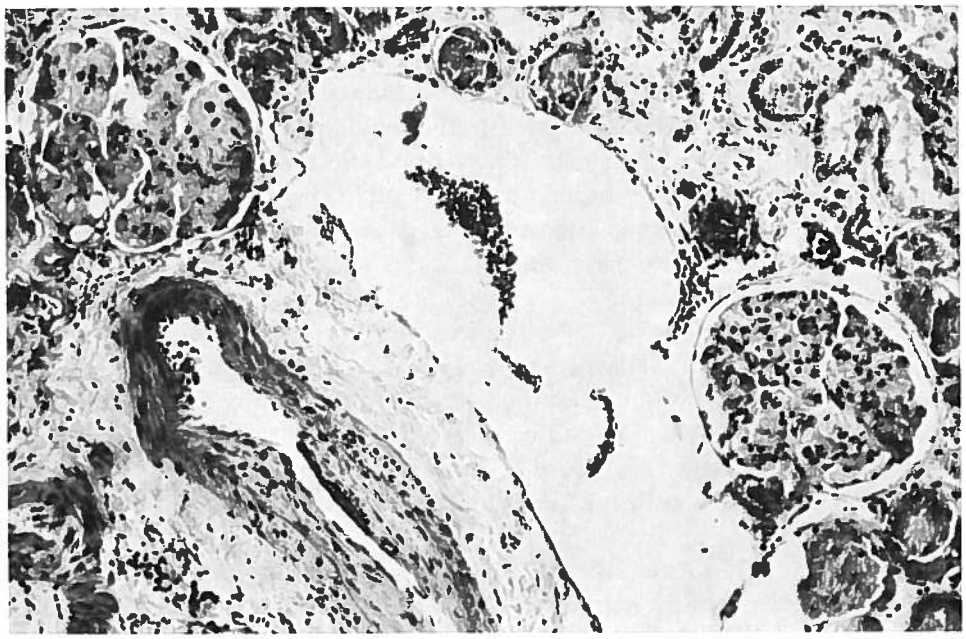

Fig. 15 Extensive changes of renal glomeruli in an allograft at 117 days following transplantation (autopsy specimen). The animal had been treated with Imuran 8 mg/kg i.p. daily, started on the day of transplantation.

Note the almost complete obliteration of the glomerular tufts accompanied by fibrosis. A large artery shows a cushion-like intimal fibrosis probably representing a healed arteritis. This was the only arterial lesion present in the graft.

Hematoxilin – phloxin – saffron × 140

On histologic examination [1]) most of the Imuran treated animals showed moderate to severe bone marrow aplasia explaining the anaemia observed at autopsy. Slight pericentral vacuolar degeneration of liver cells was present in two out of five animals of which the liver was studied. This change could be due to a toxic effect of Imuran but also may have been a consequence of the anaemia. In general, the graft rejection due to vascular necrosis and thrombosis was prevented to a larger degree than that caused by severe glomerular damage, which was observed in virtually all cases. This difference was especially noted in the animals treated with Imuran (see fig. 15). The glomerular changes together with the presence of hyaline membrane pneumonitis, probably due to uremia, can fully explain the death of the animals, although drug toxicity may have been an additional fatal factor.

Discussion

The effect of prednisolone

Prednisolone produced a definite prolongation of survival in at least 4 of 15 rats. In view of early investigations this may seem exceptional since Perski and Jacob (1951), Baker et al. (1952), and De Klerk et al. (1954) were all unsuccessful in their attempts to prolong the survival of kidney allografts in dogs treated with cortisone. Todd et al. (1964) produced only slight prolongation of 42 days in one out of 16 dogs treated with aldosterone; the remaining dogs lived for an average of 6 days, none of them longer than 13 days, which was equal to the controls. This occasional prolonged survival was thought to be due to similarity of donor and recipient on a genetic base.

Dempster (1953a), working with greyhounds, was equally unable to prolong the survival of renal allografts despite the administration of 150 to 200 mg of cortisone acetate per day. His meticulous histological and physiological studies, however, provided a rationale for the use of steroid therapy for renal homotransplants undergoing rejection. He showed that the vascular endothelial reaction was materially reduced, that the plasma cell and lymphocyte infiltration was moderated and that the arteriolar constriction characterizing the active rejection process was reduced, which thereby provided a better peripheral blood supply to the kidney while it was under immunological attack.

Since then, most studies were aimed at the use of corticosteroids in the suppression of the allograft rejection which develops during other immunosuppressive therapy. The effect of prednisolone as an additional agent has proven to be valuable in canine (Marchioro et al., 1964a) as well as in clinical cases (Goodwin et al., 1963; Hume et al., 1963; Starzl et al. 1963).

All this resulted in a scarcity of data on single immunosuppressive regimens

[1]) The histology was performed by Professor Dr. M. J. de Vries.

and made Berenbaum (1965) comment as follows: "there is a tendency to use complex systems of treatment although simple ones have not been explored adequately. This is particularly marked in renal transplantation in the dog where experimental aims are often so confounded by the temptation to imitate clinical practice, that in some large series hardly two animals have been treated alike".

However, Zukoski, Callaway and Rhea (1965) reported on prolonged acceptance of canine renal allografts following treatment with prednisolone as the only i.s. agent. Ten dogs, weighing 7–15 kg, received 30 mg prednisolone daily starting 2 days before operation. Their survival times were 16, 18, 22, 26, 29, 30, 33, 41, 477 and 1177 days, the last two dogs being still alive at the time of the report. Control allografted dogs survived 9–25 days with an average survival of 15 days.

These data correspond well with our own results and indicate that in both species long term survival is possible with prednisolone therapy alone, even in the presence of known strong histo-incompatibility, as is the case with our rat model.

The effect of Imuran

In evaluating the i.s. action of 6-MP or of its derivates one should realize that important species differences have been reported when the skin was used as allograft. Survival time of skin grafts in rabbits treated with 6-MP has been reported to be doubled or tripled (Meeker et al., 1959; Schwartz and Dameshek, 1960).

Azathioprine treatment prolonged skin graft survival in dogs from an average of 10.3 days in controls to an average of 18.8 days in the treated animals (Kisken, 1966). Additional thymectomy further prolonged survival up to 24.6 days. It should be mentioned that in the case of skin grafts in mice treated with Imuran, prolonged survival could only be obtained in the presence of weak histocompatibility differences between host and donor, whereas the drug was found to be without any effect in strongly incompatible systems (Nouza, 1966). Probably for that reason investigations on rats treated with 6-MP after skin allografting were most disappointing. Hubay et al. (1960) observed no effect at all and only a very modest prolongation of skin graft survival of a few days was achieved by Thomas et al. (1961) and Santos and Owens (1965).

In contrast to these results the pronounced effect of Imuran observed in the present experiments should be noted. It clearly demonstrates that the rejection of the allogeneic rat kidney is more susceptible to Imuran than that of the skin.

Very few exact data are available on canine renal allografts treated only with Imuran since the drug was usually administered in combination with other i.s. agents (Alexandre and Murray, 1962; Moseley et al., 1966; Diethelm

et. al., 1968). Calne et al. (1962) reported on 15 dogs treated with Imuran in a daily dose of 5 mg/kg. The average survival time was 35 days and only one dog lived longer than three months. Starzl and coworkers (1964), however, obtained better results in dogs that were given pretreatment with azathioprine. The mean survival time of animals treated with azathioprine on the day of operation was 36 days, while in those given pretreatment with azathioprine for 7–30 days before operation, the mean survival was 69 days.

Such a gain in effect by pretreatment with Imuran has never been reported by other workers in this field, while in our hands this procedure increased the hazards of operation, as drug toxicity appeared to induce oedema and more evident vulnerability of the tissues.

The somewhat better results with the rat than with the dog may be due to the fact that the incidence of infection and drug toxicity was lower in our series, and rarely accounted for the animals death. Apart from two cases of fatal infection, all deaths could be attributed to deterioration of the kidney graft, while in the above mentioned dog experiments more than half of the deaths were due to drug toxicity or infection in the presence of well functioning grafts.

Prolonged survival of kidney allografts after cessation of drug therapy as occurred in the 4 cases of Imuran treated rats, has been reported before by Pierce and Varco (1962), Starzl et al. (1964) and Sheil et al. (1968) with canine renal allografts. This, however, is not a consistent phenomenon in the dog and evidence is provided by these authors that withdrawal of therapy even after many months may lead to rejection of the graft within two weeks, while this appears to occur more frequently following cessation of drugs after only a few weeks of treatment. In our laboratory, however, three weeks of treatment of allografted rats has been demonstrated to induce prolonged survival comparable with our own results (De Bruin, 1970). The mechanism of such a prolonged acceptance of the allograft in the absence of any treatment of the host was not obvious.

In an attempt to clarify this, in one case following kidney transplantation and combined Imuran and prednisolone treatment for three weeks thereafter, the animal received a skin graft of original donor type 105 days after the kidney graft. This rat died 264 days after transplantation of the kidney while at that time the skin graft showed no signs of rejection. This is in contrast with the outcome of a similar experiment in the dog (Sheil et al., 1968), where after withdrawal of drugs, skin from a third party donor was rejected in a first set fashion and specific donor skin in an accelerated way, while the renal allograft was maintained. However, the latter experiment, as well as our own, was performed only once and such limited results hardly permit any conclusion.

In the dog, only under continuous i.s. therapy a prolonged skin graft survival can be obtained in the presence of a simultaneously grafted donor type kidney (Moseley et al., 1966). Similar drug therapy never induced prolonged survival

to that degree of skin grafts alone, indicating a protective influence from the kidney to the skin.

Apparently, following the central action of the i.s. drugs inducing non specific tolerance, once a renal transplant has been established and drugs are withdrawn, some other mechanism may become involved. The latter might well be specific immunological tolerance. However, another possibility that is recently receiving increasing support (Marquet et al., 1970), is that under these conditions enhancing antibodies induced by the kidney graft favour the continued acceptance of both kidney and skin.

Furthermore, evidence is provided by the work of Guttmann et al. (1969), that a reduction of graft immunogenecity with time may play a role in these situations.

CHAPTER VI

THE EFFECT OF LOCAL IRRADIATION OF THE GRAFT AND OF SPLENECTOMY AND THYMECTOMY OF THE HOST

Introduction

General

Since the results obtained with local irradiation of the graft and splenectomy or thymectomy of the host have appeared to be inconsistent in the experimental situation, the application of these procedures in clinical kidney transplantation has been controversial and has not been accepted as a routine procedure. Therefore it seemed of interest to evaluate these methods in the present rat kidney allograft model.

Local irradiation of the graft

Irradiation in its various forms has proved to be a useful tool for the mitigation of allograft rejection.

The immunosuppressive effect of total body irradiation has long been recognized. Dempster (1950) first demonstrated that survival of skin allograft on rabbits was prolonged by prior irradiation. It has subsequently been used in dog (Hume et al., 1960) and human kidney transplantation (Hamburger et al., 1959). The latter investigators reported on the first successful case of renal transplantation performed between dizygotic twins, treated with sublethal irradiation. However, because of the formidable problems encountered with this method, it was soon abandoned.

Local irradiation of the transplant would be highly desirable since it might selectively inhibit the effective host response to an allograft, without interfering with the normal defenses of the organism against infection. It was reported by Banks and coworkers (1961) that local irradiation of the graft up to an average total dose of 770 rads in daily doses of 100 rads failed to prolong renal allograft survival in dogs. In contrast, Kauffmann et al. (1965) reported that 150 rads given on the day of transplantation and every other day thereafter up to a total dose of 900 rads did prolong dog renal allograft survival. By examining different dosage levels it was found by Kauffmann that 150 rads was the smallest single dose which was capable of having this effect, thus perhaps explaining the negative results of Banks. The beneficial effect of fractionated local graft irradiation in the experimental animals on graft survival was confirmed by Martin et al. (1964) with dog kidney allografts and by Ono et al. (1967) and

Gergely and Coles (1970) with heterotopic heart allografts of the rat and the dog respectively.

Kauffmann and coworkers (1966) suggested that local irradiation of the graft prolongs renal allograft survival by alteration of the afferent arc of the immune response. These investigators treated groups of dogs bearing renal allografts with and without local irradiation and later challenged these dogs with an untreated second allograft. They found that in non-irradiated dogs the second kidney had a mean survival time of 1.8 days, whereas in those dogs in which the first renal allograft was irradiated, the second kidney had an average survival time of 3.3 days. They concluded that the irradiation of the first transplant had caused an inhibition of the sensibilization of the host.

Wolf and coworkers (1969) provided evidence for the hypothesis that irradiation interferes with the efferent arc as well, presumably through destruction of sensitized lymphocytes entering the graft. When two renal transplants of the same donor were placed simultaneously, of which one was irradiated, prolonged survival of the latter graft only was obtained. In these experiments each dog thus became normally sensitized and served as his own control.

Finally, the ability of irradiation to interfere with the destruction of the transplant may in part be due to non-specific effects, for it has been shown by Schlegel and Gup (1965) that radiation helps to reduce oedema consequent to ischaemic renal damage, and oedema is a conspicious feature of renal homograft rejection.

Splenectomy

The spleen constitutes a fairly large pool of lymphoid follicles, which are known to be immunologically active. As early as 1911 Luckhardt and Becht studied the immune response of splenectomized dogs immunized with a single large dose of goat erythrocytes. Nine out of ten dogs were tested within a month after operation, one animal was challenged after 275 days. All of the splenectomized dogs failed to produce as high titers of circulating antibodies as non-splenectomized control animals. Likewise, splenectomy has been shown by Saslaw and Carlisle (1964) to decrease the antibody response to small doses of intravenously administered particulate antigen in monkeys. With rats, Rowley (1950) demonstrated that splenectomy had an effect on antibody production only after the antigen was given intravenously and not if it was administered intraperitoneally or intraportally. The spleen also plays an active role in the cellular response to an intravenous antigen, as indicated by Gunderson et al. (1962).

On this basis, one could speculate that splenectomy might exert an immunosuppressive effect of its own, especially in the case of an immediately vascularized transplant. Although splenectomy, in adult mice, has been shown by Werder and Hardin (1953) to prolong skin graft survival, the negative results

of Haller et al. (1966) with mice are in contrast with these findings. According to Krohn (1953) increased skin graft survival was not seen in rabbits subjected to splenectomy. Kountz (1962) reported that splenectomy alone did not prolong the survival of renal allografts in dogs. When splenectomy was combined with splenic allotransplantation, survival of a kidney graft was increased by approximately one week, but Marchioro and coworkers (1964b) found that splenic allotransplantation without splenectomy also prolonged survival of renal allografts in dogs.

Veith and coworkers (1965) found that splenectomy in addition to azathioprine and azaserine failed to increase skin allograft survival in dogs and did not permit a higher drug dosage or protect against leucopenia and death caused by the drugs. In cases of human renal transplantation, splenectomy did not increase the functional survival of the graft in patients subjected to chemical immunosuppression. Splenectomy appeared to result in excessively high platelet counts and splenectomized patients tended to have slightly more rejection crises than those who were not splenectomized.

Thymectomy

Thymectomy in adult animals has been associated with a decrease of the lymphocytes present in the peripheral blood, the thoracic duct lymph, the lymph nodes and the spleen (Metcalf et al., 1960). However, no significant defects in the capacity for rejecting allogeneic skin homografts (Miller et al., 1963) or for producing serum antibodies have been observed in animals thymectomized in adult life and challenged within 1–2 months after thymectomy (Mac Lean et al., 1957). This is in marked contrast to the severe immunological defects which occur following neonatal thymectomy (Good et al., 1962). These observations have suggested that, during early life, the thymus is essential for the complete and normal development of certain immunological faculties, but that the presence of the thymus becomes less essential with increasing age.

Evidence that the function of the thymus in initiating immunogenesis is not necessarily restricted to early life has, however, been produced by Miller (1962). This author demonstrated in mice that irradiation with a single sublethal dose of 350 rads following thymectomy in adult life resulted in an impairment of the ability to recover the capacity for an immune response to both sheep red cells and skin allografts. Moreover, Miller (1965) found that thymectomy of adult mice resulted in a decline in immunological capacity which becomes significant only after a period of 6–9 months. It was suggested that thymectomy in the adult, unlike in the newborn, has no immediate effect on immunological capacity, presumably because an adequate pool of immunologically competent cells has already been produced. Only when the pool becomes depleted, as a result of the limited life span of its cells, do immunological defects with respect to newly encountered antigens become evident.

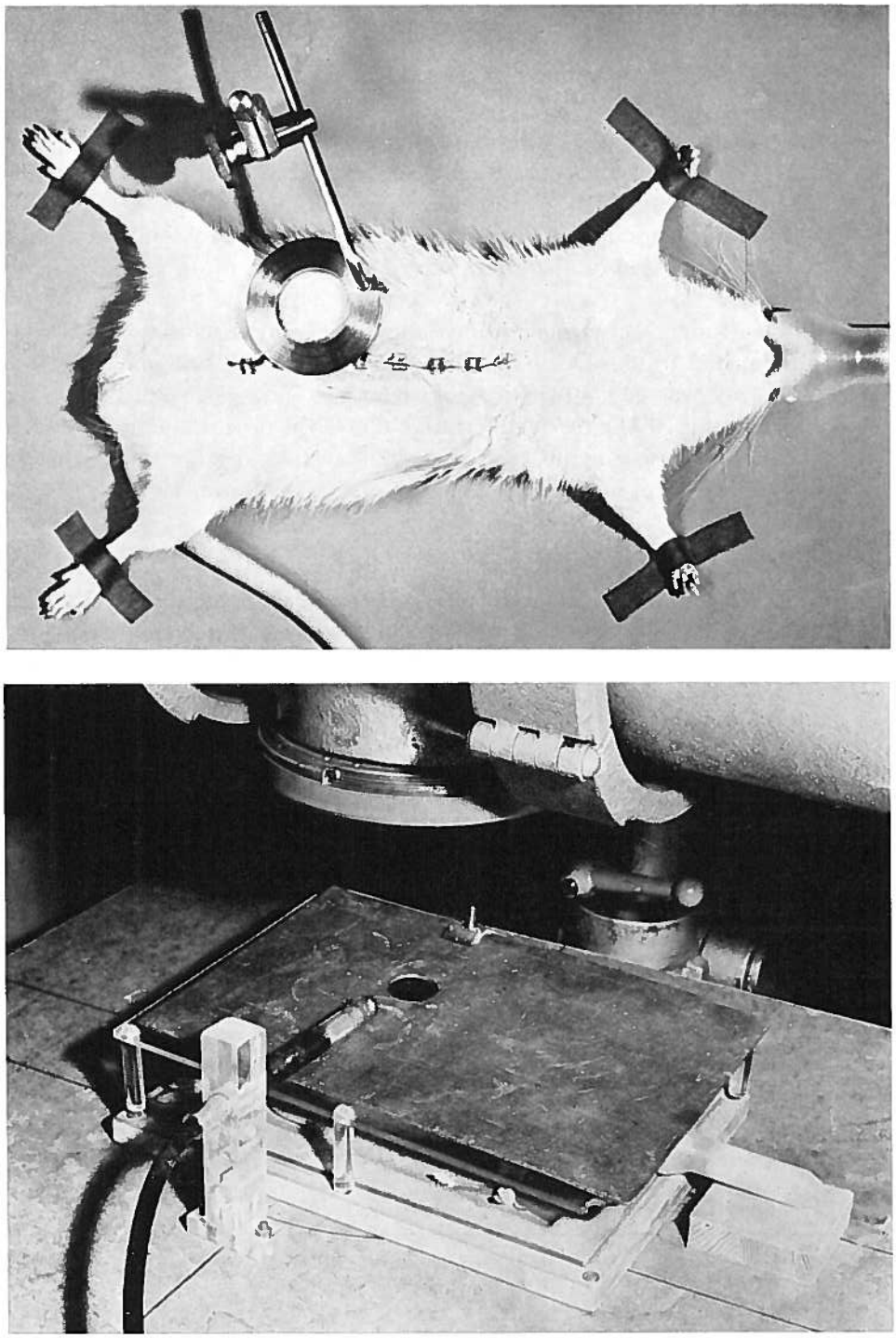

Fig. 16 Technique used for local irradiation of the graft.

The graft is localized and fixed by an external metal ring (a) after which the surrounding body is protected by a 2 mm lead plate (b).

Methods

Local irradiation of the graft

This was performed in the anaesthetized rat taped to an operating board (fig. 16a). The graft was localized and fixed by an external metal ring after which the surrounding body was protected by a 2-mm lead plate (fig. 16b), thus minimizing the total body dose. The transplant was irradiated every other day, starting on the day of operation, with 150 rads/day up to a total dose of 900 rads.

A Philips-Müller X-ray generator was used, operated at 300 kV, 10 ma, half-value layer 2.1 mm of Cu. The focus skin distance was 38 cm. The dose rate to the graft was 100 rads/min. Apart from a 1 cm zone around the graft, the remainder of the body received less than 8% of the dose to the transplanted kidney. The dose, used in the present study is well below the minimal dose which causes radiation nephritis in the dog or the rat (Redd, 1960).

Lymphatic tissue ablation

Groups of rats were thymectomized or thymectomized and splenectomized at the age of 6 weeks and kept until they were old enough to serve as recipients. Other rats were splenectomized at the time of the kidney transplantation.

Results

Table V shows the individual survival times of allografted rats subjected to the various methods of treatment.

Table V The effect of local irradiation of the graft, splenectomy or thymectomy on the survival of WAG rats bearing BN kidney allografts.

Nr. of rats	Treatment	Mean surv. time	Median surv. time	Survival times (days)
57	None	14	12	See fig. 13
15	Local irradiation of the graft	27	16	11 12 13 14 14 15 16 16 17 17 22 51 54 64 73
14	* splenectomy	81	59	9 11 14 16 27 47 58 60 67 69 73 172 194 312
4	** splenectomy + thymectomy	32		14 15 50 51
5	** thymectomy	12		10 11 12 12 14

Local irradiation of the graft with a daily dose of 150 rads was started on the day of transplantation and continued every other day up to a total dose of 900 rads.
Animals that died during transplantation or within the first week thereafter are not included.
* Performed at the time of transplantation.
** Performed at the age of six weeks of the recipient.

Local irradiation of the graft resulted in a marked prolongation of animal survival at least in 4 out of 15 rats.

Much more pronounced was the effect obtained with splenectomy performed at the time of the transplantation. Although the number of animals is small, evidence is provided that thymectomy does not add to the effect of splenectomy, and is of no effect by itself.

In the irradiated kidneys examined microscopically, the mononuclear cellular infiltration was found to be less pronounced than with the control allografts which is in accordance with findings of others (Kauffmann et al., 1965).

Some of the irradiated grafts had a haemorrhagic appearance at autopsy and showed microscopically a degree of capillary congestion and interstitial haemorrhage not usually observed in the control allografts. Presumably, this represents a certain radiation damage to the capillaries similar to that found by Fowler (1960) in dog allografts after irradiation with a dose of 3.000 rads.

Discussion

The results obtained with local irradiation of the graft are comparable to those reported by Kauffmann with dog renal allografts subjected to an identical irradiation scheme. The mean survival time of 11 dogs thus treated was 23.4 $\pm$ 15.2 days. The reduced cellular infiltrate found in the grafts is in accordance with the suggestion that the mechanism of local irradiation is interruption of the mononuclear cell – mediated effector mechanism of graft destruction (Wolf et al., 1969).

With respect to the mode of action of local irradiation on graft rejection, a recent report by Freeman and coworkers (1970) is of interest. These investigators demonstrated that rat heart allografts, derived from donors which had previously been subjected to whole body irradiation while the heart region was shielded, enjoyed a prolonged survival.

These findings support the idea of Guttmann and Lindquist (1969) that immunosuppressive pretreatment of the donor reduces graft immunogenecity by destroying "passenger leucocytes". These cells may be an important source of antigen for the induction of allograft immunity. It is unlikely however, that this mechanism plays an important role in local graft irradiation since it was demonstrated by Hume and coworkers (1960) that irradiation of the transplant in doses up to 1.500 rads prior to kidney transplantation did not abolish either the rejection of the graft or its infiltration by lymphoid cells. The effect of graft irradiation therefore, appeared not to be due to some antigenic change in the graft itself. Moreover local irradiation of the graft is only effective when given in repeated dosages.

Our results, confirming those obtained in dog experiments may give a basis to the consistent sequential use of local graft irradiation in all clinical cases by

Hume and his associates (1967). It should be noted in this respect that in an analysis of a collected series of 1.200 human renal allografts, Gleason and Murray (1967a) reported that such irradiation improves early function and survival in kidneys from unrelated donors. However, in a subsequent report of the Human Kidney Transplant Registry (1968) a more detailed analysis revealed that the difference between the irradiated and non-irradiated transplants emerges prior to the completion of the prophylactic irradiation in the immediate post-transplant period. Thus the irradiation effects were inadvertently confounded with an earlier effect altering survival, possibly the methods of procurement and preservation of the cadaver transplant.

The remarkable prolongation of graft survival obtained with splenectomy prior to transplantation has so far never been observed in the experimental situation. Apparently, the splenic portion of the R.E.S. system in the rat plays an important role in the rejection mechanism. It is of interest that the effect of various forms of resection of lymphatic tissue was shown to be less pronounced on the tempo of rejection of heterotopic heart allografts in the rat (Van Bekkum et al., 1969).

A number of harmful effects such as the additional operative stress, increased susceptibility to sepsis and to thrombo-embolic disease and decreased tolerance to drugs have been mentioned to be striking sequel as of splenectomy in man (Hume, 1966). Since that time in most clinics splenectomy therefore has no longer been performed routinely in association with renal transplantation. However, Gleason and Murray (1967b) in a report from the Kidney Transplant Registry, evaluating the function of 565 transplants performed during the 3 year period prior to March 1965, found some indications in recipients receiving a related kidney, that splenectomy resulted in a higher proportion of transplants functioning up to 3 months post-transplant. After the initial 3-month period, transplants appeared to function in about the same proportions in both splenectomized and non-splenectomized recipients. This is of interest since the hazards associated with splenectomy, that were mentioned before, may be prevented more adequately at the present time. Moreover, Pierce (1968) has reported a significant increase of survival of second kidney transplants after splenectomy in humans.

Although species variations due to differences in immunological function do not permit the extrapolation from dog or rat to man, our results may justify a more critical re-evaluation of the use of splenectomy in clinical transplantation. Similar investigations with primates which have so far not been performed, may appear to be valuable in this respect.

The fact that thymectomy prior to transplantation failed to prolong graft survival is in accordance with findings of Calne (1963) with dog kidney experiments. The possible effect of thymectomy on kidney graft survival after many months, will be difficult – if ever – to assess.

CHAPTER VII

THE EFFECT OF ANTILYMPHOCYTE SERUM

Introduction

General

Antilymphocyte serum (ALS) is the name given to an antiserum raised in members of one species by the injection of lymphocytes or lymphoid cells taken from members of another species.

The *in vitro* as well as *in vivo* leucocytotoxic properties of such antisera have long been recognized (Metchnikoff, 1899; Pappenheimer, 1917). These initial investigations provided no evidence of a specific effect of the sera against one class of leucocyte as against another.

The first indication that an antiserum could be prepared, which selectively reduced the number of circulating lymphocytes, was provided by Chew and Lawrence (1937). These authors found that a considerable degree of lymphopenia could be maintained in guinea pigs treated daily with rabbit antiserum for as long as ten days.

Cruickshank (1941) rendered rats lymphopenic by the administration of ALS and showed that complement was utilized when such serum was incubated with rat lymphocytes. Earlier workers with ALS all reported the loss of the ability to produce a lymphopenia upon chronic administration of the serum.

The first successful prolongation of allograft survival in animals treated with ALS was reported by Woodruff and Anderson (1963). In their experiments with inbred rats, a prolongation of survival of skin allografts up to 75 days was obtained by treatment with rabbit antiserum produced against thoracic duct lymphocytes from the rat. The grafts in untreated recipients were all rejected in eight days. The treatment was most effective if commenced prior to grafting. Similar observations have been reported by Nagaya and Sieker (1965), who also found that antiserum to thymocytes was more effective than antiserum to mesenteric lymph nodes.

The results originally obtained with rats have since been observed in mice (Gray et al., 1966; Monaco et al., 1966a; Levey and Medawar, 1966), while more recent studies have also indicated that ALS is capable of prolonging skin allograft survival in monkeys (Balner and Dersjant, 1967) and humans (Monaco et al., 1967).

Many reports have appeared lately to establish the valuable suppressive effect of ALS on the rejection of vascularized organ grafts. Prolongation of survival of renal allografts was demonstrated among others by Abaza et al. (1966); Huntley et al. (1966); Monaco et al. (1966b); Pichlmayr (1966) and

Clunie et al. (1968) in the dog and by Guttmann et al. (1967) in the rat. Likewise notable results were obtained with liver transplantation in the dog (Starzl et al., 1967; Pichlmayr et al., 1968), and with heart allografts in the rat (Ono et al., 1969; Van Bekkum et al., 1969).

Subsequent investigations in humans with organ transplants suggest that anti-lymphocyte antibody in conjunction with reduced doses of steroids and other immunosuppressants can effectively maintain normal graft function (Starzl et al., 1968a; Traeger et al., 1968; Shorter et al., 1968; Cooley et al., 1968).

It is generally agreed that the major part of the activity of antilymphocyte sera is located in the IgG fraction (Iwasaki et al., 1967; Betel et al., 1970). In order to produce a most potent ALS, some controversy, however, exists as to the best source of antigen and method of immunization to be used. Moreover, there are marked species differences. For instance to obtain long term survival of renal allografts in the dog, ALS prepared in the horse is much superior to that produced in sheep (Abaza et al., 1966). Furthermore, there is disagreement as to the most effective schedules of administration of the serum. Preoperative treatment alone is effective in prolonging survival of skin allografts in the mouse (Gray et al., 1966) or of renal allografts in the dog (Shanfield et al., 1968), or rat (Guttmann et al., 1967), but has been reported by Jeejeebhoy (1967) and by Starzl et al. (1967) to be without effect for rat skin or canine renal allografts, respectively.

The addition of prolonged postoperative treatment to preoperative treatment appears to be most effective in producing long term survivals, as indicated by Woodruff (1963), Starzl et al. (1967) and Russell and Monaco (1967).

In the prolongation of organ transplant function in the experimental animal ALS appears at least comparable to and sometimes even more potent than other immunosuppressive agents. Moreover ALS has the advantage that the homograft rejection which is mediated by small lymphocytes is selectively depressed without extensive effects on serum antibody production. The response to infection for instance, seems to be only mildly affected, though virus infections may occur (Abaza et al., 1966; Van Bekkum et al., 1967).

There is no generally accepted theory on the exact mode of action of ALS as an immunosuppressive agent.

The cytotoxic effect of ALS on the lymphocytes cannot fully explain its mechanism of action since many studies have indicated that the i.s. potency of ALS cannot be correlated with the degree of lymphopenia or cytotoxicity which it is capable of producing (Levey and Medawar, 1966a; Iwasaki et al., 1967; Jeejeebhoy, 1967). To account for this, it has been suggested that ALS may "blindfold" immunologically competent cells by rendering them incapable of reacting to foreign antigens or may "sterilely activate" these cells by transforming them into blasts (Levey and Medawar, 1966a).

According to Guttmann (1967) ALS may be active in the suppression of the graft rejection by its target organ specificity. He suggests that graft bound antibody may directly delay allograft rejection by a mechanism similar to that of enhancement.

All the previously mentioned mechanisms may be involved in the action of ALS but evidence is gradually accumulating that actual destruction of lymphocytes may play a decisive role. Recently, it has been found by Denman et al. (1968) and Tyler et al., (1969) that ALS selectively destroys the long-lived small lymphocyte which seems to be the principal effector cell in homograft rejection. This is not reflected in the cell counts of the peripheral blood because it is masked by the increase of short-lived lymphocytes incapable of primary immune responsiveness. Such an effect may be in accordance with the curious fact that after ALS treatment the treated subjects lymphocytes may still produce an intense infiltration in a renal allograft, yet they seem to lack the ability to cause graft destruction (Starzl, 1967).

ALS and the "second set" allograft

Since Medawar (1944) demonstrated that a second skin graft from the same donor was rejected in an accelerated manner, it has been found that various other ways of prior immunization of the host could induce the so called "second set" rejection. Primary inoculation of whole blood (Colberg et al., 1964) in rabbits or of packed erythrocytes in rats (Breyere, 1959) has resulted in accelerated rejection of subsequent skin allografts.

Investigations by Amos and coworkers (1968) have demonstrated that sensitization of human skin graft recipients can occur by blood transfusion. The mean survival time of skin grafts for subjects receiving whole blood was 6.0 days, stored blood 6.5 days and buffy coat poor blood 12.3 days. There were no significant differences in the rejection times of control (third party) grafts in the various groups (mean survival time 12.4 days).

The second set phenomenon has also been shown to occur with kidney transplantation. Accelerated rejection of kidney allografts in dogs has been observed following prior sensitization of the host by transplantation of a kidney (Dempster, 1953b; Egdahl and Hume, 1955) or skin allograft (Dempster, 1953b; Hubay et al., 1962) derived from the prospective donor. For instance Egdahl and Hume (1955) demonstrated that 9 out of 15 secondary transplants functioned for 1 day or less. Both Dempster and Hubay et al. found that the antigenic stimulus of the renal allograft was similar to that of skin in its capacity to sensitize the host.

In further studies with dog renal allografts Egdahl and Hume (1956) found that a single transfusion of 300 ml of blood given from future donor to future host from 7 to 14 days before transplantation did not alter the typical primary allograft rejection. However, cross circulation of the kidney transplant donor

with the recipient prior to kidney transplantation resulted in a very effective immunization. As short a period of cross circulation as 5 minutes was sufficient to immunize and second set phenomena were observed when transplantation was performed as late as 28 days after cross circulation. Wheeler and Gomez (1962) demonstrated with similar experiments that the effect of prior cross circulation on the renal allograft response varied from active immunization to the induction of at least partial tolerance, depending on the duration and rate of blood exchange and its temporal sequence with respect to transplantation.

With human renal transplantation, the one year survival rate with functioning allografts obtained by Gifford et al. (1967) was better after first – than after second transplantation, which made this author reluctant to recommend retransplantation in patients who have rejected their first allograft. However, Hume and coworkers (1966) reported on 57 second transplants and concluded that if a second transplant is performed after failure of the first, a better rate of acceptance is usually achieved than that obtained with first set grafts. In clinical transplantation a hyperacute rejection of renal allografts occurs occasionally in individuals who have been presumably presentized to donor antigens by previous dialysis, pregnancy or even bacterial exposure (Kissmeyer-Nielsen et al., 1966; Williams et al., 1967). It has been suggested that in some cases this may represent a Schwartzman reaction (Starzl et al., 1968b).

In the course of their initial experiments with ALS both the groups of Levey and Medawar (1966b) and Monaco et al. (1966a) reported a considerable prolongation of second set skin allografts in mice, a finding which according to the former authors, distinguishes ALS from all other immunosuppressive agents at any rate when used in a non-toxic dosage.

Such erasure of immunological memory with ALS was confirmed by Lance (1968) who also noted that the test allografts survived longest when applied near the beginning of ALS administration.

It should be mentioned that the abolition of pre-existing delayed hypersensitivity skin reactions in patients has been demonstrated within a few days after the institution of ALG therapy (Brunstetter and Claman, 1968).

ALS, however, does not have a profound effect on the secondary humoral antibody response (Monaco et al., 1966a; James, 1967).

In view of the above mentioned evidence for accelerated kidney graft rejection in experimental and clinical situations, it seemed of interest to investigate whether the effect of ALS observed with second set skin transplantation would likewise apply to organ allografts. Such an effect of ALS has so far not been studied.

In the course of the present experiments the effect of a crude anti-rat thymocyte serum from rabbits was first evaluated on the rat kidney allograft model. Subsequently, this treatment was combined with the administration of Imuran.

Furthermore, the experiments were extended by studying the effect of purified anti-thymocyte γ globulin on "second set" kidney allografts.

Methods of preparation and application of ALS

Rabbit anti-rat lymphocyte serum was prepared by a multipulse immunization schedule (see fig. 17). Flemish rabbits received 4 s.c. injections of 10^9 rat thymus cells suspended in Tyrode's solution at intervals of 10 days. Seven days after the fourth immunization, the rabbits were bled and about 50 ml of serum were collected per rabbit. Ten days later, they received a booster consisting of 10^9 rat thymus cells s.c. and, following another interval of 7 days, they were exsanguinated. Sera obtained from the two bleedings and from groups of

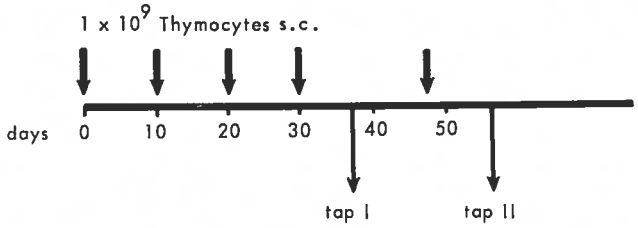

Fig. 17 Immunization scheme used for the preparation of ALS.
See text for description.

6 rabbits were pooled, and each pool was tested for its suppression of skin allografts rejection in WAG rats. To this end, the rats received 3 ml of crude ALS (or 1 ml of anti-lymphocyte globulin (ALG) containing 20 mg of γ-globulin/ml) s.c. 7 days before the grafting of BN rat skin. A second dose of 3 ml of ALS was given on the day of skin grafting and further similar doses on days 7, 14, and 21 after the transplantation. This regimen resulted in skin graft survival for periods of between 60 and 70 days, the normal rejection time being 11–12 days. The γ-globulin fraction of the serum was prepared by purification over diethylaminoethyl Sephadex columns following precipitation with ammonium sulfate. All ALS preparations were sterilized over Millipore filters before use. The cytotoxic titer for blood leucocytes of the crude serum varied between 512 and 2048.

Treatment of the kidney graft recipients was similar to that of the rats receiving the test skin grafts, except that the administration of ALS was continued weekly up to 12 weeks after transplantation. In case of a combined regimen of ALS and Imuran (4 mg/kg daily), all treatment was discontinued after 8 weeks.

In the experiments with "second set" kidneys only anti-lymphocyte globulin (ALG) was used. The treatment schedules will be presented with the results.

Results

Table VI shows the individual survival times of rats bearing allografts subjected to treatment with either ALS or with a combination of ALS and Imuran.

Table VI The effect of ALS or a combination of ALS and Imuran on the survival of WAG rats bearing BN kidney allografts.

Nr. of rats	Treatment	Mean surv. time	Median surv. time	Survival times (days)
57	None	14	12	See fig. 13
6	N.R.S.	11		9 10 11 12 13 13
14	*ALS	75	42	9 11 12 16 21 22 34 51 65 66 78 99* 275* 294*
7	**ALS + Imuran	273	299	27 54 146** 299** 307** 322** 755**

ALS (antilymphocyte serum) and NRS (normal rabbit serum) were administered s.c. in weekly doses of 3 ml., started one week before transplantation.
Imuran was administered i.p. in a daily dose of 4 mg/kg started on the day of transplantation.
Animals that died during transplantation or within the first week thereafter are not included.
* Treatment discontinued after day 84.
** Treatment discontinued after day 56.

In both groups a definite prolongation of survival can be observed while the results with the combined treatment appear to be the most promising. Control rats treated with normal rabbit serum (NRS) demonstrated a survival pattern similar to untreated allograft bearing animals. Furthermore, it is shown that continuous treatment is not mandatory for the production of long term survival.

In the case of the ALS treated rat that survived for only 9 days, death was due to pyelonephritis. No further evidence of infection was observed in the other experiments. The absence of haemorrhagic complications indicates that any thrombocytopenia that may have occurred was not severe.

Limited histological investigations (de Vries et al., 1969) have indicated that ALS prevented only slightly the vascular rejection reaction as compared to the definite susceptibility of this component of graft rejection to Imuran (see fig. 18). However, the lymphoid cell infiltration and particularly the damage of parenchymal cells, upon which Imuran had only a minor effect, appeared to be specifically inhibited by ALS.

In order to obtain a "second set" kidney allograft test model, in preliminary experiments future hosts were pretreated intravenously with prospective donor blood. This method of sensitization was chosen to imitate in a way one of the possible mechanisms of pre-immunization occasionally encountered in clinical kidney transplantation. Rather unexpectedly, this pretreatment resulted in a prolonged survival of the allografts and subsequent investigations on this subject are reported in chapter VIII.

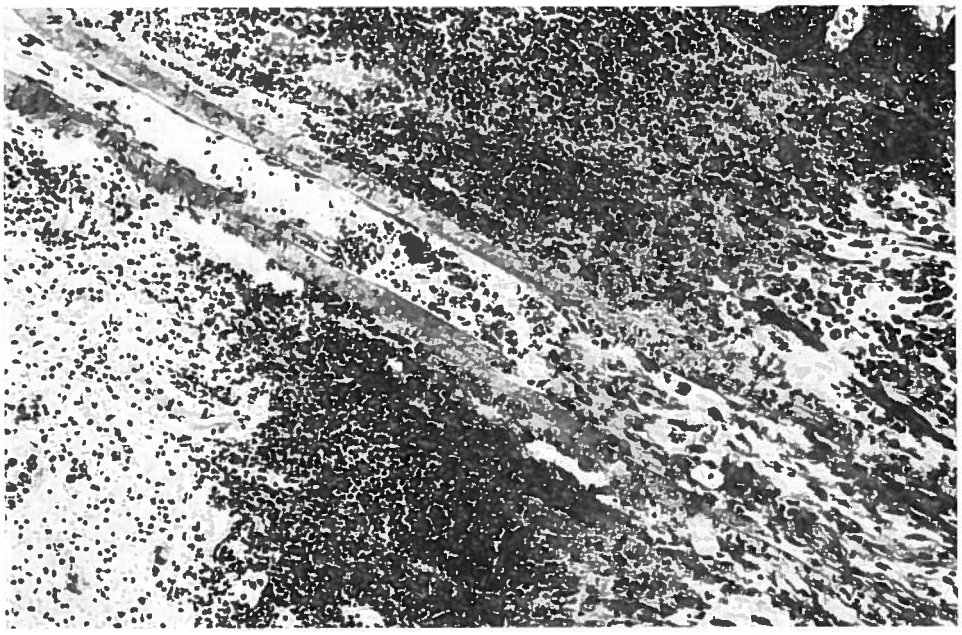

Fig. 18 Necrotizing arteritis of a large artery in a renal allograft at 11 days following transplantation (autopsy specimen).

The animal had been treated with ALS in weekly doses of 3 ml s.c., started one week before transplantation. Note the leucocytic infiltration and necrosis of the vascular media and obliteration of the lumen by endothelial proliferation. The renal parenchyma showed large areas of infarction.

Hematoxilin – phloxin – saffron × 140

Table VII The effect of ALG on WAG rats bearing "second set" BN kidney allografts.

Nr. of rats	Previous skin graft	*Treatment with ALG	Mean surv. time	Median surv. time	Survival times (days)
57	—	None	14	12	See fig. 13
8	+	None	7		5 5 6 6 7 8 8 8
4	+	1 ml on days −7; 0; +7	8		7 7 8 10
4	+	1 ml on day −7 ½ ml on days 0; +1; +3; +5; +7	10		10 10 10 11
5	+	½ ml on days −7; −5; −3; 0; +1; +3; +5; +7	40	18	14 17 18 23 130

The recipients were sensitized with a BN skin graft 18–20 days prior to transplantation. ALG (antilymphocyte globulin) containing 20 mg of γ globulin/ml. Animals that died from causes other than rejection are not included.

* In relation to the day of kidney transplantation.

A more effective sensitization was acquired by primary transplantation of a donor type skin allograft, performed 18 to 20 days before transplantation of the kidney. Circulating haemagglutinating antibodies and cytotoxic antibodies against donor leucocytes were detectable just prior to kidney grafting.

In table VII the survival times of rats bearing "secondary" kidney transplants are shown. Animals dying from other causes than rejection are excluded from the data. In untreated rats accelerated rejection of the grafts resulted in survival times about half of those found with control primary allografting. Although the series are small, evidence is provided that ALS inhibits the "second set" rejection of kidney allografts, at any rate when the treatment is started before transplantation and continued thereafter at a "steady level" regimen until one week after transplantation.

Discussion

The immunosuppressive effect of ALS on the first set kidney allograft demonstrated in the present study is comparable with and in some cases more pronounced than that obtained by many investigators with dog renal transplantation. Monaco et al. (1966b) described two out of 7 dogs surviving beyond 350 days under continuous treatment with ALS. Lawson and associates (1967) reported on 13 dogs treated with rabbit anti-canine thoracic duct lymphocyte serum. The mean survival time was 28 days. Shanfield and coworkers (1968) obtained a mean survival of 47 days among 13 dogs of which one dog died after 372 days. It was demonstrated by these authors and confirmed by Clunie et al. (1968) that a given amount of antiserum was considerably more effective when administered preoperatively than when given postoperatively.

The prolonged survival of rats bearing kidney allografts after withdrawal of ALS treatment was confirmed in our laboratory by De Bruin (1970). With three weeks of ALG therapy similar results as our own were obtained. These observations of the efficacy of a limited course of treatment of ALS may be of practical importance since long term administration has a number of disadvantages (Iwasaki, 1967).

The results with combination therapy of ALS and Imuran are the best so far obtained in the present experiments. The histological observations previously mentioned may provide a theoretical basis for these superior results.

In mice, both the lymphopenia and the immunosuppressant action initiated by a priming dose of ALS can be maintained for a prolonged period by a variety of conventional immunosuppressive drugs (Hoehn and Simmons, 1967). In dogs, however, Starzl et al. (1967) reported that the addition of small doses of Imuran to the treatment with ALG resulted in slight and statistically non significant increases of survival, but the degree of histologic injury seemed to be reduced. More recently, Weil and Simmons (1968) demonstrated that the

combination of preoperative ALS and postoperative azathioprine significantly increased the prolongation of canine renal allograft survival beyond the effect obtained with either treatment alone. These results suggest that the danger of the prolonged administration of heterologous serum may be avoided by the addition of standard i.s. drugs to short courses of ALS. Moreover, there are indications that the clinical application of ALS in the future will be predominantly in combination with Imuran, not only because the latter drug may add to the i.s. effect by an action of its own, but also indirectly by inhibiting antibody formation by the recipient to the ALS preparation.

Alternatively, the successful induction of tolerance to the effective principle of ALS preparations may become feasable (Wood, 1970). The prolonged survival produced with ALS treatment of rats receiving a "second set" kidney may have practical consequences for the acceptance of a kidney allograft in man in case of a possible pre-immunization of the recipient to antigens present on the cells of the kidney donor. It should, however, be realized that in those cases the effective dose of ALS has to be high since a treatment schedule sufficient to induce prolonged first set allograft survival was incapable of doing so with second set kidneys. In the latter situation more frequent pre- and postoperative injections were required to obtain that effect.

CHAPTER VIII

THE EFFECT OF PRETREATMENT OF THE RECIPIENT WITH DONOR SPECIFIC ANTIGEN

Introduction

General

Over the past twenty years efforts to prolong the viability of allografts by treatment of adult recipients prior to transplantation with donor specific antigen have been of increasing experimental interest. The immunological mechanisms underlying this phenomenon, although not fully elucidated, have been classified under two main categories: Tolerance and Enhancement.

Acquired immunological tolerance

Immunological tolerance may be defined as a state of specific non-reactivity to a normally effective antigenic challenge, induced in an animal by prior exposure to the antigen concerned or to a closely related antigen. Tolerance may be complete or incomplete, in the latter case representing a significantly lowered response to challenge. It may be induced in early or in adult life. In the latter situation it usually necessitates the use of large amounts of antigen.

In 1949 Felton showed that adult mice could be immunized and thereby protected against otherwise lethal doses of living pneumococci by the injection of 0.5 microgram of pneumococcal polysaccharide. The injection of larger doses, e.g. 500 microgram on the contrary failed to induce resistance: the mice became vulnerable to pneumococcal infection and could no longer be protected against it by subsequent injection of the smaller dose of polysaccharide (immunological paralysis). It is suggested that this phenomenon is related to immunological tolerance and that the terms tolerance and paralysis can be used interchangeably (Mitchison, 1967).

In 1945 Owen showed serologically that most cattle twins which are commonly synchoreal and therefore exchange foetal blood, are erythrocyte chimeras at birth. This means that they possess and may retain throughout life a certain proportion of erythrocytes of the type belonging to the opposite twin. These observations led Burnet and Fenner (1949) to postulate that an animal challenged with antigen during foetal life, prior to the development of immunological competence, would thereafter be unable to respond to further challenge with this antigen. Subsequently, Anderson and associates (1951) showed that most dizygotic cattle twins would accept skin grafts from each other and that this mutual tolerance was specific, for skin transplanted from a third party was rejected in the expected way.

Billingham, Brent and Medawar succeeded in 1953 in producing actively acquired tolerance by the injection of mouse embryo's of the CBA strain in utero with a suspension of living spleen cells of the A strain. When the CBA recipients had grown to adulthood they were regularly found to accept A strain skin grafts. Furthermore, these investigators (1956) demonstrated that the cells of the lymphoid system are directly involved in this kind of unresponsiveness, as evidenced by their experiments on adoptive transfer. They found that animals rendered tolerant by perinatal exposure to transplantation antigens could be reequiped immunologically by the injection of normal isogeneic lymphoid cells. The total absence of circulating antibodies directed against the tolerance inducing antigens was further evidence for the central failure of the immunological response, involved in the tolerant state. Moreover, Dietrich and Weigle (1964) demonstrated the inability of lymphoid cells of tolerant animals to form antibodies against the tolerated antigen after transfer to irradiated recipients.

The principle of immunological tolerance has subsequently been shown to apply to infant (Brent and Gowland, 1961) and adult animals (Shapiro et al., 1961; Marshall, 1962; Brent and Gowland, 1962). However, when suspensions of living homologous cells were employed as inocula, the repeated dosages required to overcome major incompatibilities were very large. The effect was evident only on intravenous administration. The subcutaneous, intramuscular and intraperitoneal routes of administration led to accelerated rejection (Marshall, 1962). Next to viable cells, cell homogenates as well as cell fractions have been successfully employed to induce tolerance (Martinez et al., 1963; Medawar, 1963).

The exact mechanism of immunological tolerance is unknown, but some theories have been proposed. According to the clonal selection theory of Burnet (1959), antibody forming cells are a heterogenous population and individual cells have a predetermined ability to act against a given antigen. The induction of tolerance is based on the elimination of only those cell clones that are capable of reacting against the specific antigen. This would imply the total absence of reactive cells in the tolerant state.

The stem cell theory of tolerance induction referred to by Brent and Gowland (1961) postulates that all immunologically immature cells are susceptible to the induction of tolerance, regardless of whether they are in the embryo or the adult. The exposure of adult animals to antigenic stimuli should therefore set in motion two processes: sensitization of mature cells and induction of tolerance to immature cells. Tolerance would normally be masked by the immune response but if the sensitized cells were to be removed by some means (as for example by prolonged contact with antigens) the lymphoid cell population of adult animals might be changed progressively from a predominantly sensitized to a predominantly tolerant one.

Almost all studies of antigen induced tolerance have been performed with

skin serving as the test graft. More recently however, several reports have appeared on the prolonged survival of experimental kidney allografts in animals pretreated with donor antigen (Halasz et al., 1966; Linn, 1966; Dagher et al. 1967). These results were interpreted by the authors in terms of immunological tolerance. However, in view of current information on this subject, enhancement may have been an additional factor involved.

Immunological enhancement

Immunological enhancement may be defined as the successful establishment or delayed rejection of an allograft as a consequence of the hosts' active or passive immunization against the graft (Kaliss, 1962). The presence of anti-graft antibody as the effective agent distinguishes this phenomenon from other types of immune unresponsiveness. It is produced by a paradoxic effect of immunization: pretreatment with antigenic material which stimulates transplantation immunity can, under certain circumstances, prolong allograft survival.

Immunological enhancement was probably first noted by Flexner and Jobling (1907) who observed increased susceptibility of rats to transplants of a rat sarcoma after pretreatment with a heated emulsion of the tumor. The immunologic nature of the phenomenon was not recognized however, until the demonstration of Kaliss and Molomut (1952) that the enhancing effect could be passively transferred with serum and particularly by its gammaglobulin fraction.

Most subsequent demonstrations of successful immunological enhancement have been executed with incompatible tumor grafts growing in inbred mice (Snell, 1952; Kandutsch and Reinert-Wenck, 1957). However, the phenomenon has also been shown to operate in the prolongation of skin allograft survival, but the effect has usually been limited to only a few days beyond control survival times (Nelson, 1962; Möller, 1964). This is probably due to the fact that cellular immunity plays a more decisive role in skin graft rejection as compared to tumor graft rejection.

In the case of active conditioning, there is a tendency for cellular reactivity to subside with the passage of time and a state is attained in which circulating antibodies are present but cellular immunity can no longer be elicited. It is in this setting that immunologic enhancement of a second graft may occur (see review by Hutchin, 1968).

When active conditioning was applied to skin allografts, success has been achieved with viable as well as with non-viable cell preparations. In the experiments of Heslop (1966) a prolonged survival up to 21 times the normal mean survival time was obtained by pretreatment of the recipients with non-viable spleen cells given intraperitoneally. The phenomenon was strain specific and transferable by serum. When, however, viable spleen cells were used the pretreatment gave rise to accelerated rejection. By contrast Billingham and Sparrow

(1955) found that only viable epidermal cells were effective in prolonging skin allograft survival in rabbits, while the intravenous route appeared to be obligatory in this respect.

In passive transfer of enhancement, large doses of serum must be given (Nelson, 1962; Heslop, 1966), since the half-life of gamma globulin is quite short (Rubinstein and Kaliss, 1964). Investigations by Moore (1965) demonstrated that only sera with high titers of humoral antibodies effected prolonged skin allograft survival in mice.

Several theories have been proposed to explain the phenomenon of immunological enhancement. The afferent inhibition theory suggests that the antibody combines with and blocks antigenic sites of the allograft, thus hindering their recognition by the host (Möller, 1963). In the efferent inhibition theory the antibody does not interfere with the initial events of antigen recognition but prevents the sensitized cells from exerting their destructive capacity. Evidence for this view comes from the demonstration of enhancement in preimmunized recipients (Kaliss, 1958) and the ability of adoptively transferred lymphoid cells from enhanced animals to confer immunity on untreated recipients (Linder, 1962). A central failure is unlikely, since it was demonstrated by Möller (1963) that enhancement can occur towards antibody-coated target-cells in the absence of circulating antibodies.

Peripheral whole blood, used in small doses, was found to be an effective conditioning agent in prolonging skin graft survival in a number of studies in rabbits (Billingham and Sparrow, 1955; Stark and Dwyer, 1959), rats (Marino and Benaim, 1958) and dogs (Halasz, 1963). No clear evidence, however, was provided from these studies that enhancement and not tolerance was the mechanism involved. More recent experimental data indicate that both active and passive enhancement can operate in the prolongation of the viability of kidney allografts in dogs (Wilson et al., 1969) and rats (Stuart et al., 1968; French and Batcheler, 1969; Ockner et al., 1970a, b).

The present experiments

In the course of the preceding experiments it was found that a single intravenous injection of 2 ml of donor blood administered to the recipient two weeks prior to transplantation resulted in a prolonged survival of the kidney graft. This observation led to further investigations which were varied as to the timing of the pretreatment before transplantation (2 hours – 1 month), the dosage of donor blood (0.05 ml–2 ml) and the route of administration employed (intravenous and subcutaneous). In several experiments a given pretreatment was likewise tested on skin allografts between the same rat strain combinations.

In order to exclude the possibility of cell chimerism or a graft versus host reaction, in one series of experiments the donor blood was irradiated prior to

injection. Furthermore, various blood constituents as well as thymocytes were evaluated for their respective conditioning capacity.

Animals pretreated with third party blood from a non-inbred rat strain (BROFO) available in our laboratory served as controls.

Materials and methods

Whole donor blood (citrated) was acquired by cardiac puncture of the anaesthesized animal.

Irradiated blood. This was obtained by giving 1.000 rads to the citrated blood just prior to use.

The erythrocyte suspension was prepared by dextran sedimentation of citrated blood and removal of the supernatant plasma, containing the majority of leucocytes and platelets. The remaining erythrocytes were washed three times with saline after which the suspension, containing 4×10^9 erythrocytes per ml was administered intravenously in a single dose of 2 ml two weeks prior to transplantation. The exact composition and dosage of the various cell suspensions will be shown in table XI.

The leucocyte suspension was prepared by the use of the above mentioned supernatant plasma; the cells were washed three times and the suspension in saline was used, containing 10×10^6 leucocytes per ml. The suspension was given intravenously in a single dose of 2 ml, two weeks before grafting.

Thrombocyte rich plasma. Since the leucocyte suspension appeared to be contaminated with a considerable amount of thrombocytes, the effect of these blood platelets in itself was investigated. A suspension containing 250×10^6 thrombocytes per ml. was prepared by centrifugation of citrated whole blood for 15 minutes ($200 \times$ G.) A single dose of 2 ml was administered 2 weeks before transplantation.

Pure plasma was obtained by ultracentrifugation of citrated blood for 60 minutes ($30.000 \times$ G) and given in a dose of 2 ml two weeks before transplantation.

The thymus cell suspension was prepared as follows. The intact thymus was minced with fine scissors and suspended in Tyrode's solution after which the material was squeezed through a fine nylon mesh. The resulting suspension, containing 5×10^8 living thymus cells per ml, was administered in a dose of 2 ml two weeks prior to transplantation.

Serum for passive transfer studies was harvested from recipient WAG rats which had received 2 ml of BN donor blood two weeks previously. The serum was stored at −20 °C until use.

A skin grafting technique as described by Balner (1964) was used. Recipients were anaesthetized with ether, the dorsal thoracic area shaved, covered with adhesive tape (Scotch no. 471) and lifted in a fold to punch out two adjacent circular pieces of 1.5 cm diameter. The grafts, punched similarly from abdominal skin and stripped of their panniculus with scissors, fitted snugly into the grafts, beds which maintained shape and position due to the surrounding tape. A dressing of impregnated gauze ("Carbonet", Smith and Nephew, England) and a fixing tape around the thorax were left on until the 7th day. Evaluation of graft survival was done macroscopically on the basis of hair growth, atrophy, oedema and haemorrhages.

Results

Table VIII shows the individual survival times of allografted rats which had received a single injection of 2 ml donor blood at various periods before kidney transplantation. Moreover, the effect of some treatment schedules on the survival of corresponding skin grafts is shown. Since the experimental groups are of different size and in certain groups the survival times are widely scattered, some of the presented mean and median survival times are of little value. As for the kidney allografts the conditioning effect of blood pretreatment is obvious in all experimental groups. Some indication may be derived from these data that shortening of the treatment – transplant interval reduces the protective effect achieved. The prolonged survival of rats pretreated only two hours before grafting should however be noted. Since BN→WAG skin allografts

Table VIII The effect of pretreatment of the recipient with 2 ml of donor blood at various times prior to transplantation.

Interval between pretreatment and grafting	WAG RATS BEARING BN KIDNEY GRAFTS			BN→WAG SKIN GRAFTS
	Mean surv. time	Median surv. time	Survival times (days)	Survival times (days)
30 days	270	270	270 270	9 9 10 10
14 days	85	46	11 13 29 32 60 109 182 243	9 10 10 10
7 days	41	27	8 8 11 27 65 78 93	
2 days	19	15	9 12 15 15 16 46	13 13 13 14
2 hours	22	19	10 12 15 19 26 36 36	

The donor blood was administered intravenously.
Animals that died during transplantation or within the first week thereafter are not included.

are normally rejected by day 11, a quite opposite influence of timing may be observed in the skin grafting experiments.

It is shown in table IX that no clear dose response relationship can be derived from the results obtained with different amounts of donor blood administered to the recipient 2 weeks before transplantation. Prior injection of 0.05 ml appears to be as effective as pretreatment with 2 ml of donor blood. Irradiation of the conditioning blood before administration does not seem to interfere with its effect on graft survival. Subcutaneous administration of the blood in some cases leads to accelerated rejection of the kidney graft, while it does not affect skin graft survival. Third party blood is of no effect.

Table IX The effect of various methods of pretreatment of the recipient with blood two weeks before transplantation.

				WAG RATS BEARING BN ALLOGRAFTS			BN→WAG SKIN GRAFTS
Dose (ml)	Route of admin.	Source of blood	Irr. of blood	Mean surv. time	Median surv. time	Survival times (days)	Survival times (days)
2	i.v.	BN	–	85	46	11 13 29 32 60 109 182 243	
0.2	i.v.	BN	–	164	127	8 78 111 143 157 486	
0.1	i.v.	BN	–	362		361 364	
0.05	i.v.	BN	–	106	125	20 56 125 159 170	
2	i.v.	BN	+	158	118	8 11 12 14 19 92 145 214 231 331 358 462	9 9 10 10
2	s.c.	BN	–	9		6 6 8 9 10 10 10 11	11 11 11 11
2	i.v.	BROFO	–	12		11 11 12 14	

Animals that died during transplantation or within the first week thereafter are not included.

The results obtained with pretreatment of the recipient two weeks before transplantation with various donor cell preparations is shown in table X. Both the erythrocyte and thrombocyte preparations show a distinct effect on kidney allograft survival, while the skin grafts in the latter case are rejected in an accelerated fashion. The effectiveness of leucocytes can not be evaluated from these data since the thrombocytes contaminating the leucocyte suspension by themselves are able to induce kidney graft protection.

The marked results obtained with the thymus cell suspension are of interest. As might be expected cell free donor plasma does not affect kidney graft rejection.

To examine whether enhancing antibodies might be involved in the induction of prolonged allograft survival, in 4 cases the recipients were injected intravenously at days – 1 to +7 with respect to kidney transplantation, with 1 ml of serum derived from WAG rats previously conditioned with donor blood. These recipients survived for 12, 13, 22 and more than 100 days.

Table X The effect of pretreatment of the recipient with various donor cell preparations

Conditioning agent*	Dose (cells)	WAG RATS BEARING BN ALLOGRAFTS Mean surv. time	Median surv. time	Survival times (days)	BN→WAG SKIN GRAFTS Survival times (days)
Erythrocytes	8000×10^6	37	26	12 20 25 26 27 55 96	11 11 11 11
Leucocytes+ thrombycytes	20×10^6 500×10^6	36	18	8 11 16 21 25 135	9 9 9 9
Thrombocytes	500×10^6	49	18	9 14 16 17 18 50 52 60 203	7 7 9 9
Plasma	2 ml	12		11 11 12 12 13	
Thymus cells	1000×10^6	191	163	11 41 140 186 366 406	

All preparations were administered intravenously two weeks before transplantation.
Animals that died during transplantation or within the first week thereafter are not included.
* For the exact composition and dosage of the various cell suspensions, see table XI.

Discussion

The present results demonstrate that the tempo of rejection of renal allografts can be markedly retarded by the administration of different donor cells to the recipient at various periods prior to transplantation. This alteration of allograft rejection is an immunologically specific phenomenon as indicated by the failure of blood of a different strain to induce a similar effect.

There have been a number of successful attempts to prolong allograft survival by the prior inoculation of living donor cells to the recipient. Prolonged acceptance of skin grafts across a significant histocompatibility barrier by the administration of donor strain splenic cells to adult mice has been produced under experimental conditions requiring large cell doses (Brent and Gowland, 1962; Shapiro et al., 1961). While these mouse studies suggest the production of acquired tolerance in adult animals, this is probably not so for previous attempts to prolong renal allografts (Wilson et al., 1969; Stuart et al., 1968). The latter investigators demonstrated in rats that the beneficial effect of donor spleen cells administered prior to transplantation could be increased by the additional passive immunization with anti-donor serum prepared in rats of the recipient strain.

Halasz and coworkers (1964) obtained significantly prolonged survival of kidney grafts in dogs pretreated with subcutaneous injections of 2 ml of donor blood 10 and 15 days prior to grafting. The latter results have so far not been confirmed by others and are in contrast with our findings that subcutaneous conditioning of the recipient resulted in slightly accelerated rejection of the graft.

Our results are of interest in view of a recent report by Ockner et al. (1970a, b), demonstrating that the prior intravenous injection of bone marrow cells to the host could markedly prolong the viability of rat renal allografts. It was

found in these studies that with respect to cell dosage, 10^7 or 10^8 marrow cells and with respect to the timing of the injection, 6–13 days prior to transplantation appeared to be most effective as reflected by graft function and histological signs of rejection at 7 days following transplantation. When cell dosage and timing differed from these optimal conditions, in most instances no effect was obtained or even accelerated rejection occurred. Such sensitization resulting in shortened graft survival was not observed in the present study with a great variety in cell dosages and timing.

The fact that pretreatment with blood as late as 2 hours before transplantation also was effective, although to a less extent, is of obvious interest with respect to a possible application in the clinical situation, particularly when cadaver donors are employed. Studies of Stuart et al. (1968) with spleen cells administered 18 hours before transplantation, and of Zimmerman et al. (1970) with splenic extracts support the promising aspects of this type of conditioning of the host. The latter investigators reported indefinite survival of rat renal allografts when the recipient was inoculated at the time of organ transplantation.

In the present study, a quantitative analysis of the selective conditioning effect of each individual cell type is difficult because of the variety in composition of the cell preparations used. An attempt to that end will however be made on the basis of the data shown in table XI. When the efficacy of the leucocyte suspension is compared with that of 0.05 ml of blood, it is evident that the leucocytes in itself are not exclusively responsible in this respect, since 20×10^6 leucocytes were considerably less effective than 0.7×10^6 of these cells as present in 0.05 ml of whole blood. The same applies for the thrombocytes: a less pronounced prolongation of graft survival was obtained with 500×10^6 platelets in the leucocyte and thrombocyte suspensions, than with 40×10^6 of these cells present in 0.05 ml of blood. This leaves the erythrocytes to be considered as the most important factor involved in graft protection with whole blood. The latter assumption may seem unusual but it has been shown before (Breyere, 1959)

Table XI Composition and dosage of various conditioning agents.

Conditioning agent	Erythrocytes	Leucocytes	Thrombocytes	Total number of cells	Mean surv. time*	Median surv. time*
0.05 ml blood	450×10^6	0.7×10^6	40×10^6	500×10^6	106	125
Erythrocyte suspension	*8000×10^6*	1×10^6	40×10^6	8000×10^6	37	26
Leucocyte suspension	50×10^6	*20×10^6*	500×10^6	570×10^6	36	18
Thrombocyte suspension	4×10^6	0.4×10^6	*500×10^6*	500×10^6	49	18
Thymocyte suspension	20×10^6		7×10^6	*1000×10^6*	191	163

* Survival times of WAG rats bearing BN kidney allografts, see also tables IX and X.

that rat erythrocytes do carry transplantation antigens. However, 8.000×10^6 red blood cells present in the erythrocyte suspension were less effective than 450×10^6 of these cells in 0.05 ml of blood, while both agents contain a comparable number of other cell types. For this no satisfactory explanation can be given. The possibility that $8{,}000 \times 10^6$ erythrocytes in itself have been too many cells so as to induce some degree of sensitization instead of optimal enhancement, can be excluded on the grounds that a pronounced prolongation of survival was obtained with as many as 20.000×10^6 cells present in 2 ml of blood. Because more information is lacking so far, we have to consider the possibility that the method of preparation of the erythrocyte suspension may have reduced the antigenicity of the cells. This explanation is supported by the fact that the erythrocyte suspension in contrast to the leucocyte and thrombocyte suspensions did not induce accelerated rejection of subsequent skin allografts.

It is not surprising that the thymus cells were most effective because they are known to carry a relatively high concentration of transplantation antigens. Although our data provide no information as to the efficacy of leucocytes – which in the rat consist of 80–90% of lymphocytes – there is every reason to suppose that these cells are capable of producing a conditioning effect of their own if given in sufficient quantity. Our results indicate that rat thrombocytes contain transplantation antigens, as evidenced before by work of Wilson (1962). They appear, however, to be less effective than an equal number of blood cells of whole blood, presumably because of their much smaller size.

Evidence that the effect of pretreatment with donor cells can be due to enhancement, was provided by Ockner et al. (1970b), who showed that serum, produced by immunization of normal recipient rats with bone marrow, was capable of suppressing renal allograft rejection when passively transferred to the recipient. In our own 4 cases of passive transfer, at least one animal survived for a prolonged period of more than 100 days. These preliminary results indicate that enhancement may likewise be involved in the present study. This has been confirmed in more detail in the course of extended experiments in our laboratory by Marquet and coworkers (1970).

The question remains whether the principle will ever become feasible in human transplantation. To this respect a recent report of Dossetor et al. (1967) is of interest. This study indicates that previous contact of the recipient with human blood does not necessarily result in sensitization of the host to a subsequent kidney graft. In an analysis of 29 cases of cadaver kidney transplantation a significant difference was reported by these investigators, whereby those patients with a longer period of haemodialysis treatment prior to transplantation showed less evidence of rejection activity in the first three post-transplant months. However, much of the mechanism of beneficial antigen pretreatment has to be further elucidated before extrapolation of this principle to the clinic will be justified.

SUMMARY AND CONCLUSIONS

Although the rejection process which occurs after allogeneic transplantation of organs is still a largely unexplained phenomenon, an obvious improvement can be observed in the results obtained with clinical kidney transplantation. This progress is partially due to the development of more new immunosuppressive agents together with the experience thereby gained in animal experiments.

Until recently, the dog appeared to be the most suitable test animal for such studies. However, when in 1965 Fisher and Lee developed a method to transplant the rat kidney, a model was created which seemed to offer many advantages over that of the dog. Thus it became possible to carry out renal transplantations under genetically controlled conditions because of the inbred rat strains available.

The Introduction (Chapter I) comprises a discussion of the difference in behaviour between allografts of skin, kidney and other primarily vascularized organs with similar donor-host combinations. It is concluded that the kidney should be chosen as the object of studies designed to improve the results of kidney transplantation.

The purpose of the present study was to examine whether the model system of the rat can be used to obtain sufficiently large series of kidney transplantations and at the same time, to investigate its suitability for the evaluation of various immunosuppressive agents.

Chapter II is a short summary of the developments in microvascular surgery which led to the feasability of kidney transplantation in the rat; several techniques are discussed.

Chapter III contains a more detailed description of Fisher and Lee's technique, which is also used for this study. In the course of the investigation, the procedure was changed slightly (bilateral instead of unilateral nephrectomy during transplantation). For the allografts, the donor was taken from the BN strain and the recipient from the WAG strain. Both strains are inbred and display a marked histoincompatibility with respect to one another (Štark and Křen, 1969).

From the technical results (Chapter IV), it appears that the yield of successful transplantations was rather low. The peroperative mortality was 30%, while death within the first week after transplantation varied between 10 and 70%. The latter seems to depend on a number of factors. When a bilateral nephrectomy was performed during isografting among the WAG strain, the

death rate during the first week was considerably higher than when one of the kidneys was left in situ for 4 to 7 days. This difference was much smaller when the BN rat was used as the donor. Both the rat strain and the procedure used are thus influential on the incidence of early complications.

These complications were always of a technical nature and were to an important degree inherent to the urinary tract reconstruction employed for this study.

After the first week the timing of the nephrectomy no longer influenced the mortality. Death within the first week after transplantation was therefore not taken into further consideration. Most of the animals bearing isografts survived for more than 1 year.

The majority of the untreated animals bearing allografts died on day 11 and 12 after transplantation as a result of rejection of the transplant. The death pattern of this control group, however, showed a definite spread (8 to 55 days). This variation, in contrast to the more constant behaviour of similar skin transplants, is an unexpected disadvantage of the model, which however does not jeopardize its use for the evaluation of immunosuppressive agents (see fig. 14).

In Chapters V through VII, the effect of different methods used to suppress the rejection process is evaluated on the basis of the survival time of rats bearing allografts. The results obtained with non-specific immunosuppressive agents, such as Imuran, prednisolone, local irradiation of the graft and ALS, agree in general fairly closely with the data published for dog renal transplantation. For some groups, the fact that rats survived longer than dogs can partially be ascribed to a relatively lower incidence of complicating infections. The longest survival was seen following treatment with a combination of ALS and Imuran.

From the experiments where therapy was discontinued after several weeks, it appeared that in most cases this did not interfere with long term survival.

Remarkable was the favourable effect of splenectomy on the rejection process, an observation not previously described in the experimental situation.

It was shown that the accelerated rejection which normally occurs after prior sensitization of the recipient with donor skin could be prevented by treatment with antilymphocyte globulin, a finding which may have implications for the clinical applicability of ALS.

The results obtained in case of pretreatment of the recipient with donor specific cellular antigens are discussed in chapter VIII. Conditioning with donor blood as well as with a thymus cell suspension had a clear and specific immunosuppressive effect, probably due to enhancement. This beneficial effect occurred after a single intravenous inoculation of generally large quantities of donor cells, varying in number from $5–200 \times 10^8$.

The study has led to the following conclusions:

1. The model system for kidney transplantation in rats is well suited for the evaluation of various methods of immunosuppression.
2. The transplantation technique employed results in a high rate of early mortality.
3. In spite of a large histocompatibility difference between donor and host, untreated animals bearing kidney allografts display a distinct variation in survival time due to rejection.
4. Conclusions 2 and 3 necessitate relatively large series of transplantations, which, however, are easier to perform on the rat than on other currently used test animals.
5. The results with conventional immunosuppressive agents agree closely with those obtained by others with dog experiments.
6. Antilymphocyte serum appears to be able, albeit in high dosages, to suppress the "second set" rejection process of the kidney.
7. The rat model is excellently suited for the experimental study of tolerance and enhancement with organ transplantation.

SAMENVATTING EN CONCLUSIES

Hoewel de afstotingsreactie welke optreedt bij de allogene transplantatie van organen voor een belangrijk gedeelte een nog onopgehelderd fenomeen is, valt een duidelijke verbetering waar te nemen in de resultaten verkregen met klinische niertransplantaties. Deze vooruitgang is onder meer te danken aan de ontwikkeling van steeds nieuwe vormen van immunosuppressie en de daarmee opgedane ervaring in het dierexperiment.

Tot voor kort was de hond hiervoor het meest geschikte proefdier gebleken. Toen Fisher en Lee in 1965 een methode hadden ontwikkeld voor transplantatie van de rattenier, was daarmee een model gecreëerd, dat vele voordelen leek te bieden boven dat van de hond. Zo werd het mogelijk niertransplantaties te verrichten onder genetisch gestandaardiseerde omstandigheden, doordat van de rat ingeteelde stammen beschikbaar zijn.

De inleiding (Hoofdstuk I) is vooral gericht op het verschil in gedrag van allotransplantaten van huid, nier en andere primair gevascullariseerde organen bij een vergelijkbare donor-gastheer combinatie. Ter verbetering van de resultaten van niertransplantatie dient dus de nier als studie-object te worden gekozen.

Het doel van het onderhavige onderzoek was nu om na te gaan in hoeverre het modelsysteem bij de rat bruikbaar is voor het verkrijgen van voldoende grote series van niertransplantaties en tevens of het geschikt is voor de evaluatie van verschillende vormen van immunosuppressie.

In Hoofdstuk II wordt een kort overzicht gegeven van de ontwikkeling van de microvasculaire chirurgie, welke heeft geleid tot de mogelijkheid van niertransplantatie bij de rat, waarvan enkele technieken worden besproken.

In Hoofdstuk III wordt de techniek volgens Fisher and Lee, waarvan bij dit onderzoek gebruik werd gemaakt, meer uitvoerig beschreven. In de loop van het onderzoek werd deze procedure enigszins gewijzigd (dubbelzijdige i.p.v. enkelzijdige nephrectomie tijdens transplantatie). Bij allogene transplantaties was de donor afkomstig van de BN stam, de recipient van de WAG stam. Beide stammen zijn ingeteeld en vertonen t.o.v. elkaar een sterke histo-incompatibiliteit (Štark en Křen, 1969).

Uit de technische resultaten (Hoofdstuk IV) blijkt, dat de opbrengst van het aantal geslaagde transplantaties nogal gering is. De peroperatieve mortaliteit bedroeg 30%, terwijl de sterfte binnen de eerste week na transplantatie varieerde van 10 tot 70%. Deze laatstgenoemde sterfte bleek afhankelijk van een aantal factoren. Wanneer tijdens isologe transplantaties binnen de WAG

stam een bilaterale nephrectomie werd verricht, was de sterfte in de eerste week aanzienlijk groter dan wanneer een van de nieren 4 tot 7 dagen in situ werd gelaten. Dit verschil was veel kleiner in die gevallen waarbij de BN rat als donor fungeerde. Zowel de rattestam als de toegepaste procedure waren dus van invloed op het vóórkomen van vroege complicaties.

Deze complicaties bleken altijd van technische aard en waren in belangrijke mate inherent aan de urinewegreconstructie welke voor dit onderzoek werd gebruikt.

Het tijdstip van nephrectomie was niet meer van invloed op de mortaliteit na de eerste week. Sterfte binnen de eerste week na transplantatie werd daarom verder buiten beschouwing gelaten.

Het merendeel van de isoloog getransplanteerde dieren overleefde een periode van meer dan 1 jaar.

Van de onbehandelde allogeen getransplanteerde dieren stierf de meerderheid op dag 11 en 12 na transplantatie als gevolg van afstoting van het transplantaat. Het sterfte-patroon van deze controlegroep vertoonde echter een duidelijke spreiding (8 tot 55 dagen). Deze variatie, in tegenstelling tot het meer constante gedrag van overeenkomstige huidtransplantaten, is een onverwacht nadeel van het model, hoewel het laatste de evaluatie van immunosuppresiva wel toelaat (zie fig. 14).

In Hoofdstuk V tot en met VIII wordt de werkzaamheid van afzonderlijke methoden ter onderdrukking van de afstotingsreactie nagegaan aan de hand van de overlevingsduur van allogeen getransplanteerde ratten. De resultaten verkregen met non-specifieke immunosuppressiva zoals Imuran, prednisolone, locale bestraling van het transplantaat en ALS, komen over het algemeen goed overeen met literatuurgegevens betreffende hondeniertransplantatie. Bij sommige groepen kon een langere overleving dan bij de hond onder andere worden toegeschreven aan een relatief minder vóórkomen van complicerende infecties. De langste overleving werd gezien bij behandeling met een combinatie van ALS en Imuran.

In die experimenten waarbij de therapie na een aantal weken werd gestaakt, bleek dit in de meeste gevallen geen belemmering voor langdurige overleving te zijn.

Opmerkelijk was het effect van splenectomie op het afstotingsproces, een bevinding niet eerder beschreven in het dierexperiment.

De versnelde afstoting welke normaal optreedt na voorafgaande sensibilisatie van de ontvanger met donor huid, kon door behandeling met antilymphocyten globuline worden voorkomen.

In Hoofdstuk VIII worden de resultaten vermeld, welke werden verkregen door middel van voorbehandeling van de recipient met cellulair antigeen van het donor type. Conditionering met donorbloed evenals met een thymuscelsuspensie had een duidelijk en specifiek immunosuppressief effect, vermoedelijk

berustend op enhancement. Deze gunstige werking vond plaats na eenmalige intraveneuse toediening van over het algemeen grote hoeveelheden donorcellen, variërend in aantal van $5–200 \times 10^8$.

Het onderzoek heeft geleid tot de volgende conclusies:

1. Het modelsysteem van niertransplantatie bij de rat is goed bruikbaar voor de evaluatie van verschillende vormen van immunosuppressie.
2. De gebruikte transplantatietechniek brengt een hoge vroege mortaliteit met zich mee.
3. Ondanks een sterk histocompatibiliteitsverschil tussen donor en gastheer vertonen onbehandelde allogeen getransplanteerde dieren een duidelijke variatie in overleving.
4. De punten 2 en 3 noodzaken tot het verrichten van omvangrijke series van transplantaties, welke echter bij de rat gemakkelijker uitvoerbaar zijn dan bij andere gangbare proefdieren.
5. De resultaten met conventionele immunosuppressiva zijn goed vergelijkbaar met die door anderen verkregen bij de hond.
6. Antilymphocyten globuline blijkt in staat, zij het in hoge dosering, de „second set" afstotingsreactie van de nier te onderdrukken.
7. Het rattemodel is bij uitstek geschikt voor experimenteel onderzoek betreffende tolerantie en enhancement bij niertransplantatie.

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