CHEMOTHERAPY IN A TRANSPLANTABLE MYELOID LEUKÆMIA IN BROWN NORWAY RATS

The work described in this thesis has been performed at the Radiobiological Institute TNO, Rijswijk.

This thesis is available as a publication of the Radiobiological Institute of the Organization for Health Research TNO, 151 Lange Kleiweg, Rijswijk, The Netherlands.

CHEMOTHERAPY IN A TRANSPLANTABLE MYELOID LEUKÆMIA IN BROWN NORWAY RATS

Studies on BNML as a model for human Acute Myeloid Leukæmia

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE ERASMUS UNIVERSITEIT ROTTERDAM, OP GEZAG VAN DE RECTOR MAGNIFICUS, PROF.DR. J. SPERNA WEILAND EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN. DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP WOENSDAG, 9 JANUARI 1980 DES NAMIDDAGS TE 3.00 UUR PRECIES

DOOR

Louisa Petronella Colly
geboren te Apeldoorn

W.D. MEINEMA B.V. - DELFT - 1980

PROMOTOR:	PROF.DR. D.W. VAN BEKKUM
CO-REFERENTEN:	PROF.DR. J. ABELS
	PROF.DR. C. HAANEN

Al ware het dat ik profetische gaven had en alle geheimenissen en alles wat te weten is, wist, en al het geloof had, zodat ik bergen verzette, maar ik had de liefde niet, ik ware niets

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Aan mijn Vader en Moeder

CONTENTS

CHAPTER 1

INTRODUCTION

Leukaemia accounts for less than 5% of the total number of malignant diseases in the USA (McCredie et al., 1976), while about 9% of all neoplasms in the Netherlands originate in the lymphatic and blood forming organs. Because of the relatively easy accessibility of the tumour cells in both the blood stream and the bone marrow, leukaemias have been studied extensively in attempts to gain insight into the growth pattern of malignancies. The results obtained with leukaemia have contributed considerably to the management of many other malignant disorders in which the tumour cell population is less accessible.

Of the two main categories of acute leukaemia, only the treatment of acute lymphatic leukaemia (ALL) in children has greatly improved over the last decade. The survival curve for this leukaemia has reached a plateau at a level of 55% after 5 1/2 years of continuous remission (Pinkel, 1979). However, a remarkable difference is observed between the response of children and adults with the same type of leukaemia and on similar treatment regimens. The results in adults are considerably less favourable (Clarkson et al., 1975). Improvement in the results of treatment of the other main type of acute leukaemia, acute myelocytic leukaemia (AML), has been much less impressive. For this disease the prognosis in children and adults is similarly poor today. Prior to the development of chemotherapy, the disorder showed a rapid and progressive downhill course in the majority of patients. More than circa 20% of the patients died within two weeks, circa 80% did not survive for two months and less than circa 5% of them lived longer than 6 months (McCredie et al., 1976; Frei and Freireich, 1965). Following the development of effective chemotherapeutic agents, treatment results have improved. With the presently employed combination treatments, the frequency of complete remissions (defined as a state in which the leukaemic cells in the bone marrow are below 5% and the peripheral blood counts are normal; for details see Chapter 3) is about 70% in optimal clinical situations (Clarkson et al., 1975; McCredie et al., 1976; Gale et al., 1977; Cassileth et al., 1977; Preisler et al., 1977). However, the majority of remissions is short, their median duration is less than one year and fewer than 20% exceed

two years (Clarkson et al., 1975; Spiers et al., 1977a; McCredie et al., 1976; Gunz, 1977). The fact that the duration of remissions has not significantly increased over the past few years indicates that present day therapeutic approaches are inadequate.

This necessitates comparison in a clinical trial of two or more different regimens, one of which being the most effective treatment of previous trials, the other being a modification or a similar regimen with additions. In this way combination of three or four drugs in remission induction therapy has proved to be superior to single agent therapy (Carey et al., 1975). It is obvious that this approach does not provide information concerning the interaction of the individual drugs with respect to the leukaemic cells.

Another possibility for gaining more insight into human pathological conditions is to study animals with a similar disease (animal models). During the past twenty years extensive studies have been performed with the L1210 murine leukaemia. Although the knowledge yielded by this research has greatly contributed to the basic information on leukaemic cell proliferation, the relevance of this mouse model for human AML is very limited, since similarity with the most important characteristics of AML* is not evident in L1210. One difference is that the leukaemic cell in the L1210 is an undifferentiated lymphoblastic cell type. Another perhaps more important one is that the L1210 is a rapidly proliferating leukaemia, with virtually all cells in cycle (Skipper et al., 1967; Skipper and Perry, 1970; Kline et al., 1972).

Basic studies of normal haemopoiesis (Cronkite, 1968; Killmann, 1968; Mauer et al., 1964; Metcalf et al., 1971) and leukaemic proliferation (Killmann, 1963, 1968; Gavosto, 1967; Strijckmans, 1970; Ernst, 1976) have shown that human AML cannot be considered simply as a disorder characterized by rapid and uncontrolled proliferation of one myeloid cell type. On the contrary, present day opinion is that rapid proliferation is not a characteristic property of AML, but that the unrelenting accumulation of myeloid cells has to be attributed to a differentiation and maturation arrest. In fact, a large fraction of the leukaemic cell population seems to be dormant, yet these cells can be triggered into cell cycle at any time. It is obvious that such kinetic properties require a completely different approach to achieve total eradication of the leukaemic population than does a constantly proliferating cell population like the L1210.

*In this study AML always means human AML.

1.1 THE CELL CYCLE

Generally, a cell has to traverse all stages of the cell cycle before two daughter cells are produced. A schematic presentation of cell cycle phases during cell division is presented in Fig. 1.1. In haemopoietic tissue, the cells traversing the cell cycle phases are in equilibrium with a compartment of cells not involved in cell division. This population of cells has been designated as G_0 cells, resting or dormant cells. The G_0 compartment acts as a reservoir from which cells can be randomly triggered into cycle in order to supply cells for division when required.

The sequence of changes which a multiplying cell undergoes may be divided into four stages. The time interval after mitosis (M) during which the newly formed cell is not yet engaged in DNA synthesis is known as G_1. This is followed by a period of DNA synthesis, the S phase. The interval between the doubling of the nuclear material and the cell division is known as the G_2 phase. The time necessary for passing through all cell cycle phases from one mitosis to the next is referred to as the cell cycle time or generation time. In terms of their DNA content, cells in the G_0 phase cannot be distinguished from cells in G_1. Whether these cells are distinct entities is in fact controversial.

With the introduction of stathmokinetic agents (Dustin, 1943) and the autoradiographic identification of cells synthesizing DNA on the basis of the incorporation of ^{3}H-TdR into the nucleus (Taylor et al., 1957), a quantitative description of the dynamic processes involved in cell growth was made possible (see also Chapter 4).

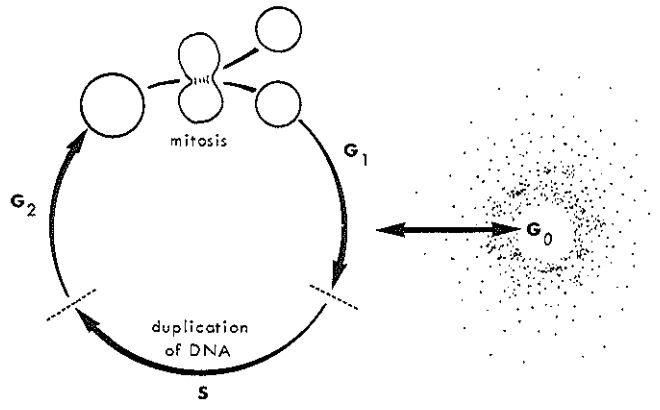

Figure 1.1

Schematic representation of the cell cycle.

1.2 HAEMOPOIETIC CELL GROWTH

Haemopoietic tissue is one of the most rapidly proliferating cell compartments in the body. The consequence is that the earliest progenitor cells represent only a small minority of the cells within the haemopoietic organs (Fig. 1.2). The most primitive cell has been designated as the pluripotent haemopoietic stem cell (HSC). By definition, this cell has the potential to give rise to all blood cells,

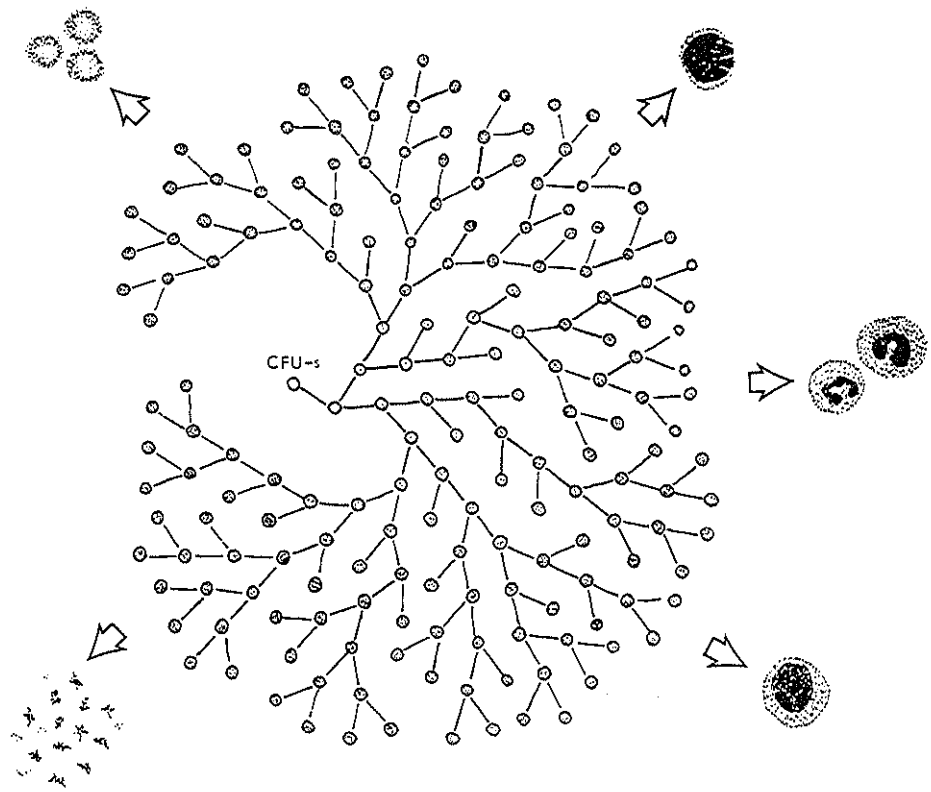

Figure 1.2

Schematic representation of stem cell differentiation.
To demonstrate the effects of exponential growth 10 cell divisions are drawn in the pathway from CFU-s to mature blood cells. In order to fit all daughter cells in a flat plane only 10% of the divisions could be allowed to continue. The figure illustrates that small changes in the proliferation/differentiation rate in an early stage can have large effects on the production of mature cells. As a consequence of exponential growth the CFU-s is a rare cell type. In reality less than 1% of the bone marrow cells is a pluripotent haemopoietic stem cell.

including the capacity to renew itself (Trentin et al., 1963; Micklem et al., 1966; Wu et al., 1968); this has been extensively reviewed by Van den Engh (1976). The technique for quantifying the number of HSC, the spleen colony assay, clearly demonstrates the pluripotential properties of the HSC; however, this assay is only available for the mouse and the rat. Several techniques are available for measuring the proportion of haemopoietic stem cells which are actively traversing the cell cycle stages, i.e., cycling cells. The fraction of cycling colony forming units spleen (CFU-s) versus CFU-s in G_0 phase for the mouse has been determined in our laboratory by the standard thymidine suicide method to be 0.2 and 0.8, respectively. It is a well known phenomenon, that under conditions such as infections and anaemia. the percentage of cells in cycle increases (Lajtha, 1975). In contrast to the HSC, the cells in the committed compartments (Fig. 1.2) are almost all in cycle. This proved to be very important in therapeutical sense. Many of the chemotherapeutic drugs used are especially active on cells in cycle; others are active in a specific phase of the cell cycle (f.e., DNA-synthesis phase). These agents have little toxicity for cells in the G_0 phase (see 1.5). When the period during which chemotherapy is given is prolonged, the fraction of HSC killed increases, at the expense of cells in the G_0 phase (Fig. 1.3). When the latter compartment is reduced to below a critical number of HSC, the repopulation of the haemopoietic system will be insufficient.

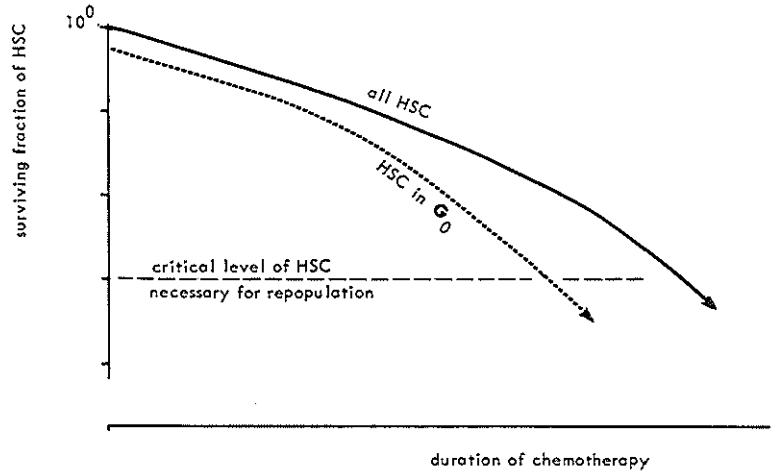

Figure 1.3

Hypothetical representation of the reduction of the number of haemopoietic stem cells (HSC) during intensive chemotherapy.

1.3 LEUKAEMIC CELL GROWTH

An overall dynamic difference between normal and leukaemic cell populations in acute leukaemia is that normal leukocytes are in steady state (cell production = cell death), whereas cell production is usually greater than cell death in untreated leukaemia. Because leukaemic cells have escaped from the orderly and well-disciplined equilibrium of cell production (including the processes of maturation, differentiation) and cell death, the tumour load may increase to 10^{12} leukaemic cells in the end phase of human acute leukaemia (Frei, 1978). The accumulation of malignant cells in bone marrow and infiltration into practically all organs are incompatible with life, because these tissues lose their normal functional properties.

Extensive studies of Gavosto et al. (1960), Clarkson et al. (1967), Killmann (1968), Cronkite (1968) and Greenberg et al. (1972) on the leukaemic cell kinetics in human acute leukaemia have shown an increased cell cycle time for the leukaemic myeloblast as compared with normal myeloblasts. However, large variations in the cell cycle times of leukaemic cells are observed (30-150 h). Apart from the lengthening of the cell cycle time, also a smaller number of leukaemic cells is in cycle as compared with normal myeloblasts. These two phenomena contribute to the fact that AML is characterized by slow proliferation of the malignant cell population. It was discussed above that the majority of the leukaemic cells are in the G_0 phase. The consequence of these kinetic properties with respect to chemotherapy is that it will be difficult to eradicate the leukaemic cell population without causing life threatening aplasia.

1.4 THE CELL CYCLE AND ITS RELATION TO DRUG ACTIVITY

Chemotherapeutic agents have been divided into three classes on the basis of the effect of these drugs on a slowly proliferating cell population with a large G_0 fraction (the HSC, quantified by means of the CFU-s assay) as compared with a rapidly proliferating population of which all cells are in cycle. Such comparative studies were performed with normal CFU-s versus a transplantable lymphoma by Bruce et al. (1966, 1968) (Fig. 1.4).

I. Nonspecific agents. There was no difference between the dose-effect curves for normal haematopoietic and lymphoma cells. Along with the exponential dose-effect curve which is linear, the therapeutic index (defined as the ratio between effect on tumour cells and normal cells)

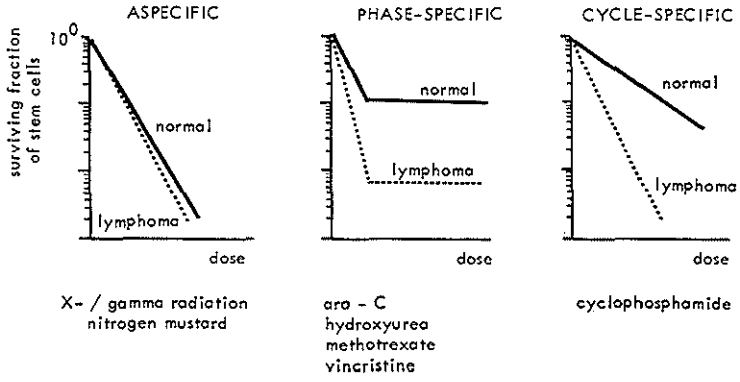

Figure 1.4

Classification of cytostatic agents based on the survival of normal haematopoietic and lymphoma colony forming cells from the marrow of mice treated with the agents during 24 h (after Bruce et al., 1966).

becomes about 1. Agents in this class are e.g. ionising radiation and the nitrogen mustard derivatives.

II. Phase specific agents. These kill proliferating cells during a specific part of the cell cycle. Resting cells do not appear to be affected by these drugs. Slowly proliferating cells are less affected because a smaller fraction of cells is in the sensitive cell cycle phase as compared to rapidly proliferating cells. In both situations the dose-effect curves reach a plateau at higher drug dosages. The therapeutic index of these types of drugs is high when the proliferative activity of the tumour exceeds that of the normal HSC. Drugs belonging to this category are ara-C, methotrexate, hydroxyurea, vincristine and bleomycin (Hill and Baserga, 1975).

III. Cycle specific agents. These drugs yield survival curves which are exponentially linear with dose. However, there is a significant difference (x6) in the slope for normal HSC and for lymphoma cells. Agents belonging to this group do not only kill cells in a specific cell cycle phase but are generally effective against proliferating tissues. The therapeutic index is high when the number of stem cells is normal and when the difference in slope between the normal and malignant populations is great. Drugs classified into this group are Cyclophosphamide, BCNU, 5-Fluorouracil and Actinomycin-D.

This classification of anticancer drugs into the three groups proposed by Bruce et al. (1966, 1968) has been confirmed and extended by Van Putten et al. (1971; 1972) using normal versus rapidly proliferating CFU-s.

In view of the large discrepancy between the kinetic behaviour of the current experimental leukaemia models, e.g. L1210 and AKR leukae-

mia in mice and given the fact that the efficacy of most chemotherapeutic agents is clearly dependent on cell kinetics, a more realistic model for AML was needed. Accordingly, attempts were made in the Radiobiological Institute to develop an animal model for human AML which is more realistic than the leukaemia models described so far. This resulted in the induction of a (pro)myelocytic leukaemia in 1971 (Van Bekkum and Hagenbeek, 1977b).

1.5 THE BROWN NORWAY MYELOCYTIC LEUKAEMIA (BNML)

The BN myelocytic leukaemia originated in the inbred Brown-Norway (BN) strain at Rijswijk (BN/Rij) in 1971 following 3 intravenous injections of 2 mg of 9,10-dimethyl-1,2-benzanthracene (DMBA) 100 days earlier. This rat leukaemia seemed to fulfill the requirements. The BNML is transplantable only in the BN strain. An inoculum of 10^7 leukaemic cells leads to the appearance of leukaemic cells in the peripheral blood at about 15 days after inoculation. Death occurs around day 25 with greatly increased spleen and liver weights and the bone marrow being completely replaced by leukaemic cells. Other important characteristics are:

- cytological and cytochemical investigations have confirmed the myelocytic nature of the malignant cell population and the leukaemic cell has been identified as a promyelocyte;
- it has a slow growth rate; about 50% of the cells are in G_0 phase at day 15 after inoculation with 10^7 leukaemic cells. This figure increases to 60% in the terminal stage of the disease (Hagenbeek, 1977b). In human AML, this fraction is slightly smaller than in the BNML;
- normal haemopoiesis in the BNML is severely suppressed during the progression of the disease; the animals die from anaemia and thrombocytopenia. This is quite similar to the situation in untreated AML (Hagenbeek, 1977a; see also Chapter 6);
- its response to chemotherapy is comparable with human AML (Chapter 3);
- the BNML cells have very slight antigenetic properties, if any;
- virological investigation did not reveal a virus as a transmissible causative agent in the BNML.

The BNML has been regularly transplanted (see Chapter 2) from 1972 to the present without a change in its characteristics.

1.6 SUBJECT OF THE PRESENT STUDY

The aim of the present study is to obtain more insight into the cell proliferation pattern of leukaemic cells and to employ this knowledge for designing chemotherapeutic schedules of maximal efficacy.

From the classification of the cytostatic drugs in general it is obvious that employing the phase specific drugs is most attractive in a situation where the number of HSC is reduced, whereas due to the linear dose-effect relationship of cycle specific drugs they are less harmful when the number of stem cells is more or less normal. However, to achieve a high therapeutic index, the effects on the leukaemic population have to be greater than the effects on normal cells. One possibility to achieve this high index is to increase the proliferation rate of the leukaemic cells without heightening the sensitivity of the normal haemopoietic cells.

This study will describe the process of stepwise reduction of the tumour load of 5×10^9 cells to a residual load of 10^3 leukaemic cells (i.e. a reduction of more than 6 decades) by means of one course of chemotherapy without unacceptable toxic side effects on normal haemopoiesis.

CHAPTER 2

ANIMALS, MATERIALS, METHODS AND EVALUATION CRITERIA

2.1 BN MYELOCYTIC LEUKAEMIA (BNML)

2.1.1 Rats

The experiments were carried out with the inbred Brown Norway (BN) rat strain maintained at the Radiobiological Institute. Generally, female rats were used at ages between 12-15 weeks and weighing 150-200 grams; one experiment was performed with male rats of 14 weeks of age and weighing 250-300 grams (see Chapter 3).

The rats were kept five per cage and were supplied with water and standard food pellets ad libitum.

2.1.2 Leukaemic cell transfer

Large frozen batches of the fifth and sixth passages of the BNML leukaemia were stored in liquid nitrogen at -196° C, using standard procedures (Schaefer et al., 1972; Hagenbeek, 1977a).

An inoculum of 10^7 leukaemic cells was used in all experiments. With this size of inoculum, it takes approximately 24 days before the rats die of leukaemia. For the purpose of standardization, the same size cell inoculum was always used. Leukaemic cells from the frozen batches were not used directly but were first injected into normal male recipients. Three weeks later, the spleens of these rats could be used for leukaemia transfer. Although both cells from the bone marrow and from the blood can be used in the later stage of the disease when these tissues contain predominantly leukaemic blasts (Hagenbeek, 1977a), the leukaemic spleen was used for leukaemia transplantation for practical reasons. In the terminal phase of the leukaemia, the spleen contains about 98% leukaemic cells. Without much effort, one spleen yields about 3×10^9 leukaemic cells. Studies of Hagenbeek (1977a) have shown that an identical growth pattern is observed when leukaemic cells from either spleen, blood or bone marrow are used for leukaemia transplantation.

To maintain a reproducible growth pattern of the leukaemia, the sequential transplantation was discontinued after two transplants and a new series was started from frozen batches.

2.2 PREPARATION OF CELL SUSPENSIONS

2.2.1 Spleen, liver and thymus

The leukaemic cells were freed from the tissue by gently cutting the organ into small pieces with scissors and pressing the fragments through multiple layers of nylon gauze. In this way, a monocellular suspension was obtained. As the suspending solution, Hanks' balanced salt solution, osmolarity 283 mOsmol (which is iso-osmotic for rat cells) was used.

2.2.2 Bone marrow

Femurs and, if necessary, tibias were excised and adherent muscles were removed. The bones were then broken in the middle and cells from each fragment were collected by repeated flushing with Hanks' solution and scraping with a bent needle placed on a syringe. The collected bone marrow cells were filtered through nylon gauze, to obtain a monocellular suspension.

The concentration of nucleated cells in suspension was determined by counting the cells in Türck's solution (0.01 % crystal violet and 1 % acetic acid in saline) in a haemocytometer.

2.2.3. Buffy coat of the blood

Blood obtained by aortic puncture (anticoagulant, 300 IU of heparin/ml blood) was centrifuged at 800 g for 10 min. The buffy coat was collected and suspended in Hanks' medium.

2.3 THE SPLEEN COLONY ASSAY FOR NORMAL HAEMOPOIETIC STEM CELLS (CFU-s)

The method of quantifying the number of pluripotent haemopoietic stem cells (HSC) was first described by Till and McCulloch (1961) in

the mouse. Bone marrow cells injected intravenously (i.v.) into lethally irradiated mice migrate to all organs; the stem cells which are trapped in the spleen give rise to colonies which can be counted macroscopically at day nine after injection. Those cells which give rise to a colony are designated as colony forming units-spleen (CFU-s). This assay has been modified for the rat by Comas and Byrd (1967). However, for the routine monitoring of rat HSC, the rat spleen colony assay is rather expensive, due to the large number of recipients required. Rauchwerger et al. (1973) succeeded in producing colonies in irradiated mice which were injected with bone marrow cells of rats, but the colony yield differed from one mouse strain to the other. An assay for quantifying the HSC of the BN rat has been standardized by Van Bekkum (1976). A linear relationship was found between the number of inoculated bone marrow cells and the number of spleen colonies in two recipient species: the BN rat itself and F1 hybrids of CBA x C57Bl mouse strains.

In this study, CFU-s were determined in suspensions of bone marrow cells, spleen, liver and in peripheral blood. The assay was initially performed in lethally irradiated F1 hybrids of CBA/ Rij x C57Bl/Rij. It was later done in the F1 hybrids of C57BL/LiRij x C3H/LwRij, because the breeding of the former mouse strain had been discontinued. The recipients received 10.25 Gy gamma total body irradiation. The colony yield was the same in the two mouse strains: 8 colonies per 10^5 rat bone marrow cells. Nine days after injection of the test suspension, colonies on the surface of the mouse spleen were counted after fixation of the spleen in Tellyesniczky's solution (ethanol 70%, formaldehyde 36%, acetic acid 100 %: 20:1:1). The absolute number of haemopoietic stem cells can be calculated by correcting the number of CFU-s for the seeding efficiency. The seeding efficiency is represented by the so-called f factor. For the BN rat, Van Bekkum (1977a) has determined that f = 0.007.

2.4 ASSAY FOR THE QUANTIFICATION OF LEUKAEMIC CLONOGENIC CELLS: LEUKAEMIC COLONY FORMING UNIT-SPLEEN (LCFU-s)

Low numbers of BN leukaemic cells (5×10^3 to 5×10^4) injected i.v. into nonirradiated recipient BN rats grow out into colonies which can be counted on the surface of the spleen at day 19 after injection. A linear relationship has been found between the number of injected cells and the number of spleen colonies (Van Bekkum, 1976). On microscopic inspection, these colonies consist of leukaemic blast cells.

The leukaemic origin of the colonies has been confirmed by injecting cells obtained from these nodules into secondary recipients.

When too many clonogenic cells are inoculated, the colonies are confluent at day 19 and cannot be counted. The same phenomenon is observed when the spleens are removed from the rats later than day 19. The f factor for BNML clonogenic leukaemic cells is 0.002 (Van Bekkum et al., 1978).

2.5 PROCEDURE FOR CONTINUOUS INTRAVENOUS INFUSION

For administering drugs by slow intravenous infusion over a certain time period, an infusion pump (B. Braun, Melsungen, W. Germany: type 871010) was used. This apparatus had been modified by the addition of a frame with twelve holes for 10 ml syringes and by elongation of the pressure plate. After the pump was gauged, it was adjusted in such a position that each syringe delivered 20 ml.24 h^{-1}. This volume represents approximately the normal daily fluid requirement of the rat. After the rat was placed in a specially designed fixation cage (Hagenbeek, 1977a), the drug solution was infused into a lateral tail vein, using a Bard I Cath infusion system (International, England) which was taped on the tail.

2.6 FLOW CYTOMETRY

2.6.1 Preparation of suspensions for flow cytometric analysis

Bone marrow, spleen or thymus cells were washed in Hanks' solution by centrifugation at 200 g and the pellet was resuspended to a final concentration of about 1×10^6 cells.ml^{-1} in a hypotonic solution containing 0.1 % sodium citrate and 5 % of the fluorescent dye propidium iodide (Calbiochem, La Jolla, Ca. 92037, USA) (Krishan, 1975). The cells disrupt in the hypotonic solution, so that a suspension of nuclei is actually stained and measured. The DNA histograms of the samples, which were stored at 4° C on crushed ice, were recorded within 48 h (Fried et al., 1976a). If the histograms could not be analysed within this period, the cells had to be fixed, because the unfixed nuclei disintegrate and this results in diminished fluorescence (Krishan, 1977). After fixation, an additional necessary step is RNAse treatment, because propidium iodide also stains double stranded RNA in the cytoplasm and this complicates pure DNA measurements (Krishan, 1975, 1978).

Therefore, it was necessary to store cells before DNA histograms were prepared, the suspension was fixed in ethanol (70%) and the fixed cells were treated with RNAse (Boehringer, Mannheim, 1 mg RNAse.ml^{-1}, pH 7.0) at 37^{o} C for 30 min. After washing in Hanks', the cells were stained with propidium iodide (0.01 mg PBS.ml^{-1}) for 20 min (Crissman et al., 1976), and then subjected to flow cytometry.

Comparison of the DNA histograms obtained from fresh cells disrupted in hypotonic medium with those of cells fixed in ethanol and treated with RNAse confirmed the conclusions of Crissman (1976) and Krishan (1977) that the two procedures resulted in similar profiles.

2.6.2 The flow cytometer

The samples were analyzed in a Bio/Physics cytofluorograph model 4802 (Ortho Instruments, Westwood, USA). In this instrument, single cells from a suspension are exposed one by one to a focused argon ion laser beam (488 nm) and the fluorescence resulting from the laser excitation of the dye-DNA complex in each cell is collected and quantified through a system of filters and photomultipliers. The resulting electrical pulses are stored in the memory unit of a pulse height analyzer and displayed as a histogram. The abcissa of the histogram is divided into 100 channels of increasing linear value from 0 to 100, while the ordinate indicates the number of cells recorded in each channel. The coefficient of variation (CV), which is a measure for the resolving power of the apparatus and the staining technique (Fried, 1976b), was rather large. The smallest CV which could be obtained with propidium iodide stained thymocytes was 5.3%.

Later a FACS II cell sorter (Becton and Dickinson Company, Mountain View, USA) became available, and was used for preparing DNA histograms. One of the advantages of this instrument is that the CV is considerably smaller (for thymocytes stained with propidium iodide, a CV of 1.4 to 1.6 % can be obtained). Samples analyzed with the Bio/Physics cytofluorograph and the FACS II cell sorter give the same fraction of cells in the various cell cycle compartments.

When used for DNA histogram analysis, the FACS II cell sorter is equipped with an argon ion laser (Spectra Physics 164-05, 1 Watt). The excitation wave length is 514.5 nm. The emission is determined by a S-20 type photomultiplier with filters: Ditric 6200 cut-on plus dichroic beam splitter (570 nm) (Melles Griot, Arnhem, The Netherlands). The fluorescence of the stained nuclei was measured in NaCl (0.9%) with a flow rate of 10^{3} cells.sec^{-1}. The data of the histograms were

stored on disks of a computer system (Data General Nova 3); the calculations of the proportion of cells distributed over the cell cycle phases were performed by means of an interactive computer program (Fried, 1976b) on an Eclipse c/330; Data General.

2.6.3 Mathematical analysis of DNA histograms

The method used for estimating the number of cells in the cell cycle phases from the DNA histograms has been described by Fried (1976b). It can be applied to data from synchronous as well as non-synchronous cell populations and is not limited to systems with very low coefficients of variation (CV) for fluorescence intensity. It is based on a mathematical model of the cell population having the following properties:

a) the population is separated into compartments, each consisting of cells with approximately the same DNA content: G_0,G_1 cells, G_2,M cells and cells in the S phase of the cell cycle. The cells in S phase are further classified into several compartments according to their position in S;

b) the intensity of fluorescence has a normal distribution in each compartment, with the mean intensity of the G_2,M compartment having a channel location twice that of G_0,G_1. The S phase compartments have means at intermediate values. The CV's are the same for all intensities. In carrying out the analysis with a computer program, the CV and the channel locations of the G_0,G_1 and G_2,M compartments are estimated from the data, and the sizes of the various compartments are computed by a least square procedure. A histogram is then constructed and compared with the experimental data points. Depending on the agreement between the two, the result is either accepted or specified parameters are altered and the program is rerun. The process is repeated until the fit of the model to the data results in a chi-square value of 20% or less.

2.7 EVALUATION OF TREATMENT RESULTS

In a study which is aimed at comparing the effect of different treatment regimens, it is essential to have sensitive evaluation criteria. A parameter which is very useful when the tumour load is low may be insufficient when the tumour load is high. For this reason, it is obligatory to have several evaluation parameters with different sensitivities (Fig. 2.1).

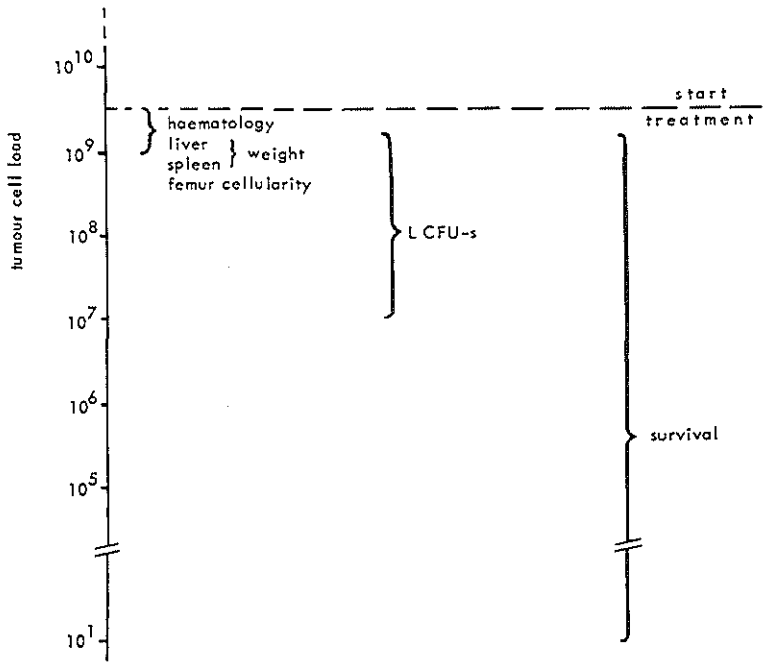

Figure 2.1

Schematic representation of the sensitivity range of the used evaluation criteria.

2.7.1 Haematological parameters

As in the routine clinical situation, the effect of therapy can be at least partly monitored in the peripheral blood. For this purpose, blood samples were obtained from the tail by cutting off its tip. The numbers of leukocytes, erythrocytes, and platelets were determined according to standard procedures. The haematocrit was determined by centrifugeing microcapillaries filled with blood for 5 min at 11,500 rpm (13.5 g). The differential counts of blood and bone marrow smears were made after staining with May-Grünwald Giemsa. Obviously, the results are of only limited value. In the phase of the leukaemia when leukaemic cells are present in the peripheral blood (i.e., the last two weeks before death), the effect of drug therapy is reflected in the decrease in the number of leukaemic cells. However, this gives no information on the tumour load in other organs.

Monitoring the haematocrit and the number of erythrocytes and thrombocytes was important for scheduling the time and extent of haematological supportive care.

2.7.2 Reduction in spleen and liver weight and total cell count per femur

During the progression of the leukaemia, the spleen, liver and bone marrow become heavily infiltrated with leukaemic cells. This results in greatly increased weights of the spleen and liver and an increase in the percentage of leukaemic blasts in the bone marrow. The latter approximates 100% in the later stages of the disease (Hagenbeek 1977a).

An estimate of the leukemic cell load in the end phase of the leukaemia in a female rat based on these three criteria is shown in Table 2.1.

By the same criteria, the minimal tumour load to be distinguished from no tumour at all is about 2-3 grams (i.e., $2\text{-}3 \times 10^9$ leukaemic cells). Thus these parameters alone only permit an evaluation of the therapeutic effect by about 1 decade of tumour cell load reduction.

2.7.3 Reduction in the number of leukaemic clonogenic cells (LCFU-s)

Determinations of the leukaemic clonogenic cells by means of the LCFU-s assay is theoretically the most attractive criterion for the evaluation of a therapeutic regimen in the BNML. The clonogenic cells are the only ones of importance owing to their capacity to resume proliferation. Experiments of van Bekkum et al. (1978) have indicated that every viable leukaemic cell of the BN leukaemia has clonogenic properties. Therefore the LCFU-s determination is not really necessary when the bone marrow is filled with leukaemic cells, but becomes very valuable for measuring the tumour load reduction with surviving fractions between 10^{-1} and 10^{-3}. However, the method has proved to be reproducible only when the number of clonogenic cells injected does not differ too much from "a standard dose" of 1×10^4 cells which results in 20 spleen colonies. These values are found in bone marrow of rats 16 days after inoculation of 10^7 leukaemic cells. The LCFU-s assay has been shown to be reproducible when the decrease in the number of LCFU-s ranges from 0.3 to 3 decades, as compared to the leukaemic controls. When the reduction is less than 0.3 decades, irreproducible results are obtained due to the large standard deviation of the assay. Unfortunately, when the reduction is more than 3 decades, the LCFU-s assay becomes unreliable, in that the values scored are too low as compared to the data extrapolated from the survival times. A

TABLE 2.1

LEUKAEMIC CELL LOAD AT VARIOUS STAGES OF THE DISEASE

Leukaemic cell load at day 15		
leukaemic liver weight (7.5 g) - normal liver weight (7 g)	= 0.5 g:	0.5×10^9 leukaemic cells
leukaemic spleen weight (1.0 g) - normal spleen weight (0.5 g)	= 0.5 g:	0.5×10^9 leukaemic cells
1 femur: 9.0×10^7 leukaemic cells (90 %); total bone marrow:		4.0×10^9 leukaemic cells
Total:		5.0×10^9 leukaemic cells
Leukaemic cell load in the end phase of the disease		
leukaemic liver weight (15 g) - normal liver weight (7 g)	= 8 g:	8.0×10^9 leukaemic cells
leukaemic spleen weight (2.5 g) - normal spleen weight (0.5 g)	= 2 g:	2.0×10^9 leukaemic cells
1 femur: $4,5 \times 10^7$ leukaemic cells (100%); total bone marrow:		2.0×10^9 leukaemic cells
Total:		12.0×10^9 leukaemic cells

Tumour load in liver, spleen and bone marrow on day 15 and in the end phase of the disease; 1 gram of leukaemic tissue represents 10^9 cells. The total bone marrow cellularity was calculated by multiplying the total number of cells per femur with 100/2.3 because 1 femur represents 2.3% of the total amount of bone marrow (P. Sonneveld, 1980). The leukaemic cell load in the blood ($60.0 \times 10^6 \times 6$ ml) and other infiltrated leukaemic organs have been neglected.

possible explanation for this discrepancy is that a larger total cell inoculum is needed when the proportion of leukaemic cells is less than 0.1%.

2.7.4 Survival

In experimental chemotherapy studies in animals, survival is always an important evaluation criterion. In the BNML, there is a linear relationship between the number of leukaemic cells injected and the survival time in the range of between 10^1 cells and 10^7 cells.

Fig. 2.2 shows this linear relationship for BN female rats. When treatment of a leukaemic animal is started at day 15 and this therapy results in a survival time of 55 days, it can be extrapolated that the increase in survival time by 40 days corresponds with a residual tumour load of 5000 leukaemic cells.

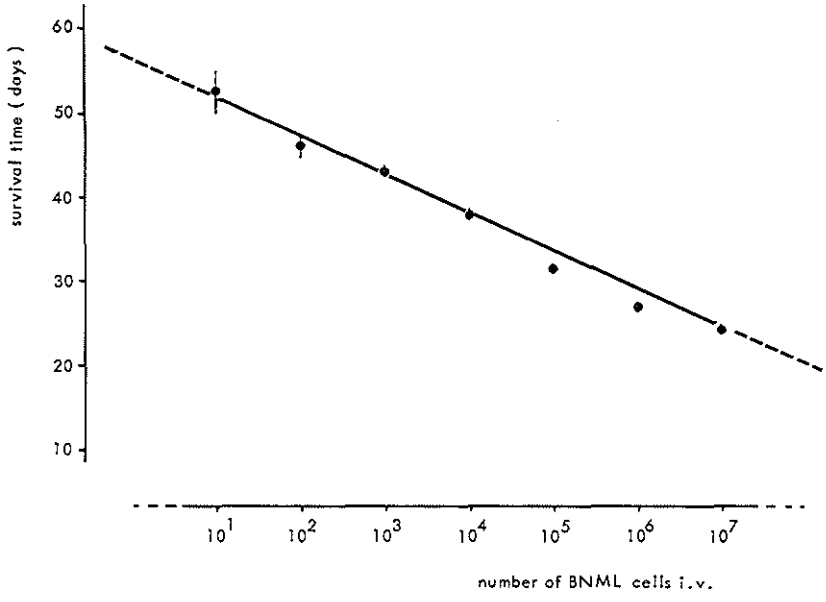

Figure 2.2

Dose-survival time curve for BN females. Each point represents means (± SE) of 10-30 rats.

2.7.5 Histology

Tissues were histologically examined for either one of two reasons: to search for leukaemic cell infiltration in various organs or to investigate the cause of death in animals succumbing during or after chemotherapy. Histological sections were prepared according to standard procedures in the Dept. of Pathology of the Institute of Experimental Gerontology TNO.

2.8 HAEMATOLOGICAL SUPPORTIVE CARE

Haematological supportive care has been of the greatest importance in our experiments, just as in human AML, for achieving long time survival after chemotherapy. Anaemia and thrombocytopenia occur in aggressively treated animals and as the disease progresses due to leukaemic cell infiltration in the bone marrow. Previous studies have demonstrated that the pluripotent stem cells (CFU-s) decrease to very low values in a relatively early stage of the disease (Colly et al., 1977). At day 15, the time that therapy is usually started, haemopoiesis is already insufficient and its further reduction becomes life threatening. For this reason, transfusions of 1.5 - 2 ml blood were given when the haematocrit was below 20 per cent and/or the thrombocytes had decreased to below $40 \times 10^{9} . L^{-1}$. The donor blood was obtained from retired BN female and male breeders which were exsanguinated by means of aortic puncture. To prevent coagulation of the blood, 300 IU of heparin per ml were mixed with the blood in the syringe. Because all animals belonged to the same inbred rat strain, transfusions of whole blood could be given without the problem of inducing antibodies to the transfused antigens.

2.9 CHEMOTHERAPEUTIC AGENTS

Instead of studying the whole arsenal of cytostatic drugs in the BNML, this study was focused on two agents which have proved to be the most effective drugs in AML: ara-C and adriamycin. The literature relating to these agents will be briefly surveyed in this section to provide a background for the experimental parts of this study.

2.9.1 Ara-C

A. Introduction

Cytosine arabinoside (ara-C, 1-β-D arabinofuranosyl-cytosine) is a synthetic nucleoside (pentose + pyrimidine base) in which the pentose is an arabinose with a furane structure and the pyrimidine base is cytosine. It was synthesized by Walwick, Roberts and Dekker in 1959 (Walwick, 1959). The antitumour activity against animal neoplasms was first reported by Evans et al. (1961) and later by Wodinsky et al. (1965), Kline et al. (1966) and Skipper et al. (1967). It has proved to be very effective in AML (Henderson et al., 1965; Howard et al., 1966; Frei, 1969; Bodey, 1969).

B. Cytotoxic activity

Before ara-C can exert its cytotoxic effect, it has to be transported across the cell membrane (Kessel et al., 1968) and to be activated to the triphosphate compound, ara-CTP (Chou et al., 1975; Kessel et al., 1967; Skipper et al., 1970; Graham, 1970a; Momparler et al., 1971). This metabolite exerts its cytotoxic effect through inhibition of DNA polymerase by competition with its natural substrate deoxycytosine triphosphate (Furth et al., 1968; Inagaki et al., 1969; Graham et al., 1970a; Matsukage et al., 1978). The consequence of the inhibition of DNA polymerase is that the cell can no longer duplicate its DNA content, which is essential for mitosis (Fig. 2.3).

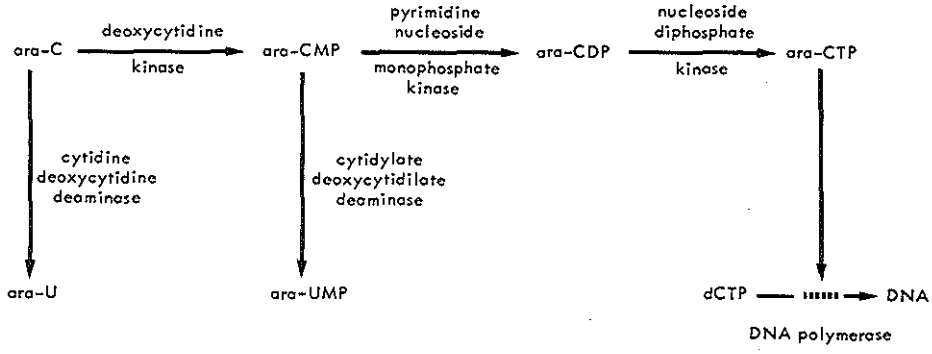

Figure 2.3

Metabolic pathway of ara-C.

C. Cell kinetics

Since ara-C is known to inhibit DNA synthesis, the action of the drug is most likely confined to the S phase of the cell cycle. Karon et al. (1969) indeed demonstrated in asynchronous Don-C cells *in vitro* that ara-C is active on S phase cells; however, an effect on late G_1 cells could not be ruled out. Bhuyan et al. (1977) showed that, in exponential growing as well as in stationary growing cultured L1210 cells, the percentage kill by ara-C corresponded with the percentage of cells in S as determined with ^{3}H-TdR.

Graham et al. (1970b) found a rapid kill of cells in S phase and a temporary blocking effect on cycle progression of cells from G_1 to S. The blocking effect on the G_1/S boundary has also been described by Yataganas et al. (1974); however, it is ignored by Karon et al.(1970).

D. Pharmacokinetics

The acute LD_{50} of ara-C for rodents, dogs and monkeys is about 1000 to 4000 $mg.kg^{-1}$ (Livingston et al., 1968). This suggests that the use of high doses of this antimetabolite is safe and, as will be shown later, this is of chemotherapeutic advantage. For eradication of all of the leukaemic clonogenic cells, a cytotoxic concentration of ara-C must be achieved in all anatomical compartments (Dedrick et al., 1972). In certain compartments which have a low rate of blood flow, the concentration of ara-C may be below the cytotoxic level even though adequate cytotoxic concentrations may be present in most of the other anatomical sites. For this reason, high doses of ara-C should be used, so that its levels will be above the minimal cytotoxic concentration in all anatomical compartments (Momparler, 1974).

The plasma half-life of ara-C in patients after i.v. injection is about 10 to 20 min (Baguley et al., 1971; Momparler et al., 1972).

The uptake and *in vivo* phosphorylation of the drug have been studied by Chou et al. (1975) in the L1210 leukaemia. They found that the ara-CTP concentration in leukaemic cells was more than 700 times that in the blood plasma, while the ara-CTP ratio of the small intestine cells to blood plasma was 24-fold, both at one hour after intraperitoneal injection of labelled ara-C.

In man as well as in animals, ara-C is rapidly deaminated to the inactive metabolite arabinoside-uracil (ara-U) (Fig. 2.3). High concentrations of the cytidine deaminase enzymes are found in the liver (Camiener et al., 1967; Creasey et al., 1966). Using labelled ara-C, 86%-96% of the radioactivity recovered in the urine was present in the form of ara-U (Papac et al., 1965; Creasey et al., 1966; El Dareer et al., 1977a).

Ara-C passes the blood-brain barrier easily, especially when it is given as a continuous i.v. infusion over two hours. The ratio of ara-C to ara-U in the spinal fluid is much higher than in the plasma, probably because of very low ara-C deaminase levels in the cerebrospinal fluid and the brain (Ho and Freireich, 1975).

Ara-C has immunosuppressive properties (Fischer et al., 1966; Gray et al., 1968a; 1968b; Mitchell et al., 1969); it abolishes the secondary immune response to antigen injection, but does not decrease preexisting antibody titers (Gray et al., 1968a).

E. Toxicity

Extensive toxicological studies in animals (Livingston et al., 1968) have indicated that exposure time and drug concentration are two important parameters with respect to the cytotoxicity of ara-C. The major harmful side effect of ara-C is on proliferating haemopoietic cells, resulting in thrombocytopenia, leukopenia and anaemia (Bodey et al., 1969; Burke et al., 1968; Carey et al., 1965; Henderson et al., 1965; Howard et al., 1966; Livingston et al., 1968; Loo et al., 1965).

Other toxic effects such as nausea and vomiting, mild hepatic and gastrointestinal toxicity are described, but these symptoms seemed not to be dose-limiting factors (Bodey et al., 1969; Carter et al., 1969). The mild hepatic toxicity was manifested by impairment in liver function (Livingston et al., 1968). Gastrointestinal lesions have been described by Slavin et al. (1978) after a three day dose schedule of ara-C infusions; the severity of the lesions is markedly increased by prior treatment with cyclophosphamide.

F. Resistance

Clinical nonresponsiveness to antineoplastic chemotherapy is one of the major problems in cancer treatment. The development of resistance in a sensitive human cell population may be natural or acquired (Bender et al., 1975).

Several mechanisms have been postulated to explain the acquired nonresponsiveness of tumour cells to ara-C. These include preventing the drugs from entering the cells, e.g., through interfering with the transport of the drugs to certain sites (Kessel et al., 1967). Secondly, factors suggesting acquired resistance are discriminated; e.g.:

a. altered intracellular phosphorylation (Kessel et al., 1967; Chu et al., 1962);
b. rapid deamination of ara-C (Steuart and Burke, 1971);
c. altered form of the target enzyme DNA polymerase (Reddy et al., 1971).

The sensitivity of tumour cells to this drug may therefore depend on the balance among a number of critically related parameters. Drug resistance studies on ara-C in the L1210 leukaemia indicate that the resistance to ara-C develops more slowly with high dose rather than with low dose therapy (Momparler, 1974; Schmid et al., 1972).

G. Ara-C (Cytosar®)

Most of the ara-C used was a generous gift of the Upjohn Company (Ede, The Netherlands).

It was dissolved in 0.9 % NaCl and adjusted to pH 5.4 with HCl.

2.9.2 Adriamycin

A. Introduction

Substances originally isolated as antibiotics have been found to exert antineoplastic activity in experimental tumour systems. One example is daunomycin which was isolated from the soil fungi Streptomyces coeraleorubidus and Streptomyces peucetius in 1962 and which has now been classified as a potent antitumour agent. In the course of a search for analogs, Arcamone of the Farmitalia Laboratories isolated from the Streptomyces peucetius variant caecius a new daunomycin-related antibiotic adriamycin (Arcamone et al., 1969; DiMarco et al., 1969). The minor difference in the molecular structure (adriamycin has an OH group where daunomycin has an H on C14) changes its antineoplastic profile. Although daunomycin is superior to adriamycin with regard to cellular drug uptake in vitro and inhibition of DNA synthesis in vitro, it has been demonstrated in both animal experiments and clinical trials that the antineoplastic effect of adriamycin is more pronounced in vivo (Skovsgaard et al., 1975; Carter, 1975).

B. Cytotoxic activity

Adriamycin is rapidly taken up into isolated cells in vitro (Silvestrini et al., 1970) and can be demonstrated in the perinuclear chromatin (Negishi et al., 1973). However, the most important effect is attributable to the inhibitory action on both DNA and nuclear RNA replication (Wang et al., 1972; Momparler et al., 1976).

The binding to DNA is postulated to inhibit the template function, either by interfering with the formation of the polymerase complex or by hindering the separation of the two polynucleotide strands in the double helix (DiMarco, 1967).

The available data on the inhibition of DNA and RNA synthesis resulting from the experiments with adriamycin differ depending on the system studied (Skovsgaard et al., 1975).

C. Cell kinetics

Studies in Chinese hamster ovary (CHO) cells by Barranco (1975) demonstrated that adriamycin cannot be considered as a phase specific drug. It kills in all phases of the cell cycle, including the nondividing state, although proliferating cells are more sensitive than resting cells in the CHO system.

Concerning the site of action of adriamycin (inhibition of DNA polymerase and intercalating with the DNA as discussed above), it is not surprising that cells in S phase of the cell cycle are the most sensitive to the cytotoxic action of the drug (Clarkson et al., 1977; Kim et al., 1972; Krishan et al., 1976). There is some controversy in the literature concerning the existence of a progression blockade in the cell cycle. Barranco (1975) found a progression delay in all phases of the cell cycle except mitosis. Clarkson et al. (1977) described a dose-dependent progression delay of CHO cells treated in mid-S phase; the same phenomenon was reported by Kimler et al. (1978), whereas Barlogie et al. (1976a) described a cell cycle progression delay in the G_1, S and G_2 phases, depending on concentration and exposure time. These cell kinetic studies were performed in a human lymphoma cell line by means of pulse cytophotometry. The observed irreversibility of the G_2 block was attributed by the latter authors to an accumulation of dead cells in G_2. These *in vitro* studies support the hypothesis that adriamycin is not strictly a cell cycle phase specific drug.

D. Pharmacokinetics

Following i.v. injection of a single dose of adriamycin into mice, rats, rabbits and humans, a rapid fall in the plasma concentration is noted. In humans, the half-life of the rapidly declining part of the biphasic curve (α-phase) is 1.5-2 hours (Benjamin et al., 1973). The disappearence curve shows a triphasic pattern (DiFronzo et al., 1971; 1973; Yesair et al., 1972; Kimura et al., 1972; Rosso et al., 1972, 1973; Benjamin et al., 1973). The initial and rapid plasma clearance corresponds with a marked accumulation in almost all tissues. Throughout 24 hours after injection, the concentration is particularly high in the liver, kidney, spleen, lungs and bone marrow; it is somewhat lower in the gastrointestinal tract, the heart, lymph nodes and thymus and very low in skeletal muscles, skin and testicles (DiFronzo et al.,

1971; Arena et al., 1971; Kimura et al., 1972; Rosso et al., 1973; Wilkinson et al., 1974). The concentration in the brain has been found not to be measurable (Rosso et al., 1973) or very low (DiFronzo et al., 1971; Arena et al., 1971; Wilkinson et al., 1974; Benjamin et al., 1974), indicating that the capacity of adriamycin to pass the blood-brain barrier is very limited.

Adriamycin is metabolized primarily in the liver to adriamycinol and several aglycone derivatives (Loveless et al., 1978); approximately half of the drug is excreted intact in the bile, with an additional 30% excreted as conjugates (Benjamin et al., 1973; Bachur et al., 1973). Patients with hepatic dysfunction have shown prolonged plasma levels of adriamycin and metabolites, which resulted in increased toxicity (Benjamin et al., 1974).

E. Toxicity

The toxic effects of adriamycin, which are generally dose-related and reversible, include myelosuppression in almost all patients, nausea and/or vomiting in 20-55 % and alopecia in almost 100 %. In contrast to these reversible effects, cardiac toxicity is a unique harmful effect of this drug and this is a severe problem in long term administration. This toxicity may lead to transient electrocardiogram (ECG) abnormalities, definitive cardiomyopathy or both. The overall incidence of congestive heart failure is 1%; however, this is deceptive, since the toxicity is related to the total dose administered. If the total dose is kept below 450 mg per m^2 in children and 550 mg per m^2 in adults, cardiomyopathy is hardly ever observed, but the frequency of this disorder is markedly increased at total doses above 550 mg per m^2 (Carter, 1975).

Cardiomyopathy is also observed in animals after adriamycin administration. It has been shown that the congestive heart disease in mice (DiMarco, 1969), rats (Lenaz et al., 1976), rabbits (Young et al., 1974), dogs (Arena et al., 1971) and rhesus monkeys (Herman et al., 1971; Sonneveld, 1978a) occurs after different total doses, indicating a species specific sensitivity. In the BN rat, congestive heart disease is observed after a total dose of 12.5 $mg.kg^{-1}$ (Sonneveld, 1978b).

The usual route of administration of adriamycin is i.v. and extravasation causes severe local irreversible damage to underlying structures. The drug has been found to exert considerable immunosuppressive activity (Isetta et al., 1971; Stacher et al., 1974).

F. Resistance

In the L1210, cross-resistance between adriamycin and daunomycin has been described by Wang et al. (1972) and Hoshino et al. (1972). In studies performed in an Ehrlich ascites tumor model in mice developed by Dano (1972) in which resistance to adriamycin and daunomycin was shown, the adriamycin resistant tumour showed cross-resistance to daunomycin and the daunomycin resistant subline to adriamycin. Cross resistance to vincristine was also observed in the adriamycin resistant group, while no change in the sensivity of the resistant tumour to methotrexate was found. The adriamycin resistant tumour proved to be somewhat more sensitive to ara-C than the original tumour; the same results were found for BCNU.

G. Adriamycin (Adriablastine®)

About half of the adriamycin used was a generous gift from Farmitalia, Milano, Italy. The drug was dissolved in 0.9 % physiological saline. It was prepared less than 14 hours before injection and stored at 4°C.

CHAPTER 3

THE EFFECTS OF CLINICALLY APPLIED AML AND ALL SCHEDULES ON THE BNML

3.1 INTRODUCTION

The life span of patients suffering from acute myelocytic leukaemia has been significantly altered by the introduction of chemotherapy. Prior to the development of effective therapy, the majority of patients showed a rapid and progressive downhill course. More than 20 % of them died within 2 weeks and 80 % within 2 months; less than 5 % of the patients lived longer than 6 months (McCredie et al., 1976; Frei and Freireich, 1965). The tumour load of the majority of patients with AML at the time of diagnosis is approximately 1×10^{12} leukaemic cells, representing a tumour mass of about 1 kg (Frei, 1972). The recent approach is that, as soon as the diagnosis has been established, remission induction therapy, which consists of a 5-7 day course of intensive clinical chemotherapy, is started. In most centers, this schedule is repeated at two to three week intervals (Clarkson et al., 1975; McCredie et al., 1976; Vogler et al., 1978; Cassileth et al., 1977; Peterson et al., 1977; Spiers et al., 1977a; Gale et al., 1977). The combinations of chemotherapeutic agents employed are capable of reducing the leukaemic cell population by approximately 1 log/course (McCredie et al., 1976). To achieve so-called complete remission (CR), the tumour mass needs to be reduced by 3 logs to 1×10^{9} cells ($\pm$ 1 gram). This can be expected after three successfully applied remission induction courses. CR is considered as a state in which the patient's health and performance status as well as the peripheral blood cell counts are normal and during which the proportion of recognizable leukaemic cells in the bone marrow remains at less than 5%. If chemotherapy is discontinued at this point, the disease rapidly reappears (Fig. 3.1).

With the combination schedules currently applied, the frequency of CR after chemotherapy is about 70 % when optimal supportive care is available (Preisler et al., 1977; Cassileth et al., 1977; McCredie et al., 1976; Gale et al., 1977). However, the majority of remissions are short; their median duration is less than one year and maximally 20%

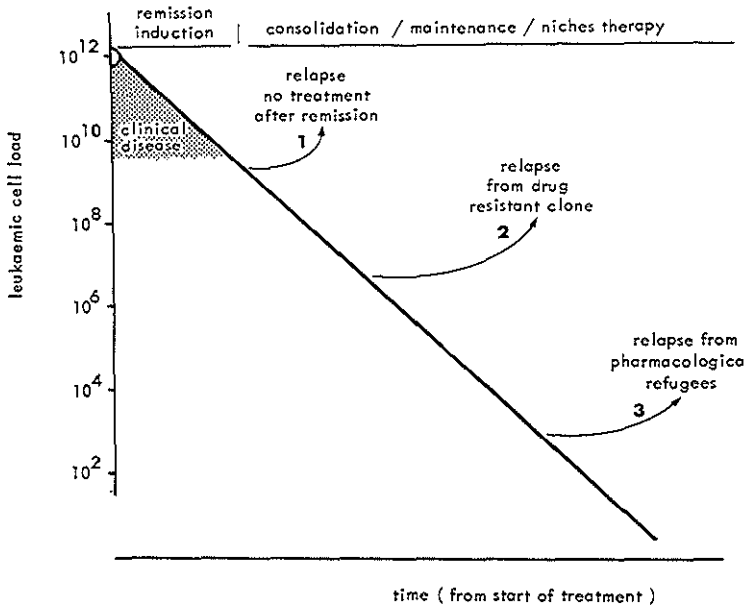

Figure 3.1

Schematic representation of the various categories of treatment of human acute leukaemia in relation to the leukaemic cell load, modified to Frei et al. (1978) (see text).

exceed two years (Clarkson et al., 1975; Spiers et al., 1977a; McCredie et al., 1976; Gunz, 1977). The fact that the duration of remissions has not significantly increased over the past years illustrates the inadequacy of present day therapeutic approaches.

In most clinics, the remission induction therapy applied in AML is followed by two additional similar courses termed consolidation therapy (Bodey et al., 1975; Spiers et al., 1977b; McCredie et al., 1976; Vogler et al., 1978; Harris et al., 1978). After this, remission maintenance therapy consisting mostly of the same combination of drugs as before but at prolonged intervals is administered (McCredie et al., 1976; Spiers et al., 1977b; Gale et al., 1977). The aim of maintenance therapy is destruction of those leukaemic cells not killed by previous treatment because of e.g., their localization in areas of the body where the concentration of the drugs was not sufficient. Such surviving cells have been termed pharmacological "refugees" (Momparler, 1974) and their locations as pharmacological sanctuaries (Frei et al., 1978). Alternatively, cells may survive (and later resume proliferation) because of resistance to the drugs applied.

As shown in Fig. 3.1, relapse of the leukaemia can originate from three sources.

1) When remission induction therapy is not followed by consolidation and maintenance therapy, it can be predicted that the remaining tumour will regrow rapidly. Complete remission is a clinical operational term and somewhat misleading, because the reality is that a considerable tumour load might still be present.
2) Remission maintenance therapy aims at eradication of the remaining 10^9-10^7 tumour cells. Since the naturally occurring frequency of resistant leukaemic cells is thought to be 1 in 10^6, a significant fraction of resistant cells is present before the start of the treatment (Fischer, 1972). A relapse may originate from a resistant clone, at least when treatment of the sensitive leukaemic cells was sufficient. For example, with respect to ara-C, the resistance is dose dependent and develops more slowly with high dose rather than low dose therapy (Momparler, 1974).
3) Leukaemia relapse may originate from places which contain pharmacological refugees. These cells are not killed by the systemic treatment and a relapse from these sites must be expected.

To obtain long lasting remissions, the treatment schedule should be focused on these possibilities. With remission induction plus consolidation therapy the tumour load should be reduced as far as possible, i.e., to the level of maximum tolerance of normal cells. After a certain period of maintenance therapy, a not previously applied potentially active drug should be added to the treatment schedule to avoid relapse from resistant clones.

Whether pharmacological refugees are a common cause of relapse in AML is not known, because most relapses originate from the bone marrow. In childhood ALL, these sanctuaries are the Central Nervous System (CNS) and the testes. If the biology of AML is comparable to that of ALL, the number of relapses originating from these sites in AML should increase when the duration of remissions is prolonged. Armitage et al. (1978) have argued against CNS prophylaxis in human AML, because CNS involvement is less frequent in the latter disease than in ALL. This could be related to the different drugs selected for induction or is due to the shorter survival time after establishing diagnosis. Ara-C is commonly employed in AML and this drug passes the blood brain barrier relatively easily, so that a sufficiently high level of ara-C is obtained in the spinal fluid to prevent a relapse from the CNS. It is known from animal studies that vincristine, the first remission-induction drug for clinical ALL, reaches only very low levels in the brain (El Dareer et al., 1977b; Castle et al., 1976). In patients no information is available on this point.

The result of activating the body's defence against its own tumour cells with nonspecific (BCG) and/or specific (allogeneic irradiated AML cells) immune stimuli have so far been disappointing with respect to remission duration (Mathé et al., 1975; Powles, 1973; 1974; 1976; Gutterman, 1974; Freeman, 1973). Somewhat longer survival times after relapse are described in patients receiving immunotherapy (Powles et al., 1977).

It is clear that an improvement of current AML treatment is badly needed. One approach which will always be followed is the investigation of new chemotherapeutic agents in AML patients. Another approach is to further study the mode of action of those drug combinations which show some efficacy at present, in the hope that more effective schedules can be obtained. A realistic animal model might be useful for this purpose. Although the BNML has shown many similarities with human AML, especially with regard to its growth characteristics (Hagenbeek, 1977a), its sensitivity to chemotherapeutic agents had not been determined.

In the study to be presented, it is investigated whether the response of the BNML to different drugs is similar to the response seen in human AML or to that in human ALL (Colly et al., 1977b).

3.2 THERAPEUTIC RESPONSE TO CHEMOTHERAPY PROTOCOLS USED IN CLINICAL AML AND ALL

A highly effective chemotherapy schedule for remission induction in clinical AML, the Ad-OAP regimen (McCredie, 1976; Fig. 3.2), was compared with an effective remission induction regimen for human ALL consisting of vincristine and prednisone (Simone, 1974; Berry, 1975; Willemze, 1975; Van der Does-van den Berg, 1976). Both schedules were adapted to the rat leukaemia. Drug dosages were calculated from the human dose by the method of Freireich (1966), which is essentially a correction for metabolism. Its principle is that the rate of metabolism in mammals increases with the ratio body surface/body weight. For chemotherapeutic agents which are completely metabolized in the body, this ratio will be a good approach, assuming that enzyme activity increases at the same ratio. However, when a drug is metabolized to a limited extent and the clearance rate is low, the thus calculated dose will be overestimated as the size of the animal becomes smaller. The calculated doses for the rat are given in Fig. 3.2.

The evaluation criteria for comparing the effects of the AML and ALL schedules were: changes in the peripheral blood counts, weights of liver and spleen determined at autopsy and survival time.

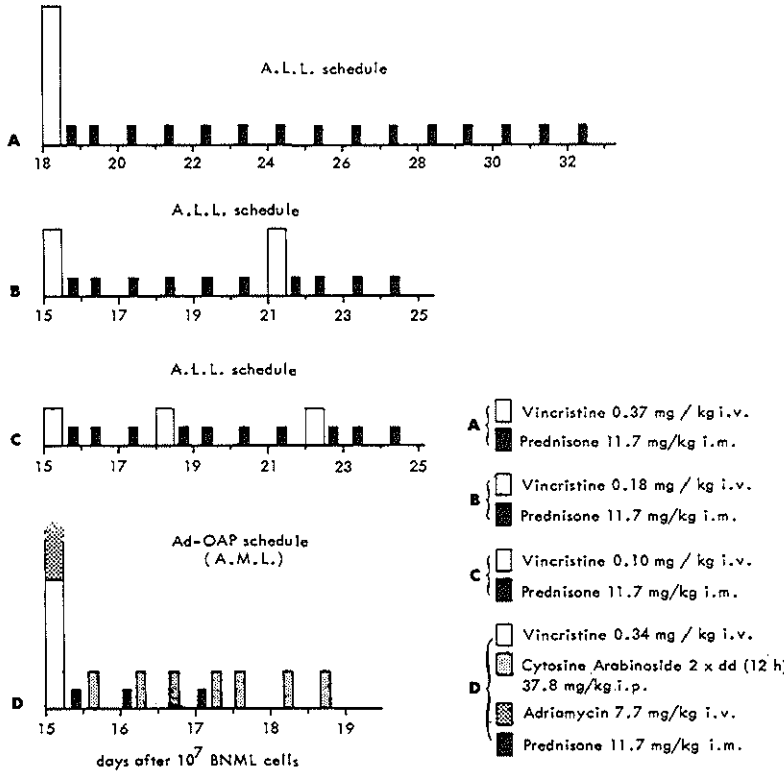

Figure 3.2

Chemotherapy regimens applied in the BNML for comparing the effect of an AML schedule (Ad-OAP) with the effect of ALL schedules consisting of vincristine and prednisone.

3.3 RESULTS

After the Ad-OAP schedule (Fig. 3.2D) was started on day 15 after inoculation with 10^7 leukaemic cells, peripheral blood was taken from the tip of the tail for routine haematologic examination. Fig. 3.3 shows the effect of this therapy on the number of peripheral white blood cells. Shortly after the start of the treatment, a rapid decrease to normal values is observed. However, the white blood cells increase about 7 days after termination of therapy and the animals die soon thereafter in relapse. The efficacy of the Ad-OAP regimen is demonstrated by the greatly reduced spleen and liver weights found at autopsy (Fig. 3.4). The survival curve (Fig. 3.5) showed that 3 out of 7 rats died during therapy, indicating that the schedule was rather toxic; the 4 remaining rats survived the leukaemic controls by about 5 days.

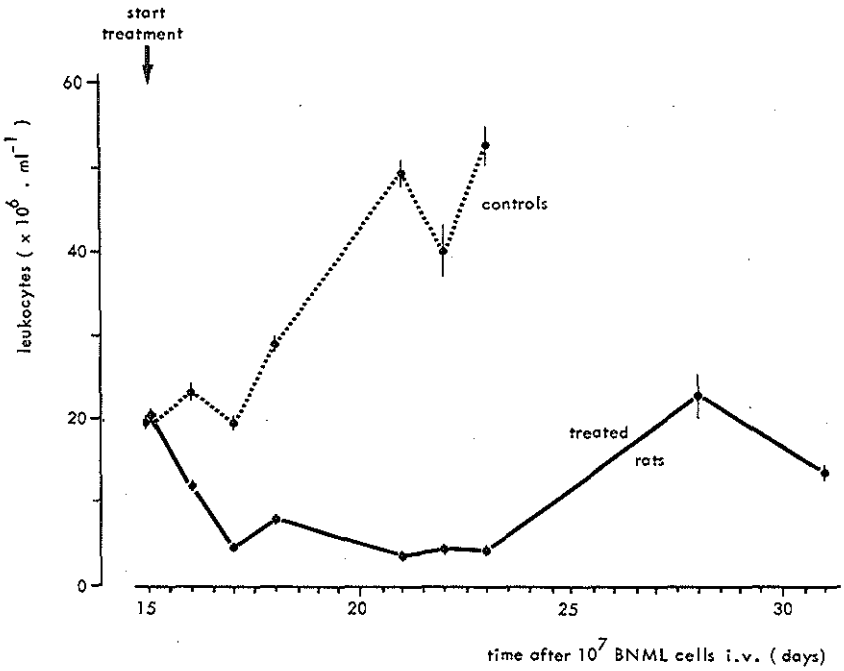

Figure 3.3

Changes in the peripheral white blood cell counts during and after the Ad-OAP schedule (mean ± SE). Treated group: n = 7; untreated leukaemic controls: n = 9.

In a pilot experiment with the ALL schedule (Fig. 3.2A), the calculated vincristine dose equivalent to the human dose of 2 mg/m^2 (0.37 mg.kg^{-1}) for the rat (Freireich et al., 1966), proved to be very toxic: 4 out of 7 rats died within 24 h after starting therapy. However, the remaining 3 animals did not survive longer than the leukaemic controls. This schedule was not toxic for normal rats; 10 female rats received one i.v. injection of 0.37 mg.kg^{-1} of vincristine and 11.7 mg.kg^{-1} of prednisone (i.m.) daily for 10 days. Only a slight decrease in the number of peripheral white blood cells was observed, but no deaths occurred.

To evaluate the effect of vincristine and prednisone on the leukaemic cell load, the vincristine dose was halved and given on days 15 and 21 after leukaemia inoculation (Fig. 3.2B). The effect on the white blood cells is shown in Fig. 3.6. Very little depression of cell counts was observed during the first few days. An increase occurred just before the second vincristine injection. Although the peripheral white blood cell counts did not increase as rapidly as in nontreated

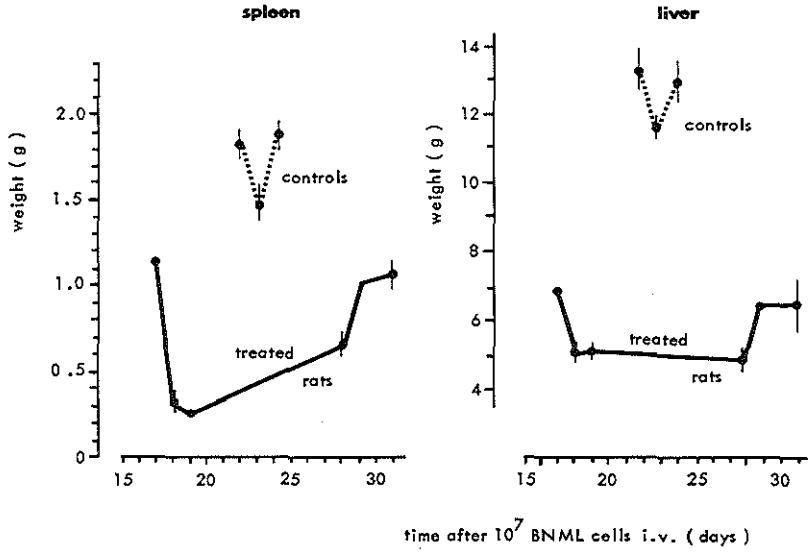

Figure 3.4

Changes in spleen and liver weight after the Ad-OAP regimen. The fresh weights were determined after spontaneous death. Each point represents either one rat or means (± SE) of 2-4 rats.

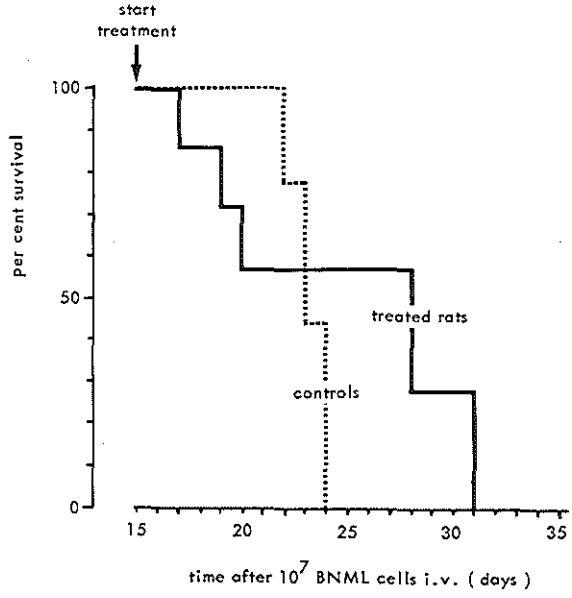

Figure 3.5
Survival curve after treatment with the Ad-OAP regimen.

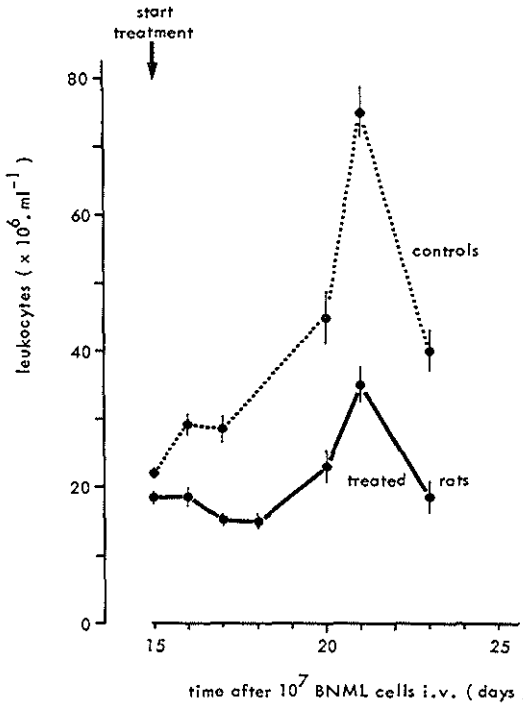

Figure 3.6

Changes in the peripheral white blood cell counts during treatment with vincristine and prednisone; schedule B (Fig. 3.2) (means ± SE). Treated rats: n = 7; untreated leukaemic controls: n = 6.

leukaemic controls, this treatment clearly had less effect than the Ad-OAP treatment previously described. Fig. 3.7 shows the weights of the spleens and the livers at the time of death. A small reduction in the spleen and liver weights of the treated groups as compared to the untreated group was observed. In both groups, these organ weight reductions occurred in the terminal phase of the disease. The survival curve after this regimen (Fig. 3.8) showed that all animals survived the first vincristine injection; however, after the second administration (day 21) about 50 % died. The overall effect was that no animals of the treated group survived longer than the nontreated leukaemic control group.

No firm conclusions could be drawn concerning evaluation of the effect of the schedule vincristine/prednisone on the tumour load, because the combination of these drugs was very toxic at the doses applied. For this reason, in a subsequent study, the vincristine dose was reduced again (Fig. 3.2C) to 0.1 $mg.kg^{-1}$ given on days 15, 18 and

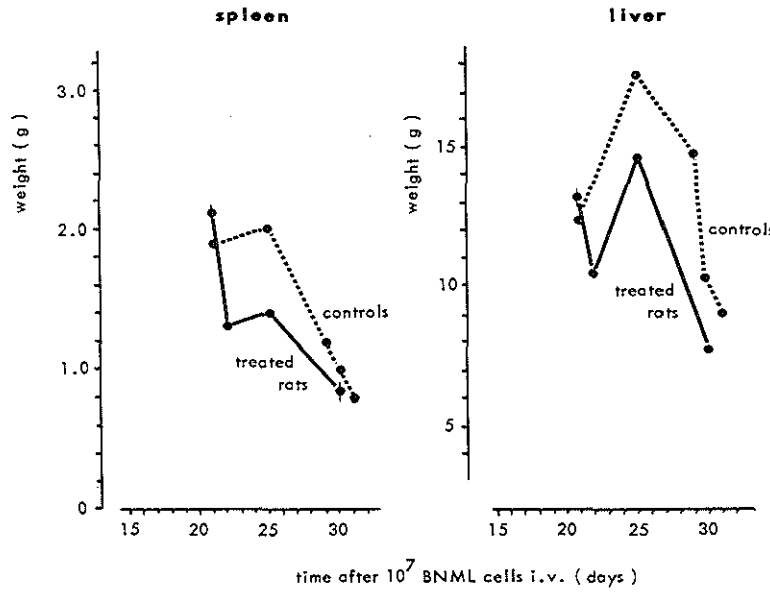

Figure 3.7

Spleen and liver weights after treatment with vincristine and prednisone; schedule B (Fig. 3.2). Fresh weights were determined after spontaneous death. Each point represents either one rat or means ± SE of 2-3 rats.

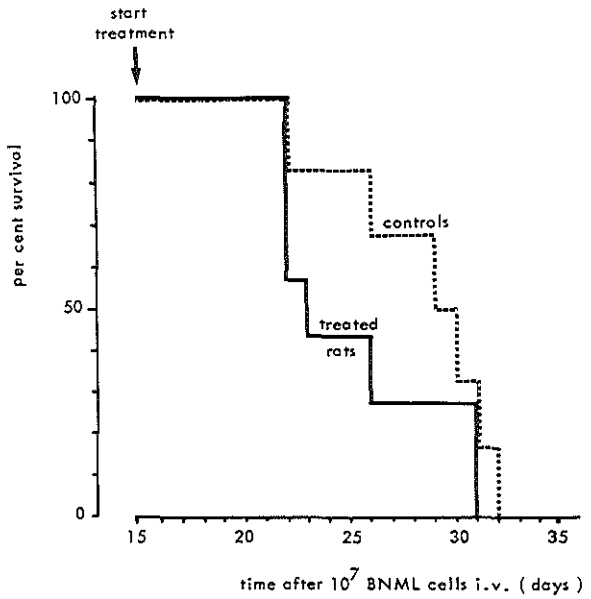

Figure 3.8

Survival curve after treatment with vincristine and prednisone (schedule B, Fig. 3.2).

22. In addition to the group which was treated with the combination of vincristine and prednisone, two parallel groups received either the same vincristine schedule alone or prednisone only. Fig. 3.9 indicates that the number of white blood cells in the peripheral blood was maintained at the same initial level in both groups receiving prednisone. In the group which was treated with vincristine, only an increase in the peripheral white blood counts, which was somewhat less than in the untreated leukaemic control group, was observed. The spleen weights in the three treated groups did not differ significantly from those in the untreated leukaemic controls. However, in the groups which received prednisone alone and prednisone + vincristine, the liver weights were much heavier as compared with the leukaemic controls (Fig. 3.10).

The survival curve (Fig. 3.11) showed that daily prednisone injection had a striking negative effect on survival, while the groups receiving vincristine and the combination vincristine and prednisone exhibited no beneficial effects as compared with the nontreated leukaemic controls.

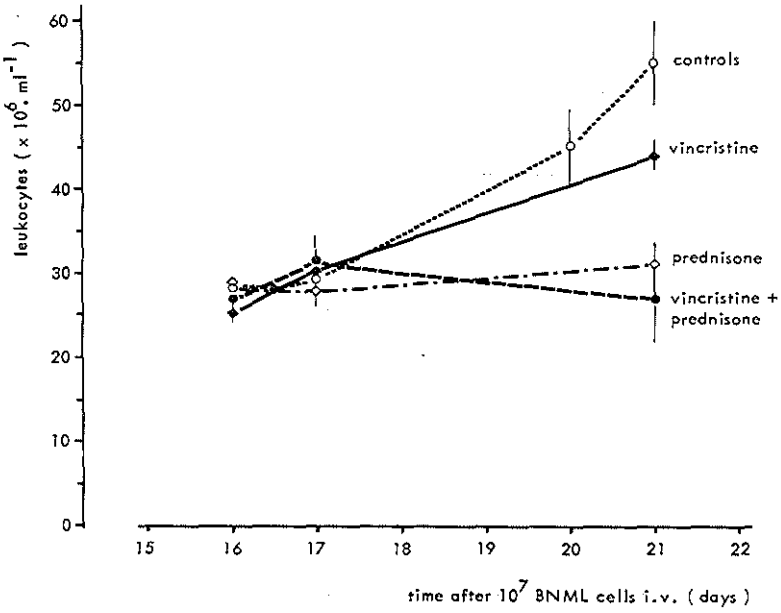

Figure 3.9

Changes in the peripheral white blood cell counts during treatment with a vincristine and prednisone schedule (schedule C, Fig. 3.2). Each point represents means ± SE of 5 rats.

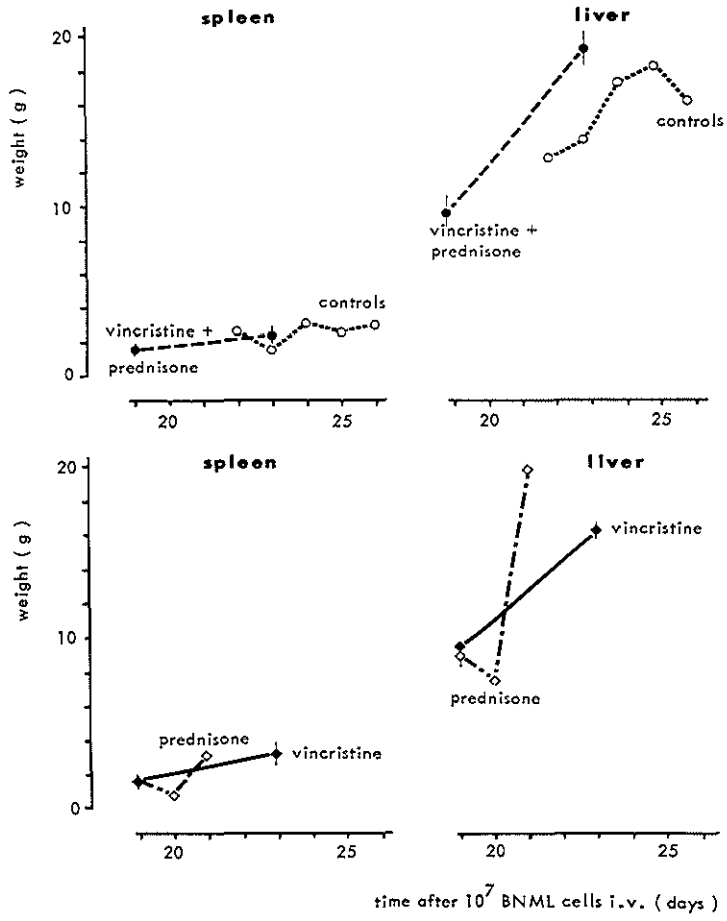

Figure 3.10

Spleen and liver weights after treatment with one or two drugs of schedule C (Fig. 3.2). Fresh weights were determined after spontaneous death. Each point represents either one rat or means ± SE of 2-4 rats.

3.4 DISCUSSION

The Ad-OAP regimen had a definite tumour load reducing effect as expressed in the decrease in the white blood cell counts and in the impressive reduction in spleen and liver weights found at autopsy. The prolongation of the survival time by about 5 days corresponds with a tumour load reduction of one decade (Fig. 2.2). This is in agreement with clinical data which show that one course of combination chemo-

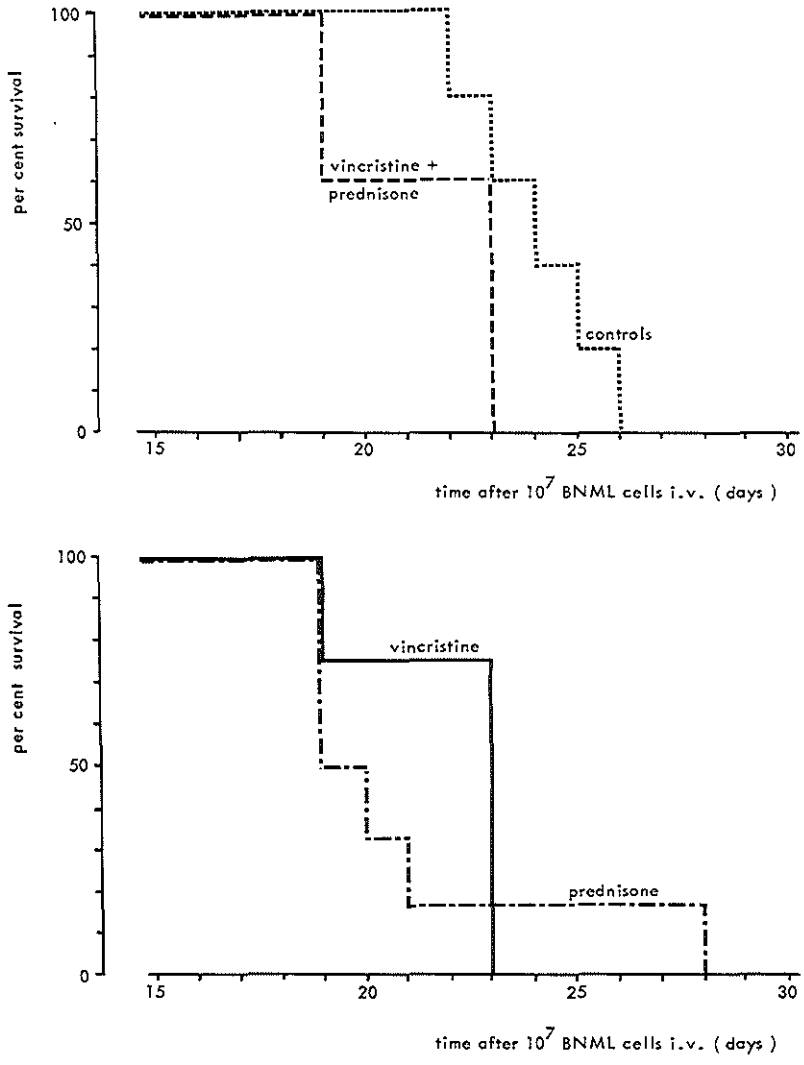

Figure 3.11

Survival curves of rats treated with one or two drugs of schedule C (Fig. 3.2) and the combination of vincristine and prednisone. Each group consists of 5 rats.

therapy reduces the tumour load by one decade (McCredie et al., 1976). The ALL regimen (vincristine and prednisone), was not at all effective in the BNML. In contrast to normal BN rats, a striking toxicity was observed in leukaemic animals.

An explanation for the vincristine toxicity may be that only less than 10% of this drug is metabolised in the rat body. In man, at least 25% of the vincristine dose is excreted as metabolites (Bender et al., 1977; Castle et al., 1976). It can be concluded that the rat metabolizes vincristine to a lesser extent than do humans; this results in neurotoxic vincristine levels for a prolonged period of time. An additional finding is that higher vincristine levels are found in brain tissue in smaller animals (rat, mice) (Castle et al., 1976) as compared to dogs and monkeys (El Dareer et al., 1977b). Based on these findings, it is tempting to explain the sudden deaths after vincristine injection as being due to these neurotoxic side effects, which are more serious in leukaemic rats than in normal ones. The reason why normal rats survive the highest applied vincristine dose and leukaemic rats do not, is not known at present.

In comparing the effects of vincristine versus vincristine/prednisone versus prednisone, it is noteworthy that the two groups receiving prednisone maintained a rather stable leukocyte count, but showed the heaviest liver weights at autopsy. The greatly increased liver weights along with the shortest survival time of these groups indicate that prednisone has an adverse effect on the leukaemia. It seems that prednisone causes no appreciable cell kill, but inhibits the migration of white blood cells from the liver. This results in lower numbers of peripheral white blood cells.

It can be concluded from these results that the BNML reacts favourably to a treatment schedule which is specifically active in human AML and does not respond to a typical clinical ALL treatment schedule. In addition to vincristine and prednisone, the Ad-OAP regimen includes the combination of adriamycin and ara-C. It follows that the beneficial effect of this regimen must be due to adriamycin and ara-C. For this reason, subsequent studies were concerned with determining the optimal effective application of these two drugs.

CHAPTER 4

EFFECTS OF ARA-C AND ADRIAMYCIN ON CELL PROLIFERATION

4.1 INTRODUCTION

Cell kinetic studies have stimulated chemotherapeutic scheduling in leukaemia in the past 20 years. This is partly based on studies in which cytostatic drugs have been subdivided into different categories according to their cell killing specificity, i.e., as non-specific and phase specific agents (see Section 1.4). The attractive aspect of the latter type of drugs is that these are supposed to kill only cells in a particular phase of the cell cycle. Another characteristic is that the toxicity will not increase with increasing dose, since the dose response curve exhibits a plateau pattern (Section 1.4). Cell kinetic studies in AML by Killmann (1968) and Gavosto (1968) have revealed that the leukaemic cell population contains a large fraction of non-cycling cells. Combining the properties of phase specific drugs and the fact that a large fraction of leukaemic cells is in the G_0 phase it can be concluded that leukaemic cells in the resting phase are insensitive to phase specific drugs such as, e.g., ara-C. Theoretically, one way to make them responsive to phase specific cytostatic drugs is to trigger them into cell cycle (recruitment; see Section 4.1.1). If the triggering procedures do not increase the cytotoxicity of the normal tissue to the same extent, a therapeutic gain can be achieved.

The effect of phase specific agents on the number of cells in S phase and in mitosis has been described by Lampkin (1969, 1971), Ernst (1971), Klein (1971) and Mauer et al. (1974). Improved chemotherapeutic effectiveness has been suggested by Lampkin (1971), Burke and Owens (1971) and Ernst and Killmann (1971; 1973) if the number of cells in a sensitive phase of the cell cycle is increased and if those cells are subsequently killed with that cycle phase specific drug.

Although some encouraging data have been published by Lampkin et al. (1971), who demonstrated an increased influx of resting cells into cycle after a single injection of ara-C, their results could not be confirmed by others (Ernst et al., 1973; Vogler et al., 1974). The conflicting results may be due to inhomogeneity in the selected pa-

tients with respect to the type of the leukaemia or phase of the disease or to differences in applied techniques, the type of cytostatic drug and dose schedules applied.

Chemotherapy schedules which were designed to change the phase distributions of the cell cycle and to subsequently administer a phase specific drug at the time of expected accumulation in that phase have been disappointing (Vogler et al., 1974; Ernst et al., 1971; Kremer et al., 1971; Gunz et al., 1977).

The availabity of a realistic rat model for AML (see Section 1.5) with a high fraction of cells in the nonproliferating phase and the recent development of a new technique for studying cell kinetics (flow cytometry) have created a challenge to reinvestigate whether recruitment can indeed be induced and usefully exploited.

Ara-C was chosen for this study because of its phase specific activity and because it is known to be an effective drug for killing myeloid leukaemic cells (Section 2.9.1).

Adriamycin was also included because it has been shown to be of great value in remission induction therapy in AML (Section 2.9.2).

In this Chapter, the principles and terminology of cell cycle kinetics along with a critical review of the various techniques for studying cell kinetics will be discussed. The results of cell kinetic studies by means of flow cytometry will also be described. The reason for selecting this technique is that it provides very reproducible results on the relative distribution of the number of cells over the cell cycle phases within one hour after the cells having been collected.

4.1.1. Cell kinetics: terminology

For many experimental (Mendelsohn, 1960) and clinical tumours, it has been shown that not all of the tumour cells are engaged in cell proliferation. Accordingly, for a morphologically homogeneous pool of tumour cells, a growth fraction may be defined by the equation:

$$GF = \frac{Nc}{N_{tot}}$$

Nc is the number of cells in cell cycle and N_{tot} stands for the total number of cells. Also, in most of the normal tissues a fraction of the cells are engaged in proliferation. The GF cannot be determined directly. An approximation of it is obtained by multiplying the percentage of cells in S by a factor of two.

The conventional method for determining the fraction of cells in S phase involves counting the labelled cells after flash labelling

with ^{3}H-TdR. Only cells which are in S phase at the time of exposure to ^{3}H-TdR are labelled. The labelling index (LI) then estimated is the ratio of labelled cells to total cells:

$$LI = \frac{N_S}{N_{tot}}$$

N_S is the number of cells in S phase.

Because mitosis can be recognized in stained microscopic preparations, the mitotic index (MI) was one of the first cell cycle parameters to be determined:

$$MI = \frac{N_m}{N_{tot}}$$

N_m is the number of cells in mitotic phase.

These two parameters, LI and MI, provide information on the fraction of cells in S phase and in mitosis, respectively. The duration of the cell cycle and cell cycle phases can be derived from a Percent Labelled Mitosis (PLM) curve. A PLM curve is determined by sampling tissue at intervals after a single injection of ^{3}H-TdR. The labelled as well as the unlabelled mitotic figures are scored in the autoradiographs:

$$PLM = \frac{\text{labelled mitosis}}{\text{labelled + unlabelled mitosis}} \times 100\ \%$$

A PLM curve is constructed by plotting the PLM against time after ^{3}H-TdR administration. The labelled cohort of S phase cells passes through mitosis, which results in halving the number of grains in the initially labelled cells. Various factors, among which are variations in phase duration and the entry of daughter cells into G_0, lead to a dampening of the curve; which is often considerable when human tissue is studied (Steel et al., 1966; 1971; Mendelsohn et al., 1971; Takahashi et al., 1971). A PLM curve of a rapidly proliferating population, GF = 1, is shown in Fig. 4.1.A; a PLM curve of a slowly growing population in which the majority of cells are in G_0 phase (GF = 0.45) is presented in Fig. 4.1.B. The PLM technique is a reliable method for determining the duration of all phases of the cell cycle. Its main disadvantages are that multiple biopsies are required and scoring of mitotic figures is laborious in slowly proliferating tissue.

The GF can be calculated from the PLM curve and LI according to a model described by Steel (1968), in which both proliferating and non-proliferating cells are assumed to be present, i.e., GF = $\alpha - 1$, where α can be calculated from:

$$N_S = \frac{1}{(\alpha - 1)} \times \varphi^*$$

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* $\varphi = N_{tot} \cdot e^{(TG_2 + M + T_S).(\ln \alpha / T_c)} - e^{(TG_2 + M).(\ln \alpha / T_c)}$
(Steel, 1968).

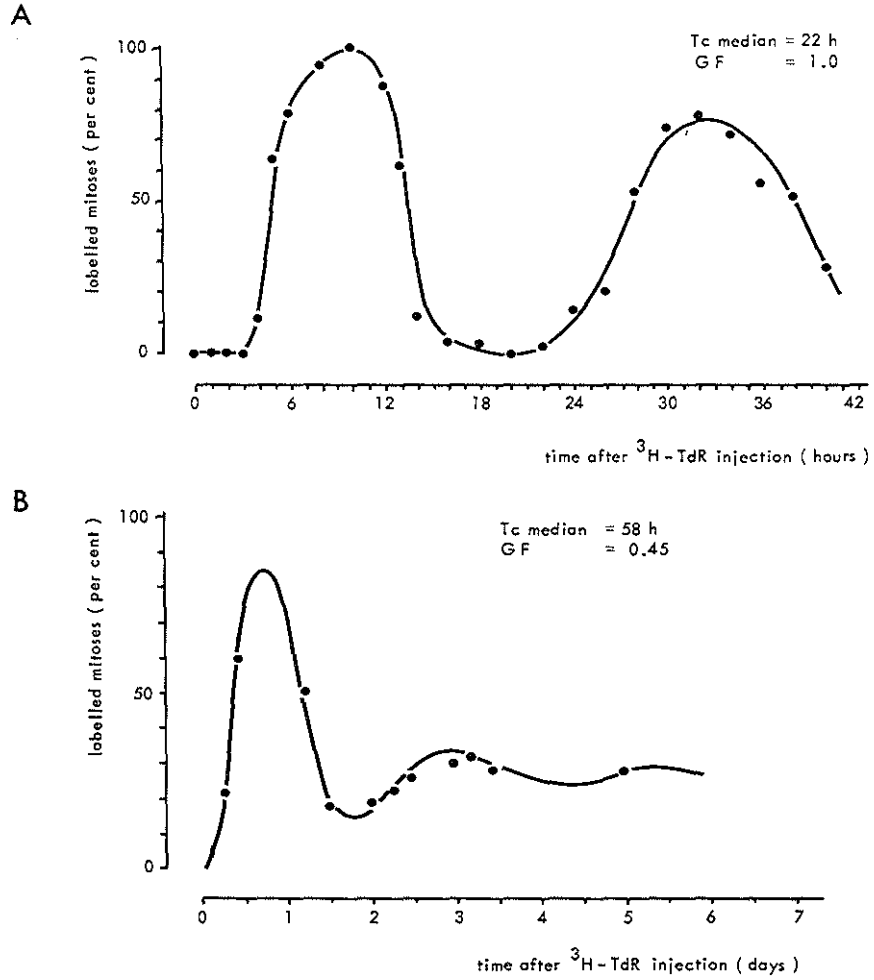

Figure 4.1 (A and B)

Examples of two PLM curves published in the literature (Fig. 4.1.A, Bootsma, 1965; Steel and Hanes, 1971; Takahashi et al., 1971; and Fig. 4.1.B, Bresciani et al., (1974). The latter figure illustrates the flattening of the second wave. Values for cell cyle times, T_c, and the fractions of proliferating cells, GF, are given in the inserts. The data shown in Fig. 4.1.A refer to experiments performed on in vitro cultured human kidney cells and those in Fig. 4.1.B to clinical studies of human squamous cell carcinoma. With respect to the accuracy of individual data points shown in these figures, it should be noted that for each value of the per cent labelled mitoses of the in vitro cultured cells, a number of at least 100 mitoses were counted, and an average number of 63 mitoses per data point were counted for the squamous cell carcinoma.

Recruitment will operationally be defined as an increase in the number of cells which move from the compartment of nonproliferating cells to that of proliferating cells (Bender et al., 1975). In a tumour system where all cells are proliferating, recruitment obviously cannot be induced. The GF is low in most human tumours.

Synchronization occurs when fractions of cells larger than normal pass simultaneously through the various phases of the cell cycle (Van Putten et al., 1976). It can be identified by a temporary increase in the fraction of cells in S phase or in mitosis.

When the cells can be investigated frequently, the chance that a temporary accumulation of cells in a cell cycle phase is overlooked becomes smaller.

4.2 METHODS FOR DETERMINATION OF CELL KINETIC PARAMETERS

4.2.1. Autoradiographic determination of LI, PLM curve and the fraction of cells in G_0 phase.

In man the LI is generally estimated in vitro by incubating cells with ^{3}H-TdR for 20 minutes at 37^{o} C. The excess of ^{3}H-TdR is removed by washing the cells with cold thymidine. Autoradiographs are prepared according to standard procedures (see Rogers, 1973) and the number of labelled cells counted.

The LI can also be estimated by injection of a pulse dose of ^{3}H-TdR (in vivo or flash labelling) and subsequent sampling of bone marrow and/or blood, usually one hour after injection. In some instances, a good agreement has been found between in vivo and in vitro labelling but in many others not (Saunders et al., 1967; Denekamp, 1973; Miller, 1975). After in vivo labelling with ^{3}H-TdR, the LI, PLM and GF can be determined.

Estimation of the G_0 compartment:

In general, the fraction of cells in G_0 can be determined only by calculation, which in its turn can only be performed when all cell cycle parameters are known. However, Clarkson et al. (1970) have attempted to investigate the fraction of cells in G_0 phase by means of administering a continuous infusion of ^{3}H-TdR to patients with acute leukaemia for a period of 8 to 10 days. They found that 88 to 93% of the leukaemic blast cells became labelled. From the 12% unlabelled cells, it was concluded that these remained in G_0 phase for the duration of the infusions. But, in using this method, it is not possible

to acquire exact information on the number of cells in G_0. Since labelled cells enter G_0 after completion of mitosis, the method seems useful to find out whether a G_0 compartment does exist in a certain cell population, but does not allow the determination of the size of that compartment.

4.2.2. Scintillation counting.

The ^{3}H-TdR uptake can be rapidly assessed by means of scintillation counting, which measures the disintegrations per minute of the incorporated ^{3}H-TdR. Unfortunately, thymidine uptake by individual cells often varies markedly, so that scintillator counts may not always reflect proliferation rates (Tannock, 1978). The scintillation counts reveal the total amount of ^{3}H-TdR accumulated in a random sample of the cell population. Besides, the scintillation method will give equal results in two widely different situations: the combination of small GF and short cell cycle times versus large GF and long cell cycle time. Because the LI, GF and cell cycle times cannot be determined with this technique, the method is useful only when these parameters are already known (e.g., in studies with cultured cell lines under ideal conditions).

4.2.3. Cell cycle analysis with a cell sorter.

Gray et al. (1977) have described a rapid method for establishing a percentage labelled S phase (PLS) curve, analogous to a PLM curve. The progress of a cohort of labelled S phase cells is monitored by measuring DNA content through a narrow window in the flow cytometer. The cells with a DNA content of mid-S phase are sorted by electronic cell sorting. The radioactivity of the sorted labelled S phase cells can be determined by scintillation counting. This method is now being developed for the solving of additional kinetic problems.

4.2.4. Flow cytometry.

Flow cytometry or pulse cytophotometry (PCP) can be used to estimate the DNA content of cells in single cell suspension (see Materials and Methods).

DNA histograms of normal rat bone marrow cells obtained with the flow cytometer can be seen in Fig. 4.2. Normally, most of the cells are diploid and have a DNA content of 2n. Those cells will be registered in one or at most in a few channels of the pulse height analyzer of the flow cytometer and are G_0,G_1 phase cells. Beside this G_0,G_1 peak in the histogram, there is a smaller second peak of cells with a double DNA value, 4n, corresponding to cells in the G_2,M phases. The cells between the two peaks, having a DNA content between 2n and 4n, represent the cells in S phase. Although a DNA histogram gives much information on the numbers of cells in the various phases of the cell cycle, it does not allow estimates of phase duration and GF, because no discrimination can be made between cells in the G_0 and G_1 phases.

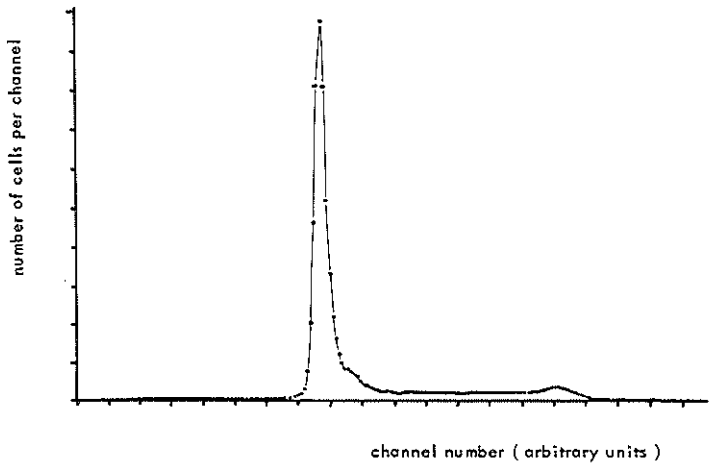

Figure 4.2

DNA histogram of bone marrow cells from a normal BN rat.

The degree of accuracy is dependent on:

1. The coefficient of variation (CV) of the flow cytometer used. The CV is a measure of the discriminative capacity of the apparatus and the staining procedure. With a high CV, there is a considerable overlap between the G_0,G_1 and S phases on the one hand and between S and G_2,M on the other. By means of mathematical analysis of the histograms, correction for overlap of the various distributions of the cell cycle phases can be made (Fried, 1976b; Chapter 2). When the cytometer has a small CV (e.g., the Phywe), the percentage of cells in a certain phase can be directly calculated from the histogram.

2. The number of dead cells present in cell suspensions. This number may increase when cytostatic treatments are given. While still being in the process of perishing they may pick up the fluorescent dye and be included in the histograms. However, cells in an advanced stage of disintegration will be lysed and can be removed as cell debris by washing and centrifugation (see 4.2.4.a).
3. Clumping of DNA fragments and the simultaneous passing of two particles with 2n DNA. This may result in a signal equivalent to 4n DNA. The magnitude of this source of error is investigated by analyzing a suspension of cells which contains cells in the resting phase, e.g., differentiated end cells. For this purpose, the buffy coat of peripheral blood cells and a suspension of thymocytes can be used. Fig. 4.3 shows a histogram of thymus cells. Since few signals are detected in the compartments containing more than 2n DNA, the clumping effect can be neglected. No particles are seen at the positions of 4n or 6n DNA, indicating that the discriminative capacity of the apparatus used was not a source of error.
4. The inhomogeneity of cell populations with respect to the DNA content of the G_0,G_1 cells. In the presence of aneuploid cells in the population, the histograms will be difficult to interpret. Only when the percentage of these aneuploid cells is small, and when these particles do not interfere with others, the percentage of aneuploid cells can be subtracted from the total number of cells and the distribution of the remaining cells over the cell cycle phases be calculated.

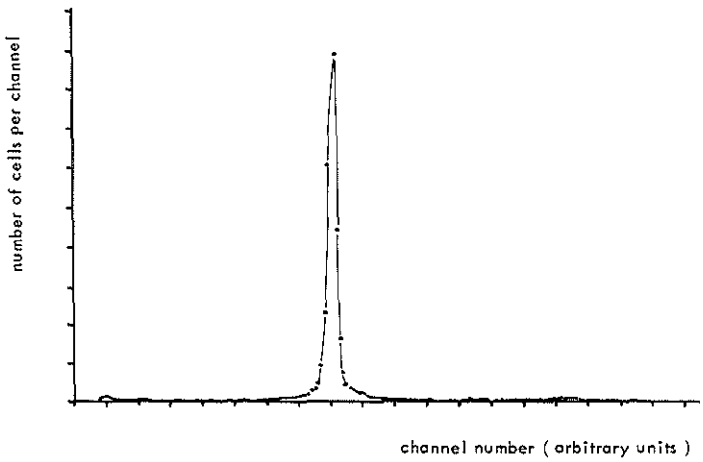

Figure 4.3

DNA histogram of thymus cells from a normal BN rat.

4.2.4.a. Interpretation of DNA histograms after cytostatic drugs.

Accumulation of cells in a certain cell cycle phase after cytostatic drug administration may result from:

1. cell cycle progression delay (the cell cycle blocking effect). In this case, the cell influx into a certain cell cycle phase continues, but the passage through that phase is impeded. When the cycle blocking influence is abruptly removed, the cells may continue through the cycle in a synchronized way. However, when the block is gradually removed, cell cycle progression is resumed in the normal asynchronous way.
2. Dead cells may accumulate in one or more cell cycle phases. A mathematical method which corrects the DNA histograms for dead cells has been described by Kim et al. (1978). In our experiments, the proportion of cells in S measured by cytometry and the LI were in excellent agreement before and after cytostatic treatment (Aglietta and Colly, 1979). Therefore, the dead cells do not seem to interfere with the flowcytometric measurements under our experimental conditions. Hillen (1975) and Barlogie et al. (1976b) also found a high degree of similarity between the results obtained by DNA histograms and those obtained by conventional labelling techniques.
3. Synchronization following recruitment (see definition in Section 4.1.1.). Differentiation between accumulation caused by cell blocking and that due to recruitment cannot be made from DNA distribution patterns alone. Although recruitment results in an increase of cells in the proliferation phase and a reduction in the G_0 compartment, one has to realize that the blocking effect in a certain cell cycle phase may result in a similar distribution over the cell cycle phases: a "reduction" in the proportion of cells in the G_0,G_1 compartment and an increase of the cells in that phase of the cell cycle which is blocked. This is due to the fact that the values obtained with flow cytometry are relative numbers.
   Evidence for the existence of a cell block in G_1 can be obtained only in rapidly proliferating populations; in slowly proliferating populations, no discrimination between G_0 and G_1 can be made with flow cytometry. For differentiation between recruitment and cell blocking in slowly growing populations, changes in the GF have to be calculated.
4. Exposure of cells to cytostatic drugs. This may alter the binding of the fluorescent dye to DNA and this may result in erroneous histograms. The interference of adriamycin with propidium iodide binding to DNA has been reported by Krishan et al. (1978) and will be discussed in Section 4.3.5.

In interpreting the changes in cell cycle distribution resulting from the administration of cytostatic drugs, the period during which the drug exerts its action is obviously a crucial factor. Drugs which are rapidly metabolized tend to induce reversible disturbances of short duration, while those which remain in an active form in the tissues for a longer period will cause more profound and longer lasting effects. The interaction of cell kinetics and pharmacokinetics will be discussed later (see Sections 4.3.2 and 4.3.5).

4.3 EXPERIMENTAL RESULTS

4.3.1. Flow cytometric follow-up of the BNML during progression of the disease

The observation of Haemmerli (Hagenbeek, 1977a) that the amount of DNA as determined in single cell Feulgen-stained smears of BNML cells was slightly increased (2.3 n DNA) has been confirmed by constructing DNA histograms in the course of the development of the disease (Fig. 4.4) (Colly et al., 1978). During the initial period when normal haemopoiesis continues while leukaemic cells are growing in the bone marrow, a small shift and broadening of the G_0,G_1 peak to the right is observed (day 4). These two populations can be distinguished at day 12. The two peaks in the G_0,G_1 area represent the normal (left) and leukaemic populations (right). In the terminal phase of the leukaemia, a relatively narrow peak corresponding with 2.3 n DNA has replaced the normal G_0,G_1 population. At the position of 2 x 2.3 n, a small accumulation of leukaemic cells representing the cells in G_2,M phases can be observed.

In all flow cytometric studies, the series were started by determining a histogram of normal bone marrow cells and an untreated leukaemic control sample. When the G_0,G_1 peak of the latter histogram contained too many normal haemopoietic cells, which was expressed as a double peak or a shoulder on the left hand of the curve, the experiment could not be evaluated and the data were discarded.

4.3.2. Flow cytometric measurements after a single injection of ara-C

The time-related effect of high dose 200 $mg.kg^{-1}$ ara-C i.v. was evaluated in leukaemic bone marrow. Fig. 4.5 shows the relative distribution of the DNA histograms obtained from measurements performed

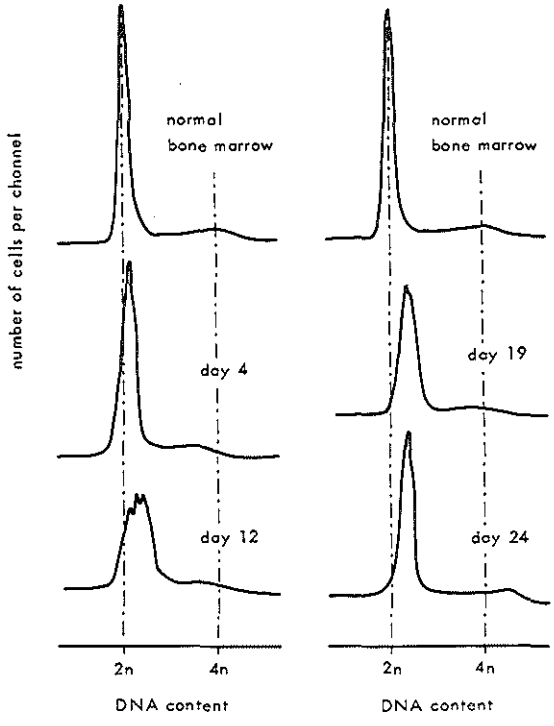

Figure 4.4

DNA histograms of bone marrow cells at various stages during the development of the BNML after inoculation of 10^7 leukaemic cells.

at regular intervals of about two hours during 0 to 28 h. By analyzing the histograms with the computer program described by Fried (1976b), the relative number of cells in the various cell cycle phases can be calculated (Fig. 4.5). These changes are in agreement with the results of LI and MI studies performed under the same conditions (Fig. 4.6) (Aglietta and Colly, 1979). However, an apparent discrepancy is observed between the nearly complete absence of labelled cells at two hours in the LI curve (Fig. 4.6) and the proportion of S phase cells in the flow cytometer curve (Fig. 4.5) at two hours, which is still about 50% of the initial value. This is explained by the fact that the assay of LI is a functional assay; only viable cells will incorporate ^{3}H-TdR. With flow cytometry, dead cells are also included in the histograms; this fraction will be high in the first hours after drug injection.

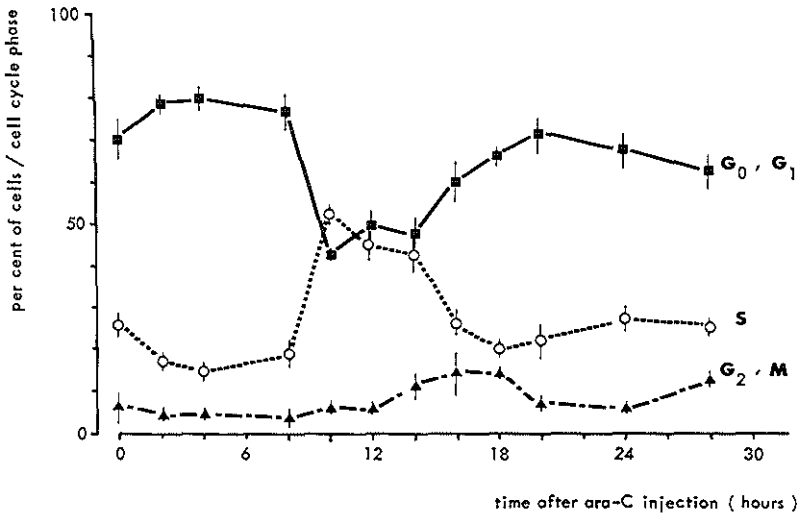

Figure 4.5

The relative distribution of leukaemic bone marrow cells over the cell cycle phases after one injection of ara-C, 200 mg.kg^{-1} i.v. at day 15 after inoculation of 10^7 leukaemic cells. Each point represents means ± SD of 5-10 measurements.

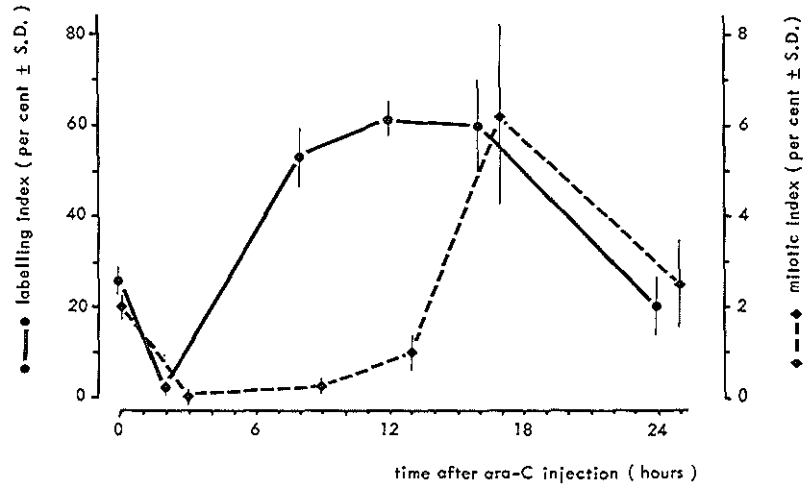

Figure 4.6

Labelling Index (LI) and Mitotic Index (MI) after a single injection of ara-C (200 mg.kg^{-1}) on the proliferation of leukaemic bone marrow cells (Aglietta and Colly, 1979).

In the curve for S phase cells, an abrupt rise is seen between 8 and 10 hours; at 10-14 h after injections, 40-50% of the cells are in S phase. The rate of progression of cells through S phase is reflected in the G_2,M curve; the cells are entering this phase at 12 hours and reach a maximum at 16 hours at a level of 14%. The mean value of the MI (Fig. 4.6) is 6% at 17 h after injection, which is lower than the number of G_2,M observed at the same interval. This has been explained by the fact that the MI is calculated from the number of cells in the mitotic phase. With flow cytometry, no distinction can be made between G_2 cells and mitotic cells. The curves for S phase and the G_2,M cells clearly show that the degree of synchronization is more pronounced during the influx of cells into S phase and is less when cells are leaving this compartment. At 24 hours after injection of the drug, the proportions of cells in the various cell cycle phases have returned to initial values. The cell cycle perturbation after administration of ara-C can be monitored very conveniently with the flow cytometric method as compared to the very time consuming determinations of LI and MI. The advantage of the LI assay is that it reflects the toxicity of the drug for S phase cells.

Fig. 4.7 shows a 3-dimensional representation of the cell kinetic perturbation induced by ara-C 200 $mg.kg^{-1}$ i.v.

The total femoral cellularity count after ara-C administration during 24 hours (Fig. 4.8) shows a decrease of 20%. This reduction is caused by the cytotoxic activity of ara-C, since the femoral cellularity counts of rats injected with 0.9% NaCl show a flat line. This reduction of 20% in femoral cellularity is in agreement with the reduction observed in the LI.

The high proportion of S phase cells found at 10-14 h after injection of ara-C requires further analysis. If this rise is the result of synchronization only, one would expect the peak value to be, at the most, two times the fraction of G_2,M cells measured at time zero, that is, 2 x 7% = 14%, since the total numbers of cells is not drastically decreased (only 20%) by ara-C. The difference between the observed 45% and the calculated 14% must be due to recruitment of G_0 cells. The evidence for the presence of a sizable G_0 fraction is derived from the larger fraction G_0,G_1 before ara-C injection (time 0 values) in comparison with the S and G_2,M fractions and the fact that the G_1 phase of the BNML is exceptionally short, namely, 0.8 h (Hagenbeek, 1977a). The reduction in the G_0 compartment after ara-C injection is nicely reflected in the curve for the G_0,G_1 cells shown in Fig. 4.5.

Theoretically, it can be expected that the cell cycle progression delay which has been described for the G_1/S boundary (Graham et al.,

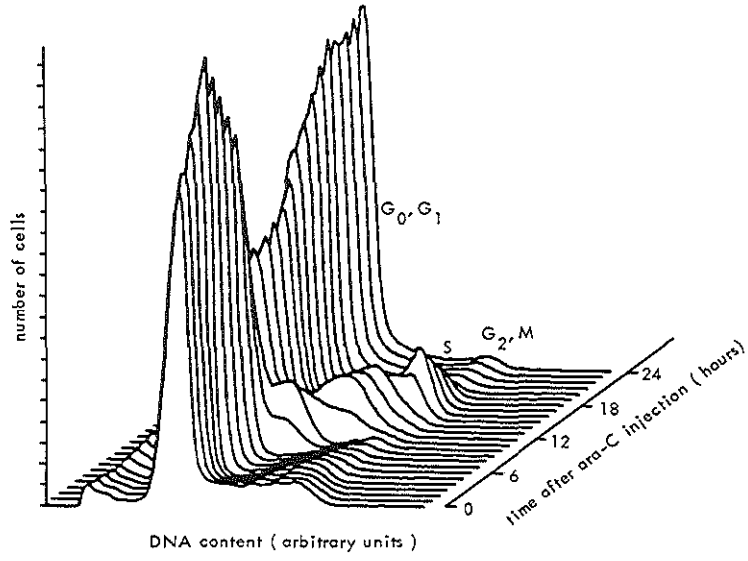

Figure 4.7

DNA content of leukaemic bone marrow cells at different time intervals after i.v. injection of 200 mg ara-C.kg^{-1}. DNA distributions were measured 0, 2, 4, 8, 10, 12, 14, 16, 20 and 24 h after ara-C treatment. The intermediate hourly DNA curves were extrapolated. The figure clearly shows the synchronizing effect of ara-C. At first, S-phase cells are depleted, thereafter the figure clearly shows a synchronized wave of cells beginning as early S, continuing as S and G_2,M phase cells through the cell cycle, and return to G_0,G_1 phase.

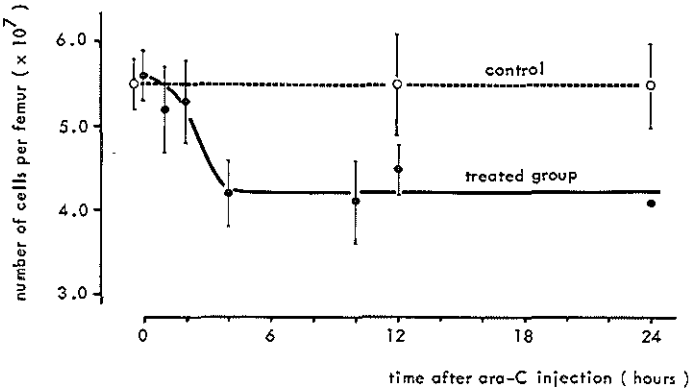

Figure 4.8

Reduction of the numbers of leukaemic cells per femur after one injection of ara-C, 200 mg.kg^{-1} i.v. as compared to the femur cellularity after one injection of 1 ml NaCl 0.9 % i.v. Each point represents either one rat or means ± SD of 2-5 rats.

1970b; Yataganas et al., 1974) will be abolished when the drug has been metabolized. The time interval between the latter process and the continuation of the cell cycle is not known. The pharmacokinetics and drug levels in plasma and in leukaemic cells after ara-C administration have been investigated in the leukaemic BN rat (P. Sonneveld, personal communication). The disappearance curve of ara-C in plasma is in agreement with data observed in humans and other animals (Creasey et al., 1966; Finkelstein et al., 1970). It shows a rapidly declining first phase, which is the result of the distribution over the whole body and the uptake in distinct organs. In the BN rat, the half-life of this "alpha phase" is 4.5 minutes; the second phase of the ara-C disappearance curve reflects the equilibrium between the metabolism in the tissues and the uptake from the plasma. The half-life of this "beta phase" is 110 minutes, resulting in undetectable levels of ara-C in the plasma and in the leukaemic cells 3 hours after injection.

It can be concluded from these pharmacokinetic data that the drug is rapidly metabolized. The mechanism responsible for the phenomenon of recruitment in the BNML still remains to be elucidated.

4.3.3. Effect of increasing ara-C doses on the phenomena of recruitment and synchronization

To answer the question whether recruitment and synchronization by ara-C are dose dependent effects, flow cytometric measurements were performed for doses of 50 mg.kg^{-1}, 100 mg.kg^{-1}, 150 mg.kg^{-1}, 200 mg.kg^{-1}, 250 mg.kg^{-1} and 300 mg.kg^{-1}. The results are shown in Table 4.1.

Comparing the groups which received increasing ara-C doses with Fig. 4.5 and the LI and MI curves of Fig. 4.6, an increase in S phase cells is observed in all groups at all dose levels during the period of 10-14 hours after administration.

The cytotoxic effect of ara-C demonstrated for the drug dose of 200 mg.kg^{-1} (Section 4.3.2) was reflected in the reduction in S phase cells during the first 6 hours (Fig. 4.5). The group which received 50 mg.kg^{-1} shows the smallest reduction in S phase cells of all groups. This drug dose is probably too low to exhibit cytotoxicity. This means that the effect on the GF is small. The consequence is that the accumulation in S phase after 10 hours of administration is partly attributed to the $S\text{-}G_2\text{-}M\text{-}G_1$ wave of the previous cell cycle and partly due to some recruitment.

Yataganas et al. (1974), in studying a human lymphoid cell line, demonstrated that the cell blocking effect occurs at a lower ara-C concentration than does cell killing.

For the other drug doses investigated, about equal degrees of cycle disturbancy were observed.

From these ara-C dose effect studies, it can be concluded that synchronization is observed for all doses investigated, whereas cytotoxicity and recruitment are seen only at doses of 100 mg.kg^{-1} and higher. This supports the idea that cell killing is required for the induction of recruitment.

4.3.4. Flow cytometric measurements after repeated ara-C injections

The experiments to be discussed here were designed to answer the question of whether cytotoxicity for S phase cells followed by recruitment and synchronization can be repeatedly induced by subsequent administration of ara-C. On theoretical grounds, it can be expected that a second injection of ara-C given at the time of maximum accumulation of cells in S phase will result in considerable cell kill. It has been shown that killing the S phase cells resulted in a decrease in the GF. The response of the resting compartment was recruitment of these cells into the proliferation phase. Analogous to the recruitment observed after the first ara-C injection, the killing of the accumulated S-phase cells due to the second ara-C injection should result in a second recruitment of G_0 cells. By repeating the ara-C injections several times, it might be possible to reduce the G_0 compartment step by step.

Flow cytometric studies were performed in animals receiving up to four ara-C injections administered at 12 h intervals in order to test this hypothesis. In order to achieve substantial cell killing, the dose of ara-C was set at 200 mg.kg^{-1} per injection. The results are presented in Fig. 4.9.

The S phase curves for the groups with and without the second ara-C injection are nearly identical. However, they represent different processes. The reduction in the S phase compartment after the second ara-C dose must be attributed to the cytocidal activity of the drug, since the G_2,M compartment does not increase, in contrast to the situation in which the animals received only one injection. The interpretation of the effect of the third ara-C injection is less clear, since the G_2,M curve does not differ significantly from the curve of the animals receiving two injections. In both cases, the proportion of

TABLE 4.1

DOSE-EFFECT RELATIONSHIP OF ARA-C DETERMINED WITH FLOW CYTOMETRY*

	h after ara-C injection	50 $mg.kg^{-1}$	100 $mg.kg^{-1}$	150 $mg.kg^{-1}$	200 $mg.kg^{-1}$	250 $mg.kg^{-1}$	300 $mg.kg^{-1}$
G_0,G_1 phase	0	72.5	72.5	72.5	72.5	72.5	72.5
	4	78.0	82.3	87.0	89.5	85.5	90.5
	6	80.0	80.0	88.5	90.0	91.0	90.0
	8	68.0	73.8	64.0	87.0	91.0	90.0
	10	59.0	54.3	49.0	46.0	52.0	50.0
	12	62.0	59.0	56.8	53.0	52.5	56.3
	14	66.0	62.5	59.0	52.5	54.0	54.3
	16	59.0	63.0	62.8	39.5	45.0	40.4
	24	63.0	65.5	66.5	64.0	69.5	65.5
S phase	0	23.5	23.5	23.5	23.5	23.5	23.5
	4	21.0	16.0	12.5	8.0	11.0	8.0
	6	17.0	10.5	10.0	10.0	8.0	7.5
	8	29.0	21.8	34.0	12.0	8.5	9.5
	10	40.0	39.7	45.0	47.0	44.5	47.0
	12	35.0	37.2	39.2	47.0	44.5	42.8
	14	35.0	37.5	41.0	47.5	46.0	45.5
	16	37.0	33.1	33.2	49.0	54.7	48.4
	24	29.0	27.0	26.5	29.0	24.5	28.0
G_2,M phase	0	3.3	3.3	3.3	3.3	3.3	3.3
	4	0.7	1.3	0.5	2.5	3.5	1.5
	6	3.0	7.7	1.5	0.0	0.5	2.0
	8	3.0	4.4	2.0	0.5	1.0	0.5
	10	2.0	6.3	6.0	7.0	3.5	3.0
	12	3.3	4.2	4.0	0.0	3.0	1.0
	14	0.0	0.0	0.0	10.0	0.0	0.3
	16	3.5	3.9	4.3	11.0	0.3	11.0
	24	8.5	7.5	6.5	6.5	6.0	6.5

*Means of 2-5 measurements

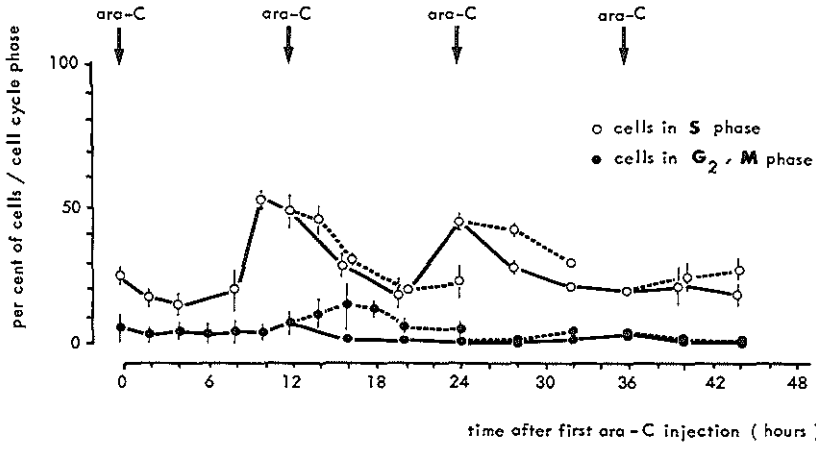

Figure 4.9

The relative distribution of leukaemic bone marrow cells over the cell cycle phases after increasing numbers of ara-C injections of 200 mg.kg^{-1} i.v. The solid line reflects the per cent of cells in S phase and G_2,M phase at the various intervals during the treatment. The broken line represents the per cent of cells in S and G_2,M phase in control animals which did not receive the previous ara-C dose. These control observations serve to evaluate the actual impact of each subsequent ara-C injection. The experimental points in the first 12 h are derived from Fig. 4.5. The other points represent means ± SD of 2-5 rats.

G_2,M cells is virtually zero. The explanation for this extremely low G_2,M compartment is that the second peak of S-phase cells (arising after 2 injections of ara-C) probably consists to a large extent of cells which die before they enter G_2. The third injection of ara-C (at 24 hours) seems to increase the cell kill even further; the decline in the S phase compartment is considerably more rapid as compared to the controls.

In studying Fig. 4.9, it should be kept in mind that the results represent relative proportions. The absolute size of the leukaemic cell population is reduced by 25% after the first ara-C injection. The remaining cells are again reduced by 50% as a result of the second ara-C injection and, after the third injection, a further reduction of 45% of the population remaining after the second injection is achieved. The expected third peak of S phase cells at 36 hours was not observed, probably for the same reason as given above for the absence of fluctuations in the G_2,M compartment. This does not imply that recruitment has no longer taken place. It is quite possible that such events are obscured by the presence of too many dying and dead cells. This can explain why recruitment and synchronization can no longer be detected.

4.3.5. Flow cytometric measurements following treatment with a single dose of adriamycin

In contrast to the consistent current information on the cell kinetic perturbation caused by ara-C, that on the action of adriamycin on cells in different phases of the cycle is controversial (see Section 2.9.2.C). The fact that most investigators have been studying a different biological system, combined with the diversity in sensitivity of different tumour cells to adriamycin, may explain many of the reported discrepancies (see Section 2.9.2).

In the present study, cell cycle perturbations following a single dose of 7.7 $mg.kg^{-1}$ adriamycin i.v. were investigated. This dose is equivalent to 40 mg/m^2 for humans, which has been reported to result in effective tumour load reduction in a variety of malignancies.

The results of flow cytometric measurements of leukaemic bone marrow cells after adriamycin during 36 h after injection of the drug is shown in Fig. 4.10. Overall, little effect on the cell cycle is apparent. These observations confirm the studies of Barranco (1975) in Chinese hamster ovary cells where flow cytometry indicated that all cell cycle phases are equally affected; no recruitment, synchronization or accumulation in a specific cell cycle phase was observed. This is in contrast with observations of Barlogie et al. (1975), and Krishan et al. (1975), who reported an accumulation of cells in G_2, even at low concentration, after in vitro incubation of cultured human

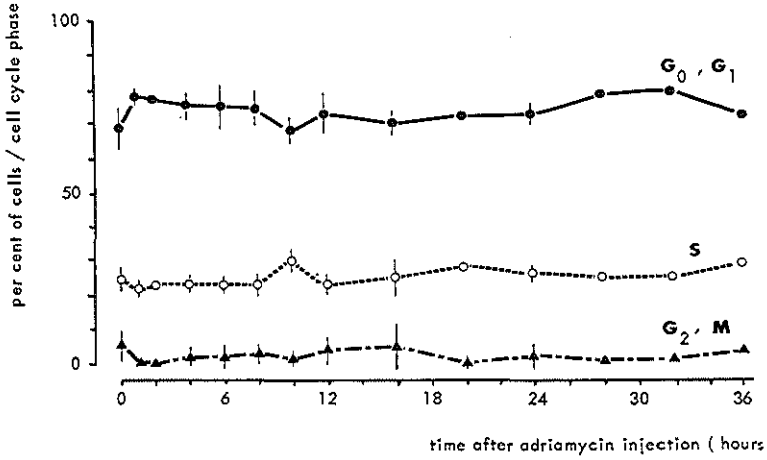

Figure 4.10

The relative distribution of leukaemic bone marrow cells over the cell cycle phases after one injection of adriamycin 7.7 $mg.kg^{-1}$ i.v. at day 15 after inoculation of 10^7 leukaemic cells. Each point represents either one rat or means ± SD of 2-5 rats.

lymphoma cells with adriamycin. Since in our studies a clear-cut cytotoxic effect of adriamycin was demonstrated in terms of both femoral cellularity reduction (30% decrease; Fig. 4.11) and of survival time (Table 5.6), it has to be concluded that adriamycin acts on cells in all phases of the cell cycle. It remains to be explained how these results are related to the observations of others (Clarkson et al., 1977; Kimler et al., 1978; Barlogie et al., 1976a) that adriamycin induces accumulation of cells in G_2. For the time being, it is assumed that those *in vitro* experiments cannot be compared directly with the *in vivo* measurements.

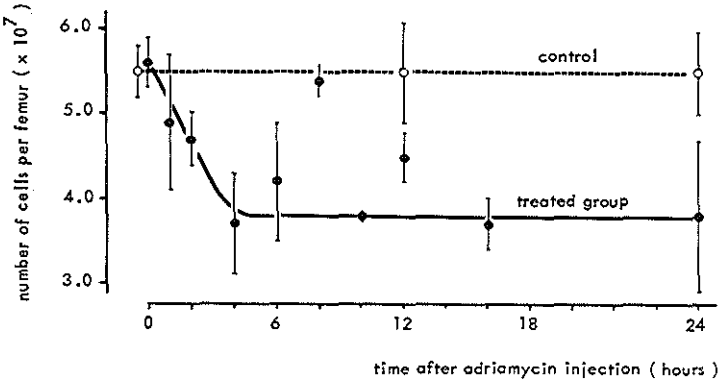

Figure 4.11

Reduction of the numbers of leukaemic cells per femur after one injection of adriamycin 7.7 mg.kg^{-1} i.v. as compared to the femur cellularity after one injection of 1 ml NaCl 0.9% i.v. Each point represents means ± SD of 2-5 rats.

4.3.6 Cytometric analysis of normal bone marrow cells after a single dose of ara-C

A therapeutic gain can be obtained with ara-C only when the critical normal tissues have either a lower proportion of S phase cells or when they are less responsive to the recruitment stimulus than the malignant cells. The most vulnerable targets for S phase specific drugs are the haemopoietic tissues and the intestinal epithelial cells. Both types of tissue consist of rapidly proliferating cells. However, in the case of haemopoiesis, the damage to the pluripotential stem cells determines the eventual chances of regeneration and it is known in rodents that a considerable proportion of those

cells is in the resting phase (G_0). If these G_0 cells are recruited and subsequently killed by the next dose of ara-C, the toxicity of such a regimen might become unacceptably high.

The percentage of haemopoietic stem cells (HSC) in normal resting rat bone marrow is 0.7% (Van Bekkum, 1977a); this means that 99.3% of the bone marrow cells are differentiating cells derived from the HSC and maturing to the various end cells (Chapter 1 and Fig. 1.1). With flow cytometry, it is obviously not possible to detect recruitment of the HSC, since their concentration in bone marrow is low. Therefore, this problem was attacked by using the CFU-s assay after repeated ara-C injection, as will be presented in Chapter 6.

4.4 DISCUSSION

Cell kinetic studies following the administration of chemotherapeutic agents are hampered by two important obstacles. The first one is technical. Before flow cytometry was introduced, the only available technique was autoradiography. The disadvantage of this method is that it is very time consuming and therefore has only retrospective significance. Nevertheless, such studies have revealed that human leukaemic blast cells exhibit large variations in cell cycle time between individual patients (Killmann, 1968; Gavosto et al., 1968; Greenberg et al., 1972). The consequence of this variation might be that each patient requires individual therapy based on the specific cell kinetic parameters of his or her leukemic cell population when the treatment employs phase specific drugs.

The introduction of flow cytometry in the early 1970's offered the possibility of determining some of the cell kinetic perturbations with greater care and more rapidly than before. However, no significant progress in the treatment of leukaemia has resulted from this approach so far. This is certainly due to the circumstances that present day therapeutic approaches to AML consist of multi-drug chemotherapy which do not provide the opportunity for a flow cytometric analysis of the role of each individual agent in cell cycle perturbation.

The second problem has been that the relevance of cell kinetics for treatment of human leukaemia has never been satisfactorily demonstrated in an animal model with characteristics similar to those of human AML.

Since the BNML was proved to be a realistic model for human acute myelocytic leukaemia, it was employed for cell kinetic studies with the purpose of designing improved remission induction schedules.

Ara-C was chosen because of its cell cycle phase specific activity and because it is known to be toxic to myeloid leukaemic cells. Adriamycin was added to ara-C treatment because it has been shown to be of great value for remission induction in AML. The effects of these drugs were analysed separately and in combination. The cell kinetic perturbation after 200 mg.kg^{-1} ara-C as observed with flow cytometry and confirmed with LI and MI studies (Aglietta and Colly, 1979) has been interpreted as recruitment of resting cells into the proliferation phase.

The recruitment could be induced repeatedly by successive ara-C injections given at 12 h intervals. Although the recruitment could not be demonstrated after the 2nd ara-C injection, because of the drastically reduced population of leukaemic cells, the results were sufficiently encouraging to investigate this phenomenon by LCFU-s and survival methods.

CHAPTER 5

SCHEDULING OF ARA-C AND ADRIAMYCIN AIMED AT MAXIMAL TUMOUR LOAD REDUCTION

5.1 INTRODUCTION

The observations on the cell cycle parameters of the BNML and their perturbations after the administration of ara-C have been used in attempts to develop schedules of treatment with ara-C and adriamycin which result in maximum reduction in tumour cell load. The effect of these treatments was evaluated by using LCFU-s, spleen and liver weights, femoral cellularity and survival as endpoints.

In the clinic, ara-C is administered by a number of different routes and schedules. Although each of these methods has its own advantages and disadvantages from the point of view of toxicity and convenience, their merits with regard to optimal tumour load reduction have by no means been established. Therefore, this question was firstly investigated in the BNML model.

For adriamycin, the only route employed in the clinical treatment of leukaemia is the intravenous one, either as a single injection or as an infusion given over a period of 2 h. In our studies, the single i.v. injection was employed for all schedules.

5.2 EFFECT OF DIFFERENT ROUTES OF ADMINISTRATION OF ARA-C ON THE TUMOUR LOAD

In discussions with clinicians*, the problem could be defined as follows: what is the most effective route of ara-C administration?:

1. daily dose ara-C given as a single rapid i.v. injection,
2. daily dose divided over 3 injections i.v. with an 8 h interval between doses,
3. same total dose given as a 24 h i.v. infusion,
4. daily dose given as a single subcutaneous (s.c.) injection,

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*Academic Hospital, Dept. of Pediatrics (Head: Prof. Dr. L.J. Dooren), Leiden University, Leiden, The Netherlands.

5. daily dose given as a single intramuscular (i.m.) injection.

In most remission induction schedules, ara-C is given either by rapid i.v. injection (Clarkson et al., 1975; Vogler et al., 1978; Spiers et al., 1977a) or by slow i.v. infusion over a period of 12 or 24 h (Bodey et al., 1975; Preisler et al., 1977; Peterson et al., 1977; Greenberg et al., 1976). For maintenance therapy, it is frequently given s.c. or i.m., e.g., in the LAM 5 protocol of the EORTC and in protocols described by Bodey et al. (1975), Preisler et al. (1977) and Carey et al. (1975).

Bodey et al. (1969) have compared the clinical response to ara-C treatment by daily rapid i.v. injection with treatment by continuous infusions, both for a period of 5 days. Their conclusion was that ara-C is more effective when administered by continuous infusion, although no clear cut evaluation parameters were employed. These results could not be confirmed by Wang et al. (1970) in acute childhood leukaemia. Ho and Freireich (1975) obtained equal results with i.v. injections every 8 h for 5 days and with continuous infusions over the same period. Both regimens were superior to the daily single injection of ara-C. The disadvantages of continuous ara-C infusions are that it causes much more nausea and vomiting and it is a more demanding treatment for both patients and nursing staff (Dooren, de Koning, personal communication).

Many studies have been performed with the murine L1210 leukaemia on time spaced scheduling of ara-C (Skipper et al., 1967; Kline et al., 1972). The best results are generally obtained when shortly spaced repeated ara-C injections are given. In comparing the effect of fractionated administration with that of continuous infusions of ara-C in the L1210, Edelstein et al. (1977) found that the tumour load was reduced to equal levels after ara-C infusion and after optimal interval treatment.

Thus, the literature does not provide dependable information on the superiority of continuous infusion versus i.v. interval treatment, nor is there reliable comparative data available on the efficacy of i.m. and s.c. administration as compared to i.v. injection.

5.2.1 Experimental design

The effect of the various modes of administration of ara-C was compared with untreated leukaemic controls using liver and spleen weights and femoral cellularity as endpoints. The two most effective ways of administration, i.e. interval push i.v. and continuous in-

fusion were also compared with regard to their toxicity on the haemopoietic system with the use of the CFU-s assay. These results will be presented in Chapter 6.

Female BN rats were inoculated i.v. with 10^7 BNML cells, weighed and divided into 6 groups on day 15 after inoculation. The method of treatment of groups 1-5 is shown in Table 5.1. The treatment of groups 1 and 2 were designed for comparing repeated interval treatment with giving the total dose in one injection. Each daily treatment was provided for a period of 4 consecutive days in order to obtain distinct effects with the chosen endpoints, as had been established in pilot studies.

TABLE 5.1

TREATMENT SCHEDULES FOR COMPARING DIFFERENT ROUTES OF ARA-C ADMINISTRATION (BN FEMALES)

Group 1	200 mg.kg^{-1}	i.v. q = 24 h	days 1-4; n = 5
Group 2	3 x 70 mg.kg^{-1}	i.v. q = 8 h	days 1-4; n = 4
Group 3	200 mg.kg^{-1}	i.p. q = 24 h	days 1-4; n = 5
Group 4	200 mg.kg^{-1}	i.m. q = 24 h	days 1-4; n = 5
Group 5	200 mg.kg^{-1}	s.c. q = 24 h	days 1-4; n = 5
Group 6	untreated leukaemic controls		n = 5

The treatment schedules for comparing interval treatment and infusion are given in Table 5.2. Male rats were used in this study because they are larger; this makes it easier to place an indwelling canula into the tail vein. Treatment was given for one day only because it was not very well possible to continue the infusion for a longer period of time. The continuous infusion was compared with administering the same amount of ara-C in two equal doses injected i.v. 12 h apart. This schedule was used for comparison because the 12 h interval proved to be the most effective schedule in the studies described in paragraph 5.3.

TABLE 5.2

TREATMENT SCHEDULES FOR COMPARING ARA-C INTERVAL TREATMENT WITH CONTINUOUS ARA-C INFUSION (BN MALES)

Group A	2 x 200 mg.kg^{-1}	i.v. q = 12 h,	1 day; n = 5
Group B	400 mg.kg^{-1}	continuous i.v. infusion,	1 day; n = 5
Group C	untreated leukaemic controles; n = 5		

In all experiments, 24 hours after the last day of the treatment, the rats were sacrificed, livers and spleens were removed and weighed and femoral cellularity was determined. All data were compared with those obtained in untreated leukaemic controls in the same stage of the disease.

5.2.2 Results

As shown in Table 5.3, the s.c. and i.m. treatments are the least effective as judged by all three endpoints. A more pronounced reduction in tumour load occurs when the drug is given i.v. and intraperitoneal (i.p.). These two routes lead to similar reductions in spleen and liver weights, but the i.v. push administration reduced the femoral cellularity more effectively ($p < 0.01$). By far, the greatest reduction in the tumour load in all 3 tissues is seen after the 8 h interval i.v. administration of the drug (group 2).

The comparative data of a 24 h infusion and 2 i.v. injections spaced by a 12 h interval are presented in table 5.4. The tumour load reduction in the spleen and liver is greater following 2 i.v. injec-

TABLE 5.3

COMPARISON OF THE EFFECTS OF DIFFERENT ROUTES AND INTERVALS OF ARA-C ADMINISTRATION ON TUMOUR LOAD REDUCTION AFTER 4 DAYS OF TREATMENT IN BN FEMALE LEUKAEMIC RATS

	spleen (g)*	liver (g)*	femur cellularity* x 10^7
Group 1 200 mg.kg^{-1} ara-C i.v., q = 24 h	1.31 ± 0.04	8.42 ± 0.11	3.5 ± 0.29
Group 2 3 x dd 70 mg.kg^{-1} ara-C i.v., q = 8 h	0.30 ± 0.007	5.37 ± 0.16	0.75± 0.12
Group 3 200 mg.kg^{-1} ara-C i.p., q = 24 h	1.34 ± 0.13	8.53 ± 0.65	4.6 ± 0.15
Group 4 200 mg.kg^{-1} ara-C i.m., q = 24 h	1.97 ± 0.15	9.71 ± 0.88	4.3 ± 0.27
Group 5 200 mg.kg^{-1} ara-C s.c., q = 24 h	1.74 ± 0.15	9.27 ± 0.48	4.2 ± 0.42
Group 6 leukaemic controls	2.20 ± 0.07	12.71 ± 0.43	5.1 ± 0.23

*Mean value ± SD

tions than after slow infusion of the same dose of ara-C, but the difference, although statistically significant, is modest. The femoral cellularity is equally reduced by the two treatment schedules.

TABLE 5.4

COMPARISON OF ARA-C INTERVAL TREATMENT AND ARA-C CONTINUOUS INFUSION. TUMOUR LOAD REDUCTION AFTER ONE DAY OF TREATMENT IN BN MALE RATS

	spleen (g)*	liver (g)*	femur cellularity* x 10^7
Group A 2 x dd 200 mg.kg^{-1} ara-C i.v., q = 12 h	1.02 ± 0.18	9.79 ± 0.59	3.4 ± 0.24
	$p < 0.05$	$p < 0.005$	N.S.
Group B 400 mg.kg^{-1} ara-C i.v. infusion during 24 h	1.26 ± 0.11	12.37 ± 0.27	3.6 ± 0.71
Group C leukaemic controls	3.22 ± 0.21	17.47 ± 0.60	7.0 ± 0.21

*Mean value ± SD

5.2.3 Discussion

After i.v. injection of ara-C, the drug is distributed over the whole body and is rapidly assimilated into the leukaemic cells, resulting in a short plasma half life (alpha phase: 10 minutes; see Chapter 4) and high tissue levels in the compartments, especially when the ara-C dosage is high (Momparler, 1974).

The explanation for the poor therapeutic response to ara-C following s.c. and i.m. administration is that the drug is inactivated by the local deaminase enzymes before effective levels can be reached (Caminier, 1967).

Comparing the effects of i.v. and i.p. administration of ara-C, the only difference was the more pronounced reduction in femur cellularity after i.v. injection. An explanation for this phenomenon may be that the leukaemic spleen and liver are bathed in ara-C after i.p. injection. This could result in a direct uptake of ara-C by the leukaemic cells in the abdominal organs, with the result of a much reduced availability of the drug to the bone marrow.

The most effective treatment in this series was shown to be the 8 h interval i.v. treatment (group 2). This can be explained by the observed perturbation of the kinetics of the leukaemic cell population following a single injection of ara-C (Chapter 4, Figs. 4.5 and 4.6). The second dose of ara-C coincides with an accumulation of cells in S phase, which is the phase most sensitive to this drug.

Subsequent studies (see section 5.3) have shown that a 12 h interval between ara-C injections results in an equal or a somewhat higher tumour load reduction.

In comparing a 24 h continuous ara-C infusion with that of optimal interval treatment, the latter proved to be more effective as determined by spleen and liver weight; femoral cellularity was equally reduced in the two groups. It should be emphasized that in these experiments treatment was limited to one day only and that the total dose of ara-C was twice as high, so that the results cannot be compared directly with those of the previous series (groups 1 to 6).

The preference for ara-C infusion therapy in the clinical situation may be based on the idea that such administration promotes the prolonged maintenance of effective tissue levels, which in turn is thought to enhance the tumour load reduction (Bodey et al., 1969). However, the experiments in the BNML indicate that spaced rapid i.v. injections are superior to slow continuous infusion of the drug. This superiority cannot be explained by a longer exposure time of the leukaemic cells to the drug, but has to be attributed to recruitment of leukaemic cells from the G_0 compartment.

5.3 EFFECT OF REPEATED ARA-C INJECTIONS ON TUMOUR LOAD

5.3.1. Introduction

The observed cell cycle perturbation after one injection of ara-C as described in Chapter 4 (Fig. 4.5) was used as the basis for further scheduling studies. Experiments were designed in which the interval between two ara-C injections was varied and the effect on tumour load in the bone marrow was estimated with the LCFU-s assay. As in the previous studies, the first ara-C injection was given 15 days after the inoculation with leukaemic cells. The LCFU-s assay was thought to be the most sensitive parameter, because it was expected that the leukaemic cells would be reduced by 0.3-1 decade (Fig. 2.1) as a result of 2 ara-C injections.

Two hours after the administration of the second ara-C injection, the rats were sacrificed. One of the femurs of each animal was dissected and graded numbers of the pooled bone marrow cells of 3 animals were injected into normal rats. This procedure in which the femurs of 3 rats per evaluating time point are used takes about 1.5-2 h. Spleen colonies were counted at day 19 as described in Chapter 2.

5.3.2. Results

Fig. 5.1 shows the surviving LCFU-s after the second injection of ara-C, expressed as a fraction of the LCFUs per femur determined after the first injection. A steep decrease is seen upon prolongation of the interval and a maximum reduction of about 90% (i.e. one decade) is reached between 10-12 h. If the interval is further prolonged, the synergistic effect diminishes and becomes close to zero at a 24 h interval.

5.3.3. Discussion

The surviving fractions of LCFU-s determined after two time spaced injections of ara-C (Fig. 5.1) agrees quite well with the observed cell kinetic disturbance after one injection of the drug (Figs. 4.5

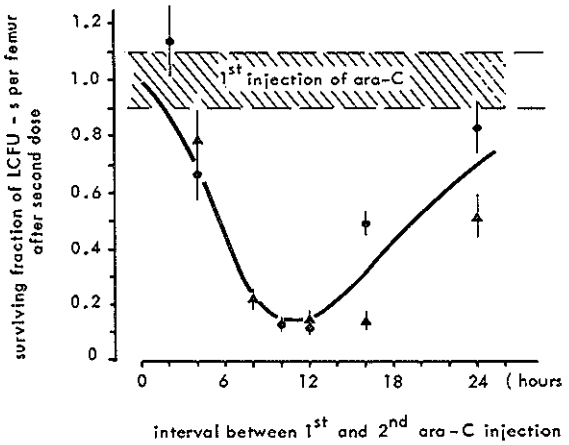

Figure 5.1

Changes in the number of LCFU-s when the second ara-C injection was given at various intervals after the first one. The animals were sacrificed 2 h after the second ara-C injection. Different symbols represent means ± SE of different experiments.

and 4.6). The magnitude of a purely additive effect of the second injection can be calculated as follows. A single injection of ara-C will kill only the cells in S phase, which is about 20-25% of the leukaemic cells (Chapter 4). Pure addition of the effect of two injections will therefore result in a surviving fraction in between 0.8 x 0.8 = 0.64 and 0.75 x 0.75 = 0.56. This is about 40% total kill. On this basis the surviving fraction in Fig. 5.1 should be 0.8. The actual maximum reduction by about a factor of 10 confirms the synchronization and recruitment induced by the first injection as was demonstrated by different techniques in Chapter 4. It might be concluded from Fig. 4.5 which shows a steep increase in S phase cells between 8 and 10 h, that a second ara-C injection given at 12 h is somewhat too late, because the cells are half way synthesizing DNA. However, in Fig. 5.1, about equal reduction in LCFU-s was observed in the period of 8-12 h. The 12 h interval was chosen for subsequent ara-C injections because it proved to give optimal therapeutic results.

5.4 COMBINATION OF SINGLE ARA-C AND ADRIAMYCIN INJECTIONS

5.4.1. Introduction

In the previous study, the effect of two successive doses of ara-C was investigated. In this section, a study of the combination of one injection each of ara-C and adriamycin is described. From the pharmacological properties of the two drugs, it is to be expected that each exerts most of its activity within 24 h. For this reason, the drug combination was investigated in both sequences (ara-C/adriamycin and adriamycin/ara-C) with the LCFU-s as endpoint. Based on the results of this experiment, the effects of ara-C/adriamycin and the reverse sequence with an interval of 12 h between the two drug injections, have been tested using survival time and reduction in liver and spleen weights as evaluation criteria.

5.4.2. Results

The reduction in the number of LCFU-s in the bone marrow as a fraction of the leukaemic controls is shown in Fig. 5.2. When adriamycin is given at 24, 16, 12 or 8 h before ara-C, the reduction in the number of LCFU-s does not differ significantly ($p > 0.05$) from the value obtained after simultaneous injection of ara-C and adriamycin.

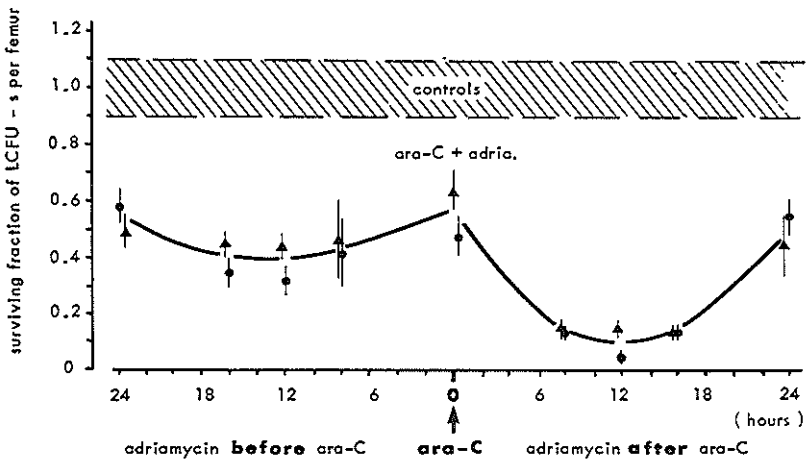

Figure 5.2

Changes in the number of LCFU-s when second drug in the combination adriamycin/ara-C and ara-C/adriamycin was given at various time intervals after the first drug. The animals were sacrificed 2 h after the second drug injection. Different symbols represent means ± SE of different experiments.

In contrast, the opposite drug sequence, i.e., ara-C followed by adriamycin, results in a more significant reduction in the number of LCFU-s, the effect depending on the time interval between the administration of the two drugs. When the interval between the two injections was 8, 12 or 16 h, a highly significant difference was calculated as compared with both the simultaneous injection of the two drugs ($p < 0.002$) and injections with a 24 h interval ($p < 0.001$). To compare the whole curve of ara-C before adriamycin with that of the opposite drug sequence, the multiple variation analysis was used. The difference between the two curves is highly significant ($p < 0.00001$).

To determine the effect of drug sequence on survival time, one group of leukaemic rats was treated with the schedule ara-C followed by adriamycin and another group with the opposite sequence. Both groups of rats received the schedule two times: on day 15 and on day 24 after inoculation of 10^7 leukaemic cells. The reason for giving two treatments was that a single treatment equivalent to a tumour load reduction of 1 decade does not result in a demonstrable increase in survival time. The superiority of the drug sequence ara-C followed by adriamycin compared to the opposite sequence is shown in Fig. 5.3 ($p < 0.001$). The schedule adriamycin followed by ara-C did not prolong survival time as compared with the untreated leukaemic control group, while the reverse sequence led to a prolongation of the median survival time of about 10 days.

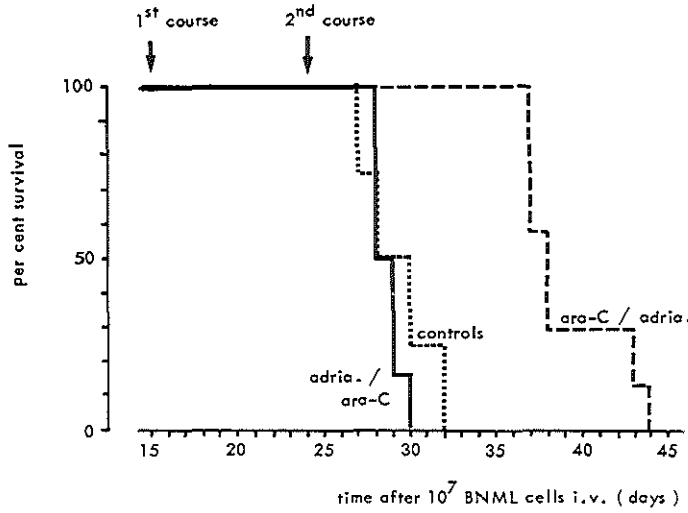

Figure 5.3

Survival curve of rats treated with two courses of the schedule ara-C/adriamycin and adriamycin/ara-C. Time interval between drug injection: 12 h. Each group consists of 7 animals (explanation in the text).

In a parallel experiment, the rats were sacrificed on day 25, i.e., 24 h after the last cytostatic drug injection. Tumour load reduction was determined by weighing livers and spleens (Table 5.5). Although the weights of the spleens and livers of the rats treated with the drug sequence ara-C followed by adriamycin were less than those of the rats treated with the opposite sequence, the differences were not statistically significant, due to large variations in the adriamycin/ara-C group.

TABLE 5.5

COMPARISON OF SPLEEN AND LIVER WEIGHTS AFTER THE ADMINISTRATION OF THE DRUG SEQUENCES ARA-C/ADRIAMYCIN AND ADRIAMYCIN/ARA-C GIVEN TO LEUKAEMIC RATS

	spleen weight (g)* mean ± SD	liver weight (g)* mean ± SD
ara-C/adriamycin (n=4)	0.31 ± 0.06	5.61 ± 0.84
adriamycin/ara-C (n=5)	0.59 ± 0.29	6.54 ± 1.47
leukaemic controls (n=3)	1.63 ± 0.59	15.72 ± 8.68

*Mean value ± SD

5.4.3. Discussion

Edelstein et al. (1974) demonstrated a schedule dependent synergism for the drug combination ara-C and daunorubicin in the L1210 murine leukaemia. A maximum reduction in the number of LCFU-s was found when ara-C was given first and followed by injection of daunorubicin about 15 h later. Although no cell kinetic measurements were performed in those experiments, the hypothesis suggested was that ara-C partially synchronizes the leukaemic cells in cell cycle, which should result in an increased reduction ("synergism") in the number of clonogenic leukaemic cells, when a second phase specific drug is given at the right moment.

The results shown in Fig. 5.2 are in agreement with the data of Edelstein et al., suggesting that populations of cycling cells of different leukaemias respond in the same way to treatment with a related cytostatic schedule. The reduction in the number of LCFU-s is maximal when adriamycin is given at 8-16 h after ara-C. From the flowcytometric analysis and LI studies after ara-C it is known that a maximum accumulation of cells in S is observed during this period. It can be concluded from the results of these in vivo experiments that adriamycin kills cells in S phase. This is in agreement with Krishan and Frei's results (1976) with synchronized cultured human lymphoblasts.

The left hand part of the curve in Fig. 5.2 shows a more or less flat pattern, indicating that the phenomenon of recruiting cells into cycle is not a result of the property of the drug to kill cells in S phase. In the absence of other information, it has to be concluded that induction of recruitment is specific to ara-C.

The maximal reduction in the number of LCFU-s (Fig. 5.2) for the drug sequence adriamycin/ara-C is 60% and for the drug sequence ara-C-adriamycin 90% (1 decade). Assuming that such an effect can be repeated after 9 days (Fig. 5.3) the calculated tumour cell survival for two courses for the group adriamycin/ara-C is 0.4 x 0.4 = 0.16 (16%), which should correspond to an increase in survival time of 1 day. For the combination ara-C/adriamycin (group A) the calculated reduction of tumour load after two courses is 2 decades which corresponds with an increase of survival time of 10 days. Both calculated figures fit well with the observed prolongation of the mean of the survival time (Fig. 5.3); for the adriamycin/ara-C group no detectable difference in survival time is observed as compared with the untreated leukaemic controls, whereas the survival time of the group treated with the combination ara-C/adriamycin has been prolonged with 9 days.

Evaluating the results described in sections 5.3 and 5.4, it can be stated that after an initial injection of ara-C, maximum reduction in the tumour load is observed when the second drug (ara-C or adriamycin), is administered at the time of maximum accumulation of cells in S phase.

5.5 ENHANCED TUMOUR LOAD REDUCTION AFTER REPEATED ARA-C INJECTIONS FOLLOWED BY ADRIAMYCIN

5.5.1. Introduction

The specific property of ara-C to recruit G_0 cells into cell cycle was exploited in further attempts to obtain maximum reduction in the leukaemic cell load with combinations of ara-C and adriamycin. It has been shown that a second ara-C injection also induces recruitment from the G_0 phase with subsequent synchronization again after about 12 h (Fig. 4.9). However, it was not possible to demonstrate this effect with flow cytometry after a third ara-C injection (see discussion Chapter 4), presumably because the remaining cell population is only 1% of the original population. To test the effect of three or more ara-C doses, survival studies seem to be the only sensitive method. Such tests will be described in this section.

The tested schedules consisted of increasing numbers of ara-C injections followed by one injection of adriamycin. In each schedule, adriamycin was given as the last drug because the specific property of ara-C, i.e., that of recruitment, was utilized to its maximum capacity and in this final stage recruitment is obviously unwanted. An additional consideration was that cells which are resistant to ara-C could be killed by a final dose of adriamycin.

5.5.2. Experimental design

In each experiment generally 30 leukaemic rats were used. The animals were divided into three groups, of which two were treated and one served as control. This procedure was necessary for practical reasons: interval treatments of rats and the accompanying supportive transfusions could not be performed with large numbers of animals at the same time. The various treatment schedules investigated are listed in column 1 in Table 5.6.

TABLE 5.6

COMPARISON OF OBSERVED REDUCTION OF TUMOUR LOAD AFTER INCREASING NUMBERS OF ARA-C INJECTIONS WITH THE CALCULATED THEORETICAL TUMOUR LOAD REDUCTION
(for explanation, see text)

	number of rats	survival time (days) mean ± SD	decrease of tumour load (decades) extrapolated from survival time	theoretical
Controls	7	23.6 ± 3.2		
1x ara-C	10	25.1 ± 1.7		
1x adriamycin	10	28.4 ± 2.6		
Controls	10	25.6 ± 2.6		
ara-C+adriamycin	12	28.9 ± 2.8		
ara-C/adriamycin q = 12 h	12	34.0 ± 2.6	1.6	1
Controls	10	25.0 ± 5.1		
2x ara-C/1x adriamycin, q = 12h	9	38.9 ± 3.9	2.6	2
3x ara-C/1x adriamycin, q = 12h	8	46.5 ± 6.5	4.0	3
Controls	10	23.3 ± 1.2		
4x ara-C/1x adriamycin, q = 12h	10	41.8 ± 5.9	3.0	4
5x ara-C/1x adriamycin, q = 12h	9	45.6 ± 9.8	4.0	5
Controls	16	23.1 ± 4.9		
6x ara-C/1x adriamycin, q = 12h	60	57.3 ± 19.7	6.6	6

Because intensification of therapy caused increased suppression of normal haemopoiesis, four blood transfusions of 1.5 - 2 ml given on days 1, 3, 5 and 7 after termination of chemotherapy, were required to keep the animals alive. Although this supportive care was necessary only for the most intensively treated groups, all groups, including the untreated leukaemic controls, received the same number of transfusions in order to provide uniform conditions in all experimental animals.

In about half of the animals which died, liver and spleen weights were determined post mortem. Large variations in these organ weights were observed; therefore, two extensive bioassays were performed in two groups of 10 treated leukaemic animals (6x ara-C/1x adriamycin) at 48 h after termination of treatment. These assays involved the injection of samples from suspensions of pooled cells from the spleen, liver and bone marrow into normal recipients. The percentage of recipients dying from leukaemia and their survival time provided an indication of the original leukaemic cell load of the tissues assayed.

5.5.3. Results

The effects of intensification of ara-C/adriamycin therapy on survival time are shown in Table 5.6 and Fig. 5.4. The most extensive treatment consisting of 6x ara-C/1x adriamycin was repeated 6 times, each time in a group of 10 rats, because a small population of the treated rats survived for long periods of time ($>$ 100 days). This resulted in very large variations, so that several repeats were required to obtain a reliable average.

In Table 5.6 the survival time in column 3 of the groups treated with increasing ara-C injections followed by adriamycin is shown. Because survival time is dose related with tumour load, the reduction of the leukaemic cells can be calculated as has been shown in section 2.7.4. The extrapolated tumour load reduction in column 4 can be calculated by subtraction of the extrapolated tumour load reduction from the initial tumour load at day 15: 5×10^9 leukaemic cells. The data in column 5 (theoretical reduction) are based on the LCFU-s reduction (Figs. 5.1 and 5.2), assuming that each subsequent ara-C injection results in an additional tumour load reduction of 1 decade.

The survival curve for the entire group of rats treated with the schedule 6x ara-C/1x adriamycin is shown in Fig. 5.4. Eight out of 60 rats survived longer than 70 days, and died without macroscopic signs of leukaemia. Unfortunately, these animals were not suitable for histological examination. From a parallel experiment, two rats were

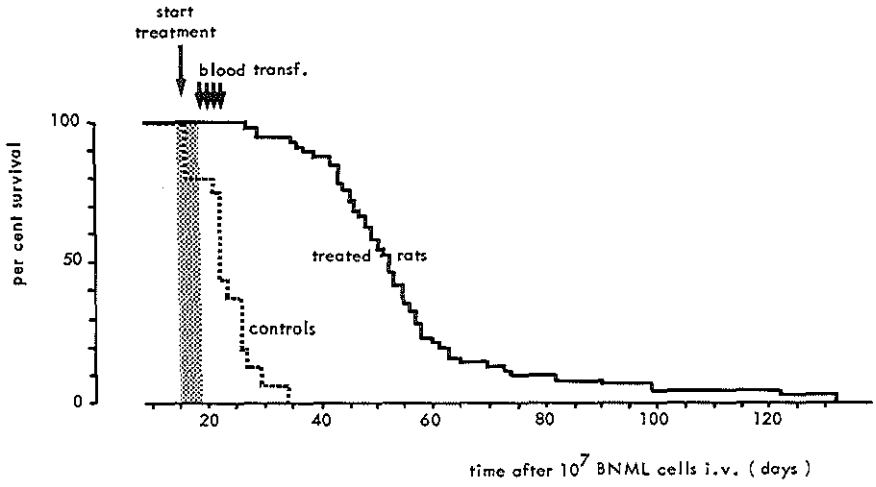

Figure 5.4

Survival curve of 60 leukaemic rats treated with the schedule 6x ara-C/1x adriamycin, interval between drug injections: 12 h, leukaemic controls (n = 16). Treatment was started at day 15 after inoculation.

sacrificed for histological examination at 90 days after inoculation. Foci of leukaemia were observed in the bone marrow of both animals.

At autopsy, the spleens and livers showed large variation in weight (Figs. 5.5 and 5.6). As can be seen, most rats dying around day 50-60 showed increased liver and spleen weights, whereas animals dying after day 80 had less than normal liver and spleen weights.

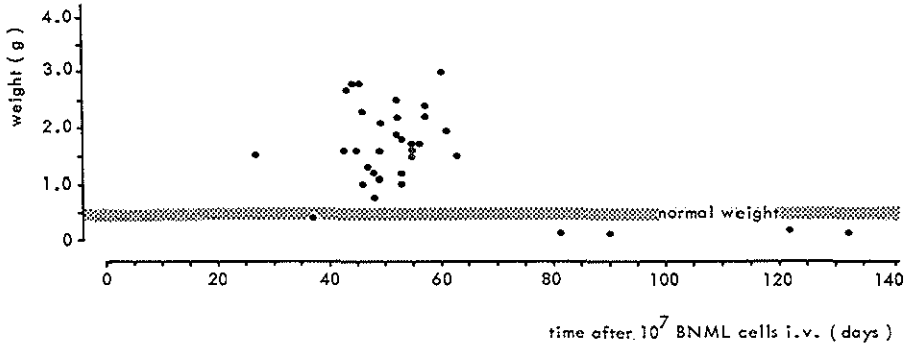

Figure 5.5

Spleen weights of leukaemic rats treated with the schedule 6x ara-C/1x adriamycin, determined after spontaneous death (Fig. 5.4).

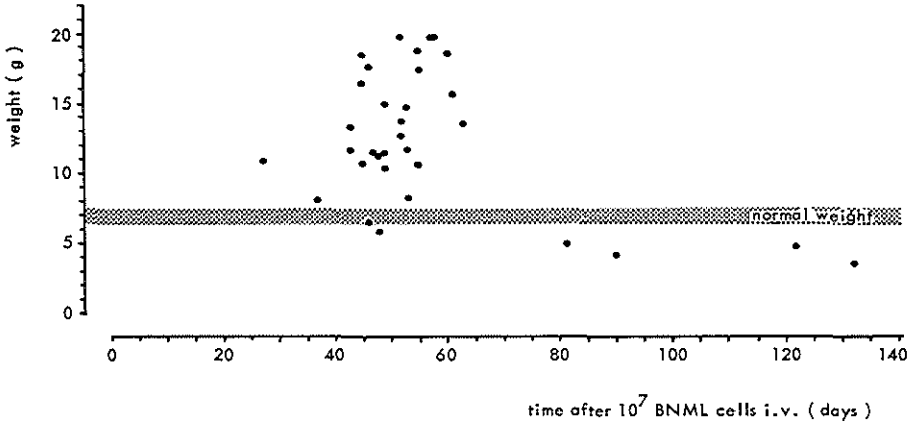

Figure 5.6

Liver weight of leukaemic rats treated with the schedule 6x ara-C/1x adriamycin, determined after spontaneous death (Fig. 5.4).

The results of the bioassays are listed in Table 5.7. It should be emphasized that the cell samples for bioassay were drawn from strictly comparable groups of rats, as justified from the survival time of the nonsacrificed treated animals, namely 52.9 and 54.3 days respectively. However, in bioassay "2", 10^3 residual leukaemic cells were calculated to be present in the bone marrow against none in the other bioassay. The liver in both bioassays contained about 10 leukaemic cells per 10^7 cells and no leukaemia cells could be detected in the spleen.

5.5.4. Discussion

The scheduling of ara-C and adriamycin which was based on the perturbation of the cell cycle induced by ara-C has resulted in increased tumour load reduction, which was correlated with the number of ara-C injections (Table 5.6). The calculated tumour load reduction which can be predicted if it is assumed that every ara-C injection was capable of recruiting the same fraction of G_0 cells (column 5, Table 5.6) agrees well with the tumour reduction calculated from survival time (column 4). Extrapolation of the average survival time after the treatment with 6x ara-C/1x adriamycin results in about 1000 leukaemic cells surviving. This number is somewhat smaller than the surviving fraction of leukaemic cells which is calculated on the assumption that each dose of ara-C kills an identical fraction of the population surviving after the previous dose of ara-C, namely $(0.1)^6$. Thus, from the tumour load at day 15: 5×10^9 cells, only $(5 \times 10^9).(0.1)^6 = 5000$ cells survive. However, this difference is well explicable by the large standard deviation of the various assays. These results suggest that the leukaemic cell population outside the bone marrow reacts similarly to that inside the bone marrow to repeated ara-C injection; in other words: it implicates that the concentration of ara-C in liver and spleen was sufficiently high to induce recruitment and subsequent leukaemic cell kill.

The survival times of the 60 rats which were treated with the schedule 6x ara-C/1x adriamycin show large variations. The values for spleen and liver weights at autopsy (Figs. 5.5 and 5.6) were also greatly varied. Although some animals died with normal or reduced spleen and liver weights throughout the whole period of observation, there were some early deaths (around day 45) where increased spleen and liver weights were observed. Statistical analysis of the survival curve of the group treated with 6x ara-C/1x adriamycin has revealed that the curve is not normally distributed, so that the long survival

TABLE 5.7

ESTIMATION OF THE NUMBER OF LEUKAEMIC CELLS LEFT AFTER TREATMENT OF LEUKAEMIC RATS WITH THE SCHEDULE 6x ARA-C/1x ADRIAMYCIN

bioassay 1

inoculated cells	survival time (days)	tumour load (cells)
1×10^7 BM cells n = 8	> 300 all 8	0
1×10^7 liver cells n = 8	n = 4: 60,5 (40-91) n = 4: >300	< 10
1×10^7 spleen cells n = 4	> 300 3 out of 4*	0

Survival time of non-sacrificed group 52.9 ± 6.8

bioassay 2

inoculated cells	survival time (days)	tumour load (cells)
1×10^7 BM cells n = 10	41.3 (37 - 45) all 10	10^3
1×10^7 liver cells n = 9	n = 2: 65.5 (46-77) n = 7: > 200	< 10
1×10^6 spleen cells n = 9	> 200 all 9	0

Survival time of non-sacrificed group 54.3 ± 5.8

*One rat died without macroscopical signs of leukaemia

time cannot be explained by a normal variation in the group. Further evaluation of the possible toxicity of the separate drugs used in the schedule 6x ara-C/1x adriamycin in normal rats has shown that adriamycin might be a damaging factor. For a group of normal rats injected 6x with ara-C was compared with a second group injected with the schedule 6x ara-C/1x adriamycin. Furthermore, a third group of normal rats received only one injection of adriamycin. The preliminary result of this experiment in terms of survival time was that rats receiving 6x ara-C lived for more than 200 days, while both the group receiving 6x ara-C/1x adriamycin and the group on the schedule 1x adriamycin alone died after a mean survival time of 103 ± 36 and 92 ± 7 days, respectively. The cause of death is so far unknown, but further histological and biochemical investigation is certainly required. The question whether the long term survivors in the treated leukaemic rats (Fig. 5.4) eventually died from a relapse or from the effects of adriamycin after a possibly effective cure of the leukaemia still remains to be settled.

The variation in the therapeutical response is also expressed in the two bioassays. The bone marrow suspension of bioassay number "2" clearly contained bone marrow of one or more rats which will have been rather less responsive to therapy. In both bioassays in which liver cells had been injected, a number of rats (4 out of 8 and 2 out of 9) developed leukaemia after 60.5 and 61.5 days. It can be concluded from these figures that a very small number of leukaemic cells, probably less than $\pm 10^7$, were present per 10^7 cells of the original liver suspensions. In contrast, both bioassays of the spleen suspensions contained no leukaemic cells in the injected cell suspensions. Although in the first assay one rat died, no macroscopical signs of leukaemia, e.g. increase of spleen and liver weight, could be observed.

It can be concluded from these bioassays that a relapse originating from the bone marrow is a distinct possibility, more so than from the liver, while a relapse originating from the spleen is unlikely.

The reason why repeated ara-C treatment was not continued to the point at which the leukaemic cell load should be totally eradicated, was a practical one. The rats which had been treated with the schedule 6x ara-C/1x adriamycin had to be anaesthesized 11 times, and, since their condition deteriorated, it could be expected that additional periods of anaesthesia would result in nonspecific mortality. Besides, it was very difficult to perform intravenous injections into the tail veins for more than 11 times in the same animal.

Summarizing the results, it is shown that very effective tumour load reduction was obtained by means of recruitment and synchronization of the leukaemic cells, by repeated treatment with ara-C. The final injection of adriamycin proved necessary because it does not have the characteristic of recruitment, and recruited cells are obviously unwanted at the end of treatment.

CHAPTER 6

THE NORMAL HAEMOPOIETIC COMPARTMENT

6.1 INTRODUCTION

6.1.1. Quantification of normal haemopoietic stem cells

The quantitative assays for colony forming cells, either *in vivo* (CFU-s) or *in vitro* (CFU-c), are powerful tools for studying the cell renewal capacity of normal haemopoietic tissue.

Considerable knowledge of the haemopoietic system has been gained from studies in the mouse. By combining the CFU-s assay (Till and McCulloch, 1961) with haemopoietic stem cell (HSC) identification by electron microscopy, the existence of the HSC has been both qualitatively and quantitatively substantiated (Van Bekkum et al., 1971).

A similar CFU-s assay has been developed for the BN rat (Van Bekkum et al., 1976): firstly, in the isologous system by injecting bone marrow suspensions into lethally irradiated BN rats; and, secondly, by using lethally irradiated F1 hybrid mice of C57Bl/LiRij x C3H/LwRij as recipients. Unfortunately, no assay for monitoring the human bone marrow for CFU-s content is yet available.

However, the clonogenic capacity of haemopoietic cells can be determined in many species (mouse, rat, dog, monkey), including man, by use of an *in vitro* culture system in semisolid medium which monitors precursor cells of the granulocytic series (CFU-c) (Pluznik et al., 1965; Bradley and Metcalf, 1966; Ichikawa et al., 1966). Extensive studies in the mouse have demonstrated a good correlation between the numbers of CFU-s and the numbers of CFU-c in haemopoietic cell populations (see also 1.2). Several other culture systems have been developed to detect erythropoietic progenitors, BFU-E (Axelrad et al., 1974; Wagemaker, 1978), CFU-E (Stephenson, 1971) and megakaryocyte precursors, CFU-M (Nakeff, 1972; Metcalf et al., 1975). Colonies composed of B and T lymphocytes can be induced in culture (Metcalf et al., 1975; Rozenszajn et al., 1975); however, these colonies seem to originate from relatively mature cells, in contrast to colonies arising from haemopoietic progenitor cells.

6.1.2. Quantification of the leukaemic clonogenic cells

One of the advantages of studying animal models is the possibility of quantification of the clonogenic capacity of the leukaemic tumour load *in vivo*. For the BNML, the numbers of clonogenic leukaemic cells in a suspension can be determined with the LCFU-s assay (Van Bekkum et al., 1976) as described in Chapter 2. This has been shown to be a sensitive parameter for evaluating the leukaemic cell load down to approximately 5×10^6 in the rat, which corresponds to a detection level in leukaemic bone marrow of 0.1 % leukaemic cells.

A similar clonogenic assay is available in the L1210 leukaemia for comparing tumour load differences (Edelstein et al., 1974; 1977).

Because of the high costs of *in vivo* cloning assays, tissue culture systems which allow a quantitative estimate of clonogenic leukaemic cells *in vitro* have been developed for certain transplantable leukaemias. Despite numerous efforts, no such system is available for AML models at present; the BNML cells do not grow in any of the several culture systems investigated.

Much confusion has arisen concerning the colony forming capacity of bone marrow from patients with AML, probably due to differences in culture systems, number of days of incubation, handling and colony size criteria.

The culture system for growing haemopoietic precursor cells of the mouse has been modified for the human system (Robinson et al., 1971). Moore et al. (1973) found that leukaemic cells from patients with acute or chronic myeloid leukaemia proliferate in agar and form clusters or, more rarely, colonies. In some samples, karyotypic studies of those clusters and colonies showed that they originated from the leukaemic cell population of the patient. Because of the general similarity between normal and leukaemic colonies, it was assumed that the leukaemic cells initiating *in vitro* colonies were neoplastic equivalents of normal colony forming cells (CFU-c). However, no evidence has been provided to show that the numbers of colonies or clusters can be considered to be representative of the number of clonogenic leukaemic cells in the test suspensions. In 1975, Dicke et al. described a culture system in which marrow from AML patients formed colonies. The essential stimulator phytohaemagglutinin (PHA) induced colonies, the leukaemic origin of which has been proved by ultrastructural morphology and cytogenetics. However, this method requires the removal of T cells prior to culturing, since PHA induces T cell colony formation under the same conditions. Park et al. (1977) developed a quantitative assay for colony forming human leukaemic cells where the essential

factor was daily feeding with conditioned medium containing L-ascorbic acid and glutathione. McCulloch (1978) described an assay for colony formation in which blast cells from patients with AML could be stimulated with phytohaemagglutinin and leukocyte conditioned medium (PHA-LCM).

Bone marrow and peripheral blood of patients with CML easily give rise to a large number of colonies in the conventional agar culture system for detecting normal human CFU-c (Robinson, 1971; Moberg et al., 1974). In CML, the granulocytes are produced from stem cells containing the Ph' abnormality (Nowell and Hungerford, 1960) of the G_{22} autosome (O'Riordan et al., 1971) which persists in busulphan induced remission, in relapse and in the acute transformation phase (blast crisis) of the disease (Moberg et al., 1974).

6.1.3. Normal haemopoiesis during progression of the leukaemia

One of the major problems in AML is the severely suppressed haemopoiesis in the terminal phase of the disease, which results in anaemia, thrombocytopenia and granulocytopenia. This is reflected in many patients by the fact that CFU-c are abnormally low in number or completely absent in the bone marrow.

The *in vitro* growth characteristics of cells from patients with untreated acute leukaemia have been shown to be of prognostic value (Moore et al., 1974; Van Oosterom and Van Bekkum, 1974; Spitzer et al., 1976) (Table 6.1). A similar therapeutic response was observed by the three groups of authors in patients whose bone marrow gave rise to large or small clusters. However, their results show several discrepancies. Firstly, Moore et al. (1974) observed colony formation in a number of patients, while Van Oosterom and Spitzer never observed this

TABLE 6.1

COMPARISON OF THE PROGNOSTIC VALUE OF THE NUMBER OF CFU-c OBSERVED IN PATIENTS WITH AML

	Moore et al. (1974)	Van Oosterom (1974)	Spitzer et al. (1976)
Colony forming cells (> 40 cells)	CR 39 %	not observed in AML	not observed in AML
large cluster forming cells (< 40 cells)	CR 12 %	poor progn.	CR 21 %
small cluster forming cells (1-20 cells)	CR 53 %	good progn.	CR 75 %
no growth	CR 15 %	poor progn.	CR 76 %

in any of their AML bone marrow samples. The explanation is probably that the series studied by Moore et al. included samples of peripheral blood, since the collections were done by others. Secondly, the prognosis of patients when bone marrow produced no clusters or colonies was different in the series of Moore and Van Oosterom as compared to the series of Spitzer; this is most likely due to the fact that the investigations of Moore and Van Oosterom were carried out with patients who received no intensive supportive care during treatment, while the patients of Spitzer at the M.D. Anderson Hospital in Houston did. The absence of any *in vitro* growth in the bone marrow strongly suggests that this group of patients had a very small reserve haemopoietic capacity. In addition, the remission induction therapy employed for treating the patients in the Houston study was more advanced than that in the earlier studies in Australia and The Netherlands.

6.1.4. Normal haemopoiesis during progression of the BNML

In the BNML, all differentiated end cells of normal haemopoiesis (erythrocytes, thrombocytes, granulocytes and lymphocytes) decrease during the last 10 days of the disease (Hagenbeek, 1977a). A decrease in the progenitor cells as measured as CFU-c was observed by Van Bekkum et al. (1976) as early as 10-15 days after inoculation of 10^7 leukaemic cells.

The fate of the pluripotent stem cells as determined by ascertaining the numbers of CFU-s in the haemopoietic tissues (bone marrow, blood, spleen and liver) during the development of the disease has been described elsewhere (Colly et al., 1977a). These results, as presented in Fig. 6.1., show a similar decline in bone marrow as was previously found for CFU-c.

The shape of the CFU-s curves suggests, but does not prove, a migration of the HSC from the bone marrow to the blood as the marrow cells are being replaced by leukaemic cells. In the second week after inoculation of 10^7 cells, extensive signs of extramedullary haemopoiesis is observed in the spleen and the liver (Hagenbeek, 1977a). The reason why leukaemic rats were not able to maintain their normal haemopoiesis while still possessing considerable numbers of CFU-s has not been discovered (see also 6.3.5).

The suppression of normal haemopoiesis in the leukaemic bone marrow might be due to several mechanisms:

1. Direct cell-to-cell inhibition of normal cells by leukaemic cells.
   This possibility has been ruled out by performing mixed culture

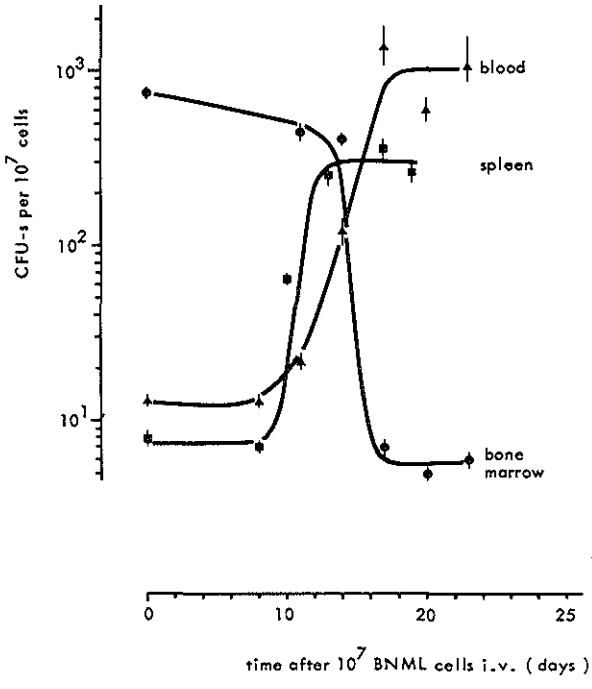

Figure 6.1

Changes in the number of colony forming units spleen (CFU-s) in the bone marrow, the blood and the spleen during progression of the BNML. Each point represents means (± SE) of 2-3 experiments with 8-10 recipient mice per spleen colony assay. Day 0: non-leukaemic controls.

studies on leukaemic and normal bone marrow cells (Van Bekkum, 1976);

2. The production by leukaemic cells of humoral factors that inhibit proliferation of normal CFU-s. Diffusion chamber experiments did not support this hypothesis (Van Bekkum, personal communication). Hence, no evidence was found in the BNML for the presence of humoral inhibiting factors similar to those reported to be present in human AML cells (Broxmeyer et al., 1978a; 1978b; Chiyoda et al., 1976). This does not necessarily mean that the BNML differs in this respect from human AML. Apparently, the pathophysiological processes of severe suppression of haemopoiesis are operative in both the BNML and human AML. It is unlikely that totally different mechanisms would be responsible for this phenomenon in the BNML and human AML. Therefore, reinvestigation of this question is required before any conclusions can be drawn;

3. Competition between leukaemic cells and normal haemopoietic cells for specific sites. Studies of Prins and Van Bekkum (1979) have shown that BNML cells tend to concentrate at sites near the endosteum following i.v. inoculation. These are exactly the locations identified by Shackney (1975) and Lord et al. (1972; 1975) as the optimum microenvironment for stem cell proliferation. According to Shackney, the haemopoietic cells at those sites migrate to the central parts of the bone marrow as they differentiate, although this is disputed by Maloney et al. (1978). The observations of Prins and Van Bekkum could explain why a severe suppression of haemopoiesis occurs relatively early in AML. Apparently, the leukaemic clonogenic cells tend to occupy sites required by HSC. Since the leukaemic cells do not differentiate, they will not migrate to the central part of the bone marrow. This results in rapid blocking of all available sites for normal stem cells. The distribution pattern of BNML cells is different from that of the cells of a lymphocytic leukaemia model (the L4415) in the rat which was recently developed in the Radiobiological Institute. In this lymphocytic leukaemia, the malignant cells are randomly distributed over the bone marrow following i.v. inoculation (Prins, personal communication). These observations, if applicable to human leukaemia, could adequately explain the difference in suppression of normal haemopoiesis between AML and ALL.

The fate of CFU-s during the progression of the disease is not the same in the various transplantable leukaemia models. In the myelogeneous leukaemia in RFM mice, a decrease in the number of CFU-s is observed in bone marrow and spleen when the leukaemia progresses, although to a lesser extent than in BNML (Husseini, 1976). Hoelzer (1974) found in the L5222 leukaemia a reduction to less than half of normal values by determining CFU-c in bone marrow and no increase in CFU-c in peripheral blood. In the AKR transplantable leukaemia of mice, no changes in the numbers of CFU-s and CFU-c in the bone marrow have been observed at any stage of the leukaemia (Nakeff and Valeriote, 1978).

6.2 CFU-s REDUCTION AFTER i.v. ARA-C INJECTION COMPARED TO CONTINUOUS i.v. INFUSION

6.2.1. Introduction

In Section 5.2, different routes of ara-C administration were evaluated with respect to tumour load reduction. The myelosuppressive side effect of the two most effective schedules and routes of ara-C administration will be described in this section. These studies were initially performed in normal rats, since the normal haemopoiesis in leukaemic animals is already very severely depressed at the time the chemotherapy studies are started (in general, day 15 after inoculation). Further reduction cannot be estimated with an acceptable degree of accuracy. Nevertheless, measurements were also performed in treated leukaemic animals (see 6.3) and compared with the results of the present experiments in normal rats. The reduction in the total femoral cellularity as well as the changes in the numbers of the haemopoietic stem cells have been monitored by using the CFU-s assay. The chemotherapeutic schedules studied were: 1) treatment with two i.v. ara-C injections (200 $mg.kg^{-1}$) with a 12-h interval; 2) 24-h continuous ara-C infusion with a total dose of 400 $mg.kg^{-1}$.

6.2.2. Results

The reductions in the number of nucleated cells per femur and in the CFU-s during and after ara-C infusion are shown in Fig. 6.2.

During the 24 hours that the rats received ara-C infusion, the total cell number shows a smooth decline (Fig. 6.2). The CFU-s curve also shows a declining pattern; at 10 hours after termination of infusion, the total reduction in CFU-s is found to be 70%.

After ara-C interval treatment, the number of cells per femur also shows a decrease (Fig. 6.3). The number of CFU-s is reduced by about 25% two hours after each ara-C injection but no further decrease occurs during 4 to 12 hours thereafter. The maximum CFU-s reduction observed is about 50 % after two injections of ara-C.

6.2.3. Discussion

Both the continuous ara-C infusion and the interval treatment result in an equal reduction in the femoral cell number of about 50 %.

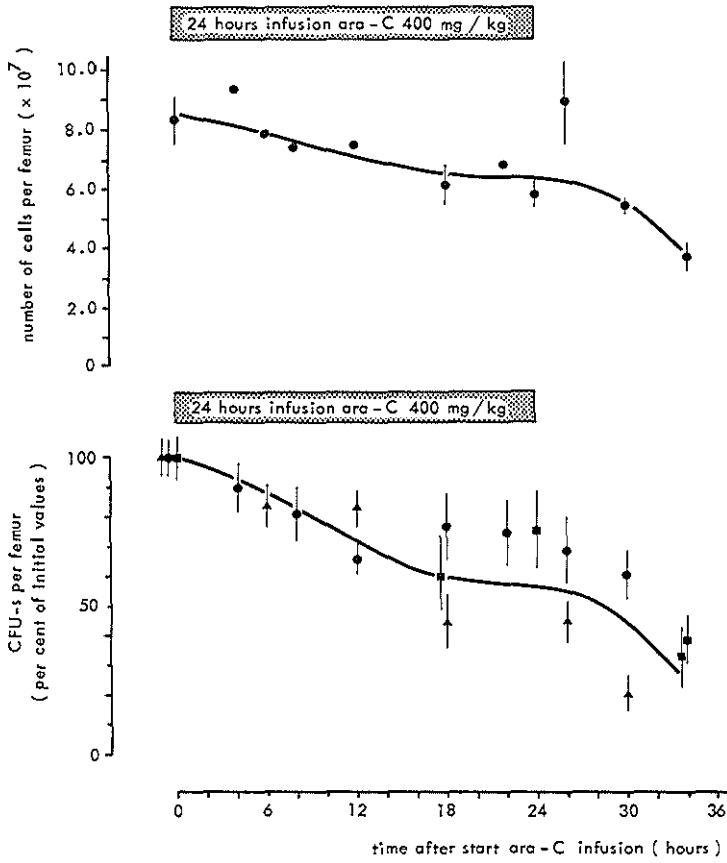

Figure 6.2

Changes in the femoral cellularity and number of CFU-s during and after ara-C infusion of 400 mg.kg^{-1} during 24 h. Different symbols represent means of three different experiments (± SE).

In normal haemopoietic bone marrow cells, ara-C exerts its cytotoxic action on proliferating cells of the stem cell compartment and the immature stages of the erythroid, myelocytic and megakaryocytic cell lines.

As previously discussed (see Section 1.2), a very small fraction of the reduction in the number of femoral cells is due to stem cell loss. Since the stem cell compartment has to restore the total haemopoietic organ after intensive cytostatic therapy, the relatively small differences observed after one day of treatment are important. It can be expected that these differences become more pronounced when the treatment is continued for longer periods of time.

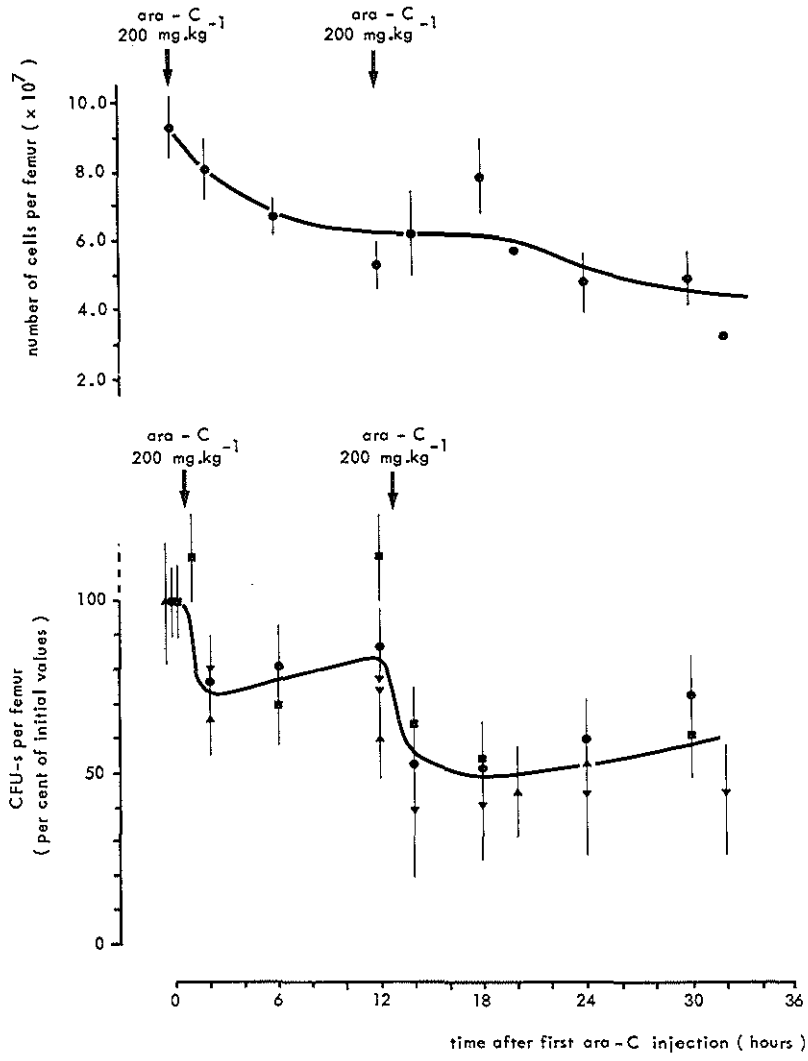

Figure 6.3

Changes in the femoral cellularity and number of CFU-s during interval treatment with ara-C 200 mg.kg^{-1} per injection. Different symbols represent either one or means (± SE) of 2-4 different experiments.

Frei et al. (1969) have described a steep dose response curve for marrow toxicity in humans when ara-C was given as a continuous infusion. Toxicity increased if the duration of the infusion period was prolonged. No severe bone marrow toxicity was observed (even at doses as high as 4200 $mg.m^{-2}$) when the drug was given as a rapid i.v. injection.

Taking into account the more pronounced reduction in tumour load by interval treatment as described in Chapter 5.3, all available evidence indicates that rapid i.v. interval treatment is therapeutically superior in terms of both antitumour activity and less haemopoietic toxicity.

6.3 QUANTIFICATION OF CFU-s AND FEMORAL CELLULARITY AFTER TREATMENT WITH ARA-C AND ADRIAMYCIN

6.3.1. Introduction

In Chapter 5, it was shown that 6x ara-C followed by one injection of adriamycin had a remarkable effect on the leukaemic cell load: a reduction from $5x10^9$ to 10^3 cells could be calculated. As far as the effect of this intensive chemotherapy on the total number of CFU-s is concerned, it appeared that the normal haemopoiesis could be completely restored by the surviving stem cell fraction. The aplastic period after treatment could be alleviated by only four blood transfusions. Since the number of CFU-s in the transfused blood from normal rats is negligible (Fig. 6.1), it follows that a sufficient number of CFU-s must have been preserved in the treated animals to allow autoregeneration of haemopoiesis.

In this section, the individual effect of the two cytostatic drugs on the stem cell pool will first be described. The reduction in the number of CFU-s calculated from these results will be compared with the observed surviving CFU-s fraction in normal and leukaemic rats treated with the complete schedule.

6.3.2. Effect of ara-C on CFU-s and femoral cellularity

The effect of increasing ara-C doses on femoral cellularity and the number of CFU-s in normal rats is illustrated in Fig. 6.4. The animals were sacrificed two hours after drug injection. This interval was based on the time course of changes in CFU-s numbers after ara-C

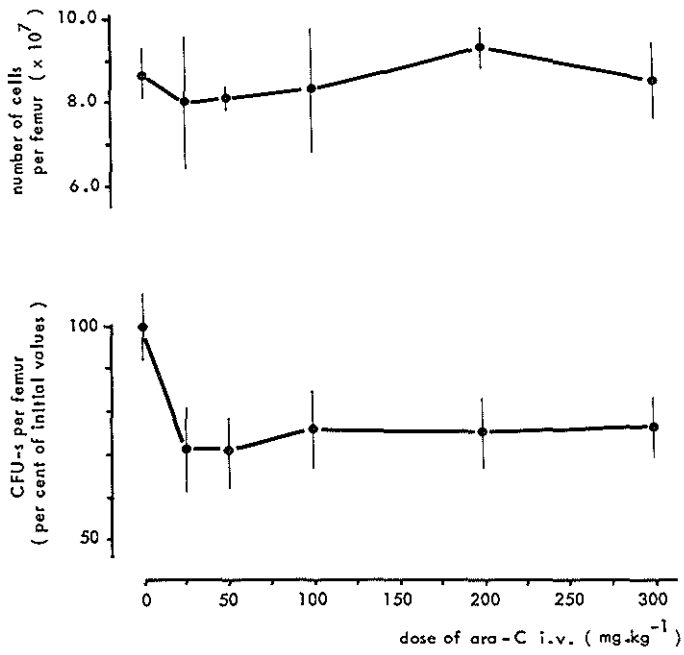

Figure 6.4

Effect of different single doses of ara-C on femoral cellularity and number of CFU-s. Each point represents means ± SE of 2 experiments. The animals were sacrificed 2 h after drug injection.

as shown in Fig. 6.3. The CFU-s curve shows a plateau for increasing drug dose; this shape is characteristic for cell cycle phase specific drugs (Bruce et al., 1966; 1968; Van Putten, 1974; Hill et al., 1975). Maximal CFU-s reduction is achieved with ara-C doses as low as 25 $mg.kg^{-1}$. A reduction of 20-30 % in the CFU-s population has been observed. This plateau curve was not obtained when the effect on femoral cellularity was measured.

6.3.3. Effect of adriamycin on CFU-s and femoral cellularity

The adriamycin dose applied in this study was extrapolated from the clinical dose used in AML protocols, specifically the Ad-OAP schedule (Fig. 3.2). From the pharmacokinetic data (see Chapter 2.9.2.d), it was expected that adriamycin would exert its activity for 24 h after administration. For this reason, the animals were killed 24 h

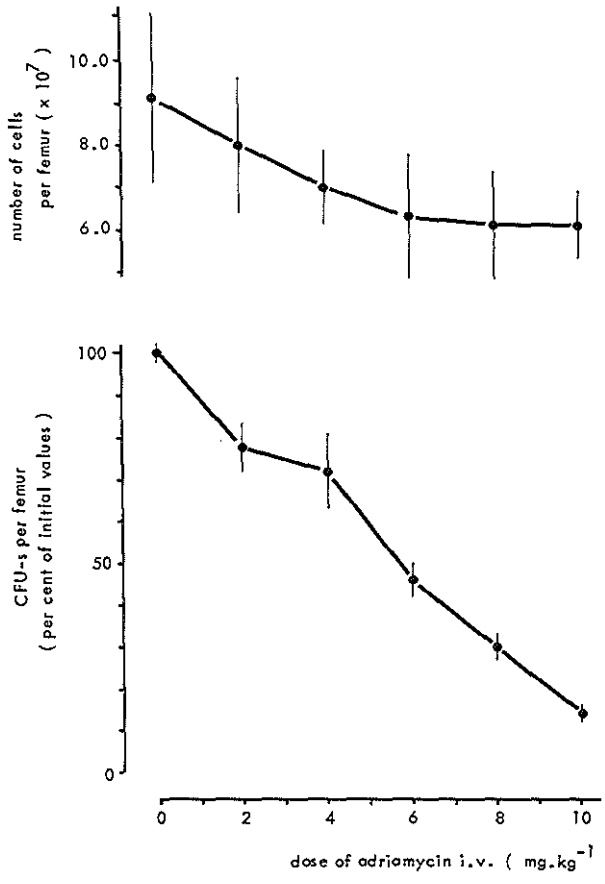

Figure 6.5

Effect of different single doses of adriamycin on femoral cellularity and number of CFU-s. Each point represents means ± SE of 2 experiments. The animals were sacrificed 24 h after drug injection.

after injection. The dose response curve (Fig. 6.5) indicates a dose dependency for CFU-s reduction: increasing the dose results in more pronounced CFU-s kill. This general nature of the relationship is not clear for the effect on femoral cellularity.

Changes in CFU-s numbers determined between 2 to 30 h after injection of the "standard 7.7 mg.kg^{-1}" dose adriamycin show a two-phase response (Fig. 6.6): an initial steep decline during the first two hours, resulting in a 60 % CFU-s reduction, and a much slower decline in the following 28 h. At 30 h after injection, only 10 % of the ini-

tial number of CFU-s is left in the bone marrow. The reduction in femoral cellularity is much less pronounced; at 30 h after injection, about 50 % of the initial cell number was preserved.

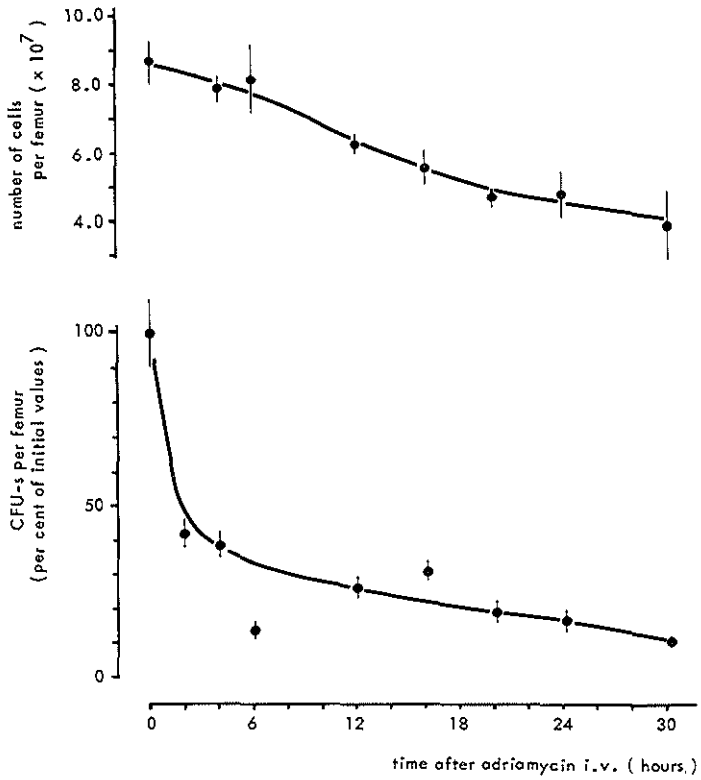

Figure 6.6

Effect of a single injection of adriamycin (7.7 mg.kg^{-1}) on femoral cellularity and number of CFU-s during 2 to 30 h after injection. Each point represents means ± SE of 2 experiments.

6.3.4. CFU-s reduction after repeated ara-C injections followed by adriamycin in normal and leukaemic rats

The pattern of the CFU-s reduction after two injections of ara-C separated by a 12-h interval (Fig. 6.3) suggests that each ara-C injection results in a constant fractional CFU-s reduction of 25% per injection. Fig. 6.7 shows the decline in CFU-s after 6 injections of

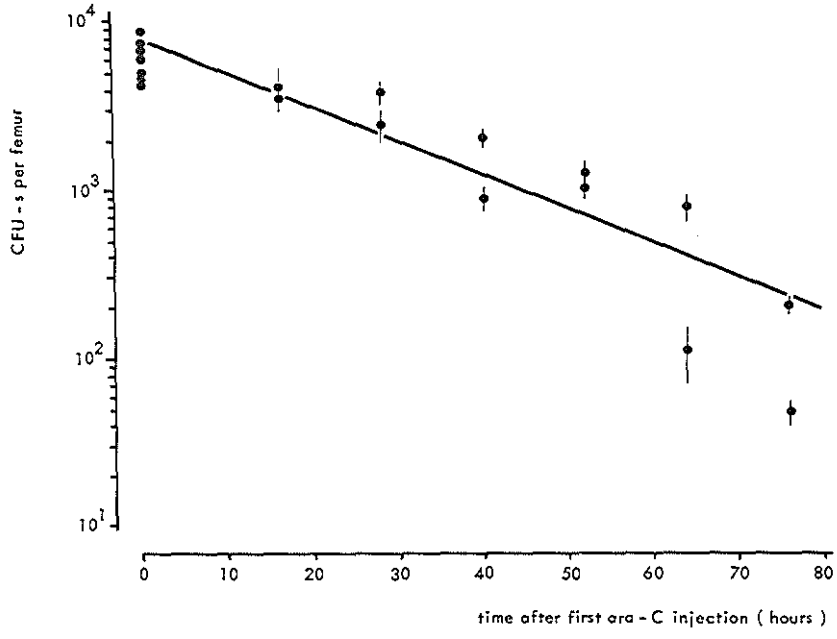

Figure 6.7

Reduction of the number of CFU-s after 6 injections of ara-C (200 mg.kg^{-1} each) given with 12 h interval. The animals were sacrificed 4 h after the last drug injection.

ara-C given 12 h apart in normal rats. Four hours after the last injection, only 3 % of the original CFU-s was left.

To evaluate the effect of therapy on the total numbers of stem cells, calculations were made in which the number of CFU-s determined in the several tissues was used to ascertain the total number of HSC per tissue. The initial total number of HSC per female rat can be calculated by using Fig. 6.1, which gives the number of CFU-s in normal rats (day 0) in bone marrow, blood and spleen. The cells per femur (8.5×10^7) represent 2.3 % of the total number of bone marrow cells per rat (P. Sonneveld, 1980). For every 10^7 nucleated cells, 750 CFU-s are detected. To calculate the absolute number of stem cells in the bone marrow, one has to make a correction with the f factor, which has been determined for the rat (Van Bekkum, 1977a) to be 0.007. Total number of CFU-s in bone marrow:

$$8.5 \times 10^7 \times \frac{100}{2.3} \times \frac{750}{10^7} \times \frac{1000}{7} = 400 \times 10^5$$

The number of CFU-s circulating in the blood can be calculated as follows: 13 CFU-s were detected per 10^7 nucleated cells. The blood volume of a female rat is 8 ml, with a nucleated cell count of 10×10^6 .ml^{-1}. Total number of CFU-s in blood:

$$8 \times 10 \times 10^6 \times \frac{13}{10^7} \times \frac{1000}{7} = 0.15 \times 10^5$$

In the normal rat spleen weighing 0.4 gram (1 gram of tissue contains 10^9 cells), 8 CFU-s were determined per 10^7 spleen cells. The total number of CFU-s in the spleen is calculated as follows:

$$0.4 \times 10^9 \times 8 \times \frac{1000}{7} = 0.46 \times 10^5$$

These values are listed in Table 6.2.

For determining the number of HSC at day 20 after inoculation of 10^7 leukaemic cells, similar calculations were made by using the numbers of CFU-s determined in the various tissues (Fig. 6.1).

The results of CFU-s determinations in bone marrow of normal and leukaemic rats which were treated with the schedule 6 x ara-C/1x adriamycin are presented in Table 6.2. The number of stem cells detected in normal rats after treatment with this complete schedule agrees well with the calculated additive effect of 6x ara-C (3% residual CFU-s) and of one injection of adriamycin (a reduction of 90%), namely, $0.1 \times 0.03 \times 400 \times 10^5 = 1.2 \times 10^5$.

During the 5 days of treatment with the schedule 6x ara-C/1x adriamycin, the HSC in the leukaemic animals almost disappeared from the blood (reduction factor 7000) and the spleen (reduction factor 820), while the number of HSC in the femur was similar to that in untreated animals at day 20. It may be assumed that the number of CFU-s at day 15 was substantially higher. Thus, some reduction of CFU-s in the bone marrow was induced by the chemotherapy.

In normal rats, the treatment schedule reduces the HSC in bone marrow by a factor 400, in blood by a factor 200 and no HSC can be detected in the spleen after treatment.

For 100 % protection of a lethally irradiated rat, the transplantation of 3×10^7 injected bone marrow cells.kg^{-1} is needed (Van Bekkum, 1977a). For a female rat of 200 gram, this amounts to 6×10^6 bone marrow cells containing 4×10^4 stem cells. The number of stem cells left in both normal and leukaemic rats after the schedule 6x ara-C/1x adriamycin is about a factor 2 or 3 more than the numbers needed for restoration by grafting after lethal irradiation.

TABLE 6.2

NUMBERS OF HSC IN NORMAL AND LEUKAEMIC RATS (DAY 20 AFTER INOCULATION) AND NUMBERS OF HSC LEFT AFTER THE SCHEDULE 6x ARA-C/1x ADRIAMYCIN

	untreated groups		after treatment with 6x ara-C/1x adriamycin	
	CFU-s detected	total stem cells	CFU-s detected	total stem cells
	NORMAL RATS		NORMAL RATS	
Bone marrow	one femur, 750 per 10^7 cells	400×10^5	one femur, 19 per 7.3×10^6	1.2×10^5
Blood	13 per 10^7 cells (1 ml)	0.15×10^5	0.6 per 3.0×10^6 cells (1 ml)	0.007×10^5
Spleen	8 per 0.4×10^9	0.45×10^5	not detectable	
		400.6×10^5		1.2×10^5
	LEUKAEMIC RATS (day 20)		LEUKAEMIC RATS (treatment started at day 15)	
Bone marrow	one femur 6 per 10^7 cells	1.3×10^5	one femur, 16 per 4×10^6	1.0×10^5
Blood	1000 per 10^7 cells	70×10^5	1.0 per 1.2×10^6 cells (1 ml)	0.01×10^5
Spleen	230 per 10^7 cells	82×10^5	54.6 per 18.2×10^8 cells	0.1×10^5
		153.3×10^5		1.1×10^5

The numbers of HSC in the untreated normal and leukaemic rats were calculated from the CFU-s numbers given in Fig. 6.1 (see text).
The treated rats were sacrificed 24 h after the last drug injection.

6.3.5 Discussion

The bone marrow is the most critical normal tissue during remission induction with ara-C and adriamycin. The damage to normal haemopoiesis is best evaluated by determining the number of CFU-s. Because of its S phase specificity, ara-C can be expected to cause relatively less damage to CFU-s, since a larger proportion of those cells are in G_0.

Ara-C has been shown to be a very effective drug for the treatment of the BNML because it induces recruitment of the leukaemic cell population. As discussed earlier, this effect is of advantage only when the normal haemopoietic stem cells are not recruited to the same extent.

The observed reduction in CFU-s after increasing ara-C injections as shown in Fig. 6.7 is compatible with a constant reduction after each injection. If recruitment had occurred, the fractional kill would have increased upon increasing the number of injections and this should have resulted in a downward bending of the regression line. The slope of the calculated linear regression line indicates a fractional reduction of 43% $\pm$ 3.4 (mean $\pm$ S.E.) after each ara-C injection. This is significantly ($p < 0.01$) different from the reduction determined after one injection (25% $\pm$ 6; Fig. 6.3) whereas the reduction after the second injection (38 $\pm$ 4%) does not differ significantly ($p > 0.05$) from that after the first (25 $\pm$ 6%). Even if the fractional reduction of 43% indicates that some recruitment of CFU-s occurs, it is much less than the degree of recruitment observed in the leukaemic population as described in the previous Chapter.

The time course of the effect of adriamycin on the CFU-s shows a continuous slow restoration during the period between 2 and 30 h after injection of 7.7 $mg.kg^{-1}$ adriamycin (Fig. 6.6). This can be explained on the basis of the pharmacological characteristics of the drug. The long half-life of adriamycin results in persisting tissue levels, with the consequence of prolonged cytotoxic activity. For this reason, the animals were always sacrificed 24 h after drug injection in experiments involving adriamycin administration. The dose dependency determined under these conditions is characteristic for cycle specific drugs. This suggests that adriamycin has similar toxicity for normal CFU-s and leukaemic cells. This has to be taken into special consideration when the haemopoietic compartment is already critically reduced.

During the development of the leukaemia, the total number of HSC decreases from 400×10^5 to 153×10^5 in the terminal phase of the disease, which is a reduction of only a factor of 2.5 (Table 6.2).

Yet, haemopoiesis is already severely suppressed relatively early in the development of the disease, resulting in anaemia, thrombocytopenia and low numbers of normal white blood cells (Hagenbeek, 1977a). This suggests that extramedullary haemopoiesis is a very ineffective process, possibly because the microenvironment outside the bone marrow is less favourable.

From the results presented in Table 6.2, it can be seen that a considerable number of HSC is preserved in the bone marrow of the treated leukaemic rats, whereas the number of HSC in the extramedullary tissues is greatly reduced. To what extent migration from these extramedullary sites to the bone marrow occurred during the five days of treatment is not known. It is likely that the empty bone marrow shaft - due to leukaemic cell kill - is an optimal microenvironment for restoration of the normal haemopoiesis.

CHAPTER 7

GENERAL DISCUSSION

The suitability of the BNML as a model for human AML has been described by Hagenbeek (1977a; see 1.5). In this study, it has been shown that, from a chemotherapeutic point of view, the BNML is much more responsive to a therapy schedule which is successfully applied in AML than to an ALL schedule (Chapter 3). The beneficial effect of the AML schedule has been attributed to the drugs ara-C and adriamycin (see 3.4). For these reasons, the efficacy of these agents were further investigated. To gain insight into the properties of the drugs, the effect of the single agents on cell kinetics, their tumour reduction capacity and toxic effects on normal haemopoiesis were investigated. The results of these studies have been discussed in detail in Chapters 5 and 6. In this Chapter, the two drugs ara-C and adriamycin will be compared with each other with respect to their efficacy in tumour load reduction and their toxic effects on normal haemopoiesis.

The effect of ara-C and adriamycin on the leukaemic population and normal haemopoietic tissue is shown in Table 7.1. As discussed earlier, phase specific drugs are characterized by a plateau curve for dose effect relationships. It can be concluded that both ara-C and adriamycin act as phase specific drugs as far as the femoral cellularity is concerned. However, when the effect of these drugs on normal haemopoiesis is studied, there are differences depending on the endpoint employed: CFU-s or femur cellularity. The characteristics of ara-C fit those of a phase specific agent with both endpoints, but the action of adriamycin resembles that of cycle specific agents for the CFU-s endpoint. On the other hand, adriamycin seems to behave in a phase specific manner when the femoral cellularity is concerned. However, it should be realized that the CFU-s assay is a more sensitive parameter for measuring haemopoietic toxicity as compared with monitoring femoral cellularity.

Summarizing the effect of ara-C on leukaemic and normal cells, it can be concluded that this is a strictly phase specific drug. Due to the increased reduction in the number of leukaemic cells by means of recruitment, the therapeutic index of ara-C interval treatment is > 1.

TABLE 7.1

COMPARISON OF THE EFFECTS OF ARA-C AND ADRIAMYCIN ON LEUKAEMIC AND NORMAL HAEMOPOIETIC TISSUE

	leukaemic bone marrow cells		normal bone marrow cells			
	femur counts		femur counts		CFU-s	
treatment	nature of the curve	per cent reduction	nature of the curve	per cent reduction	nature of the curve	per cent reduction
Ara-C 200 $mg.kg^{-1}$ time course	plateau	30	plateau	20	plateau	25
Ara-C dose-effect relationship 50 - 300 $mg.kg^{-1}$	plateau	20 *	no reduction		plateau	27
Adriamycin 7.7 $mg.kg^{-1}$ time course	plateau	30	linear regression	55	biphasic	90
Adriamycin dose-effect relationship 2 - 10 $mg.kg^{-1}$	plateau	25 *	plateau	33	linear regression	85

*unpublished observation

The effect of adriamycin on a leukaemic population previously recruited and synchronized by ara-C is identical with that of ara-C, namely, a 1 decade reduction. However, the cell killing effect of adriamycin to normal stem cells is also 1 decade, so that the therapeutic index for this drug is about 1. These findings indicate that attempts at eradication of AML by using high doses of adriamycin should be made only when its toxicity to the haemopoietic stem cells can be moderated by haematologic supportive care or bone marrow transplantation.

7.1 RELEVANCE OF THE BNML STUDIES FOR CLINICAL AML

Several results presented in the previous chapters seem to justify further clinical evaluations. These are:

1) the rationale of using prednisone in combination schedules for remission induction in AML;
2) the differential therapeutic value of various routes of ara-C administration;
3) the capacity of ara-C to induce recruitment of resting leukaemic cells in human AML.

The first two points have received extensive comment in Chapter 3 (3.4) and Chapter 5 (5.2) respectively, and only general impressions emerging from these studies will be discussed here.

In general, remission induction schedules can be divided into protocols with prednisone (McCredie et al., 1976; Spiers et al., 1977a; Ohno et al., 1975) and protocols without prednisone (Gale et al., 1977; Preisler et al., 1977). The clinical results obtained with the first mentioned protocols do not seem to be consistently different from those of the second (without prednisone). Since the results in the BNML have shown that this drug causes an accumulation of leukaemic cells in the liver, a clinical reevaluation of prednisone is indicated.

Concerning the route of ara-C administration, our experiments have shown that s.c. and i.m. administration are inferior to the i.v. route. Besides, rapid i.v. injection was more effective than a continuous infusion over 24 h. Subsequent cell kinetic analysis provided a solid biological basis for this observation.

The problem in the chemotherapy of AML is that a substantial proportion of the leukaemic cells are in G_0 and therefore not susceptible to the cytotoxic action of phase specific agents. Ara-C has been

shown to possess the particular property of recruiting cells from G_0 into cell cycle. This recruitment can be exploited to bring resting leukaemic cells into cell cycle and thereby make them susceptible to a subsequent dose of ara-C or other phase specific drugs. When second and subsequent ara-C injections are given at the right time interval, that is, during the peak of recruited and synchronized S phase cells, an optimal cell kill is obtained. However, the time interval between ara-C administration and the peak of S phase cells cannot be extrapolated easily from the rat model to the human patient, in view of differences in cell cycle parameters between the BNML and human AML. The most simple approach to solve this problem is to analyze leukaemic cell populations by means of flow cytometry at repeated intervals after high dose i.v. ara-C injection. The generally applied ara-C dose in the BNML was 200 $mg.kg^{-1}$ which equals 30 $mg.kg^{-1}$ for humans (Freireich et al., 1966). Although recruitment has also been observed for a lower ara-C dose of 100 $mg.kg^{-1}$ (for the ara-C dose of 50 $mg.kg^{-1}$, recruitment was questionable), the general tendency in this study was (Table 4.1) that the higher the ara-C dose, the more pronounced the recruitment phenomenon. The commonly applied ara-C dose in leukaemic patients is 100-200 mg per square meter; this equals 3-5 $mg.kg^{-1}$. This dose may be far too low to induce recruitment.

Data from the literature concerning the cell cycle parameters in human AML have shown substantial variations in the cell cycle time. Values between 20-152 h have been reported in different studies (Greenberg et al., 1972; Killmann, 1968; Ogawa, 1967; Ota, 1964). Some of these variations may be due to inadequacies of the methods employed such as overestimation of cycle times by using mean grain count disappearance curves (Greenberg et al., 1972). This method is valid only if all the daughter cells are in cell cycle, as is the case in a rapidly proliferating cell population. This requirement does not seem to be fulfilled in AML. By using the double labelling technique, the variations observed in cell cycle time were much less: 30-50 h (Gavosto et al., 1968; Greenberg et al., 1972). These values are slightly longer than those observed for the cell cycle time of normal myeloblasts: 30-38 h (Cronkite et al., 1960; Todo, 1968). The latter values are perhaps somewhat overestimated, since they were based on the rate of decrease in the mean grain counts.

The variations in cell cycle time of human AML cells are largely due to the variations in duration of the G_1 phase, i.e. 12-30 h in the patients studied by Greenberg and Gavosto. The duration of S phase was 14-16 h in the same studies. One approach to determine the optimal interval between successive ara-C injections for AML is to assume that

recruitment of G_0 cells in human AML occurs at comparable cell cycle related intervals as in the BNML. Using the results published for human AML with the double labelling technique, the minimal time interval necessary for cells to move from G_0 to half S is: G_1 + 1/2 S: 12 + 7 h = 19 h. The maximal period will be 30 + 8 = 38 h. When the same calculation is made for the BNML, the time interval G_0 to half S is: G_1 + 1/2 S = 0.8 + 5 h = 5.8 h. However, our experiments have shown that 12 h was the optimal interval in the rat leukaemia. Therefore, the above assumption does not prove to be useful. A better approach might be to relate the length of the optimal interval with the total length of the cell cycle. In the BNML, the optimal interval of 12 h is compared with the total cycle time of 14 h. Using this ratio, the calculated optimal interval for human AML lies between 12/14 x 30 h = 26 h and 12/14 x 50 h = 43 h. In clinical practice, it may be difficult to exploit recruitment most effectively in view of this variation in optimal intervals. Therefore, repeated bone marrow examination by means of flow cytometry after ara-C administration will be necessary, firstly, to reveal whether recruitment occurs and, secondly, to obtain more information on the optimal interval for administration of the second ara-C dose.

Our experience with the BNML has shown that this model lends itself excellently to the experimental approach of chemotherapy in AML because a number of dependable methods have been developed which allow accurate measurements of several interacting parameters of the leukaemic as well as of the normal haemopoietic cell population. Thus, the disturbances in each cell population caused by chemotherapeutic intervention can be quantitatively evaluated. The experiments described above were limited to two agents: ara-C and adriamycin. A number of observations were made which cast doubt on the way these drugs are being used in the clinic. Some possibilities of improved leukaemic cell kill have been postulated if the principles investigated can be applied to human AML. A systematic analysis of the action of other agents on cell cycle parameters and on cell population kinetics of the BNML may be expected to provide rationales for a more effective clinical use of current antileukaemic chemotherapy.

SUMMARY

In Chapter 1, the present situation concerning the treatment of human acute myeloid leukaemia (AML) is briefly described. Although complete remission is achieved in 60-75% of the patients, about 80% of these remissions result in a relapse within one year, which proves to be more difficult to treat therapeutically. The experimental approach to this problem was difficult, because the known leukaemia models in animals differ widely from human AML as far as the pattern of growth and some other factors are concerned. In recent years, a rat model for AML was developed in the Radiobiological Institute, Rijswijk, The Netherlands. It shows a much greater similarity to the human disease. The research described in this thesis was aimed at developing in this leukaemia model a rational basis for effective remission induction schedules.

The origin of the leukaemia in the BN rat and the generally used experimental procedures are discussed in Chapter 2. The evaluation criteria and the technique used for studying the cell kinetic parameters (flow cytometry) are extensively discussed. There follows a description of the biological and pharmacokinetic properties of the drugs investigated in this study (ara-C and adriamycin).

In Chapter 3, the sensitivity of the BNML to chemotherapeutic treatment is shown by comparing the effect of a clinical schedule for remission induction applied to patients with AML (the Ad-AOP regimen consisting of adriamycin, vincristine, ara-C and prednisone) with the effect of the remission induction schedule generally applied to patients with ALL (vincristine and prednisone). From the reduction in the numbers of cells in the peripheral blood, the weights of the liver and spleen and the survival time, the conclusion is made that the ALL schedule has little or no effect on the leukaemia. On the other hand, treatment with the Ad-OAP regimen resulted in a remarkable leukaemic cell reduction. In this respect, the response of the BNML is similar to that of AML. Because this schedule includes adriamycin and ara-C in addition to vincristine and prednisone, the favourable effect of the treatment must be attributed to the former drugs. Ara-C is known to

exert its specific toxic effect on the DNA synthesis phase (S phase) of the cell cycle. Because of this characteristic, the influence of ara-C on the distribution of the cells over the cell cycle was investigated in the BNML.

The theoretical background of cell kinetics and the methods available for studying cell kinetic parameters are reviewed in Chapter 4. Flow cytometric studies of bone marrow cells after 200 mg.kg^{-1} ara-C i.v. in the rat showed an initial decrease of cells in S phase. This was followed by an increase, reaching a plateau value of about 50% cells in S phase during 8-14 h after injection. The subsequent increase in cells in the G_2,M compartment indicates that the S phase cells continue the cell cycle in a synchronous way. The LI and MI studies after 200 mg.kg^{-1} ara-C agreed qualitatively and quantitatively with the flow cytometric measurements. This phenomenon was explained as follows. The initial decrease in cells in S phase is a result of the cytotoxic activity of ara-C on S phase cells. Calculation showed that the subsequent increase in these cells was due to an increased migration of cells from G_0 into the cell cycle (recruitment).

Analogous flow cytometric measurements after increasing ara-C doses ranging from 50-300 mg.kg^{-1} suggested that the recruitment phenomenon was most pronounced at the highest ara-C dose. In a subsequent study, it was investigated whether second and third injections of 200 mg.kg^{-1} ara-C at the time of maximal accumulation of cells in S phase would again induce recruitment. An accumulation of about 45% of cells in S phase was observed after the second ara-C dose at 12 h after injection. However, after a third ara-C injection, no additional increase of cells in S phase was measured. This is explained by the fact that the total number of leukaemic blast cells in the bone marrow had been reduced by about one decade by the previous ara-C injections; therefore changes in the percentage of blasts in a cell cycle phase could not be detected by flow cytometry.

In contrast to the very pronounced changes in the distribution of the cells over the cell cycle phases after ara-C injection, no cell cycle perturbations were observed in the DNA histograms of leukaemic bone marrow cells after injection of 7.7 mg.kg^{-1} adriamycin. However, the total number of leukaemic blast cells per femur was reduced by 30% after this injection. From this observation the conclusion could be drawn that adriamycin should be considered as a cycle specific drug, but later experiments showed that it acts on leukaemic blasts as a phase specific drug.

The effect of various routes of ara-C administration on leukaemic cell load is discussed in Chapter 5. The s.c. and i.m. routes of administration resulted in only a small tumour cell reduction, while i.v. interval treatment was the most effective. On comparing ara-C interval treatment with continuous ara-C i.v. infusion, the i.v. interval treatment was significantly more effective. In addition, it was investigated whether the observed recruitment and synchronization induced by one i.v. injection of ara-C resulted in increased leukaemic leukaemic cell kill when the second ara-C injection was given at the time of maximal accumulation of cells in S phase. By means of the sensitive LCFU-s technique the optimum interval for the second ara-C injection was determined; this proved to be 12 h, which coincides with the measured maximum of accumulated cells in S phase. The second ara-C injection given at that time results in a 90% reduction (one decade). The optimum interval and sequence for the combination ara-C and adriamycin were determined by means of the same LCFU-s assay. The most pronounced reduction in the number of LCFU-s was found when adriamycin was given 12 h after ara-C administration, namely, 90% or 1 decade. This study has also shown that adriamycin does not have a recruitment effect on leukaemic blast cells in G_0. Applying these data (i.e., interval between subsequent ara-C injections and for the combination ara-C/adriamycin: 12 h), schedules in which the specific property of ara-C, i.e., recruiting G_0 cells into cycle, was used to the utmost were established. The increase in the survival time was directly proportional to the number of ara-C injections. However, it must be emphasized that the final injection should not consist of ara-C, because of its very characteristic of recruiting cells. The theoretical calculation of the increase in survival time while assuming that each ara-C injection reduces the tumour load by 1 decade was in agreement with the observed survival time of 60 rats treated with the schedule 6x ara-C/1x adriamycin, all at intervals of 12 h. The number of leukaemic cells is reduced from 5×10^9 before treatment to 10^3. The short period of aplasia at the end of the treatment could be overcome with four blood transfusions given on alternate days.

Chapter 6 is concerned with how the normal stem cell (measured as CFU-s) is affected during the progression of the BNML. It was observed that, at the end of the second week after inoculation, the number of stem cells in the bone marrow was drastically reduced and that greatly increased numbers of CFU-s appeared at the same time in the blood and spleen. The total number of stem cells in bone marrow, spleen and blood was reduced from 400×10^5 to 150×10^5 in the terminal phase of the leukaemia.

Because of the drastic reduction in stem cells in the bone marrow at the very time that chemotherapy is normally started in the BNML (on day 15 after inoculation), it is impossible to evaluate the detailed effects of the cytostatics in leukaemic rats. Therefore, the effect of the individual drugs on the stem cells of normal rats was investigated. It was studied whether the normally applied routes of administration of ara-C, i.e., infusion and i.v. interval treatment, would have a different effect on the reduction in the number of stem cells. It proved to be impossible to infuse rats for more than 24 h. The reduction at h 34 after the start of a 24-h infusion of 400 mg.kg^{-1} ara-C is 70%, while the reduction at the same time after 2x dd mg.kg^{-1}, q = 12 h, is 50%. Although the changes in CFU-s numbers during these 34 h periods do not differ significantly in the two treatments, the pattern still strongly suggests a higher toxicity of the ara-C infusion which increases with the duration of the infusion.

Because the recruiting effect of ara-C on leukaemic blast cells yields a therapeutic gain only if the stem cells are not affected or are so to a much lesser extent, the number of stem cells was determined after a progressive number of ara-C injections up to 6x ara-C, q = 12 h. The reduction in the number of stem cells per ara-C injection calculated from the linear regression line is 43%. This is just significantly above the reduction after one injection (25 %). However, the reduction resulting from the second injection (38%) does not differ significantly from either of these values. The conclusion was drawn that ara-C possibly has a weak recruiting effect on normal stem cells, whereas it has a strong recruiting effect on leukaemic blast cells.

The reduction in the number of stem cells was subsequently determined in normal and leukaemic rats, both groups treated with the schedule 6x ara-C/1x adriamycin, q = 12 h. It appeared that about the same number of stem cells remained in the bone marrow of the normal and leukaemic rats, while the numbers in blood and spleen were reduced to nearly zero. The number of stem cells (ca. 1.2 x 10^5) remaining after this schedule was 2-3 times greater than the number which gives the BN rat 100% protection (i.e., 4 x 10^4) after lethal body irradiation.

Chapter 7 compares the effect of ara-C and adriamycin on the leukaemic population and on the normal haemopoietic stem cells. Ara-C acts on both leukaemic cells and normal stem cells as a phase specific drug. On the other hand, adriamycin acts as a phase specific drug on leukaemic cells, while affecting normal stem cells as a cycle specific agent (this is evidenced by the linear dose-effect relationship).

Furthermore, the Chapter deals with the clinical relevance of the BNML studies to AML. Three findings which need further clinical evaluation are successively discussed:

1. prednisone proved to be an ineffective agent in the remission induction schedule in the BNML;
2. the s.c. and i.m. routes of administration for ara-C appeared to have little effect as compared with the i.v. administration.
3. ara-C was found to induce recruitment in the BNML.

Clinical investigation of the recruiting property of ara-C requires repeated bone marrow punctures.

According to the literature, there is a wide variation in the cell cycle times in human AML. Therefore, individual therapeutic treatment is possibly required. By means of the recently developed flow cytometric technique, the optimum interval between ara-C injections could be determined. In the BNML, this interval proved to be 12 h. The combination of this fact with the data on the cell cycle times in AML reported in the literature, makes the interval of 12 h, which is generally applied in clinical treatment, suspect. An interval of 24 h might be more adequate.

SAMENVATTING

In hoofdstuk 1 wordt in het kort de huidige situatie beschreven betreffende de behandeling van acute myeloide leukaemie (AML) bij de mens. Ondanks het bereiken van complete remissie in 60-75% van de patienten blijft het feit dat 80% van de remissies binnen een jaar recidiveert en vervolgens slechter therapeutisch is te beinvloeden. De experimentele benadering van dit probleem werd belemmerd doordat de gangbare leukaemie modellen bij proefdieren sterke verschillen vertonen met de humane AML onder meer wat betreft het groeipatroon.

In de afgelopen jaren is in het Radiobiologisch Instituut een model voor AML in de rat ontwikkeld, dat een veel betere overeenkomst met de ziekte bij de mens vertoont. In het hier beschreven onderzoek is getracht met behulp van dit leukaemie model een rationele basis te vinden voor effectieve remissie inductie schema's.

De ontstaanswijze van de leukaemie in de BN rat wordt besproken in hoofdstuk 2, alsmede de algemeen gebruikte experimentele procedures. Er wordt uitvoerig ingegaan op de gebruikte criteria voor evaluatie, en de technieken voor het bestuderen van celkinetische parameters (flowcytometrie). De biologische en farmacokinetische eigenschappen van de in deze studie toegepaste farmaca (ara-C en adriamycine) worden beschreven.

In hoofdstuk 3 wordt de gevoeligheid van de BNML voor chemotherapeutische behandeling onderzocht door het effect van een klinisch schema gebruikt voor remissie inductie bij patienten met AML, het Ad-OAP schema bestaande uit adriamycine, vincristine, ara-C en prednison, te vergelijken met het effect van het bekende schema gebruikt voor remissie inductie bij patienten met ALL, vincristine en prednison. Op grond van celaantallen in het perifere bloed, het gewicht van lever en milt en de overlevingsduur, werd geconcludeerd dat het ALL schema bestaande uit vincristine en prednison, weinig of geen effect had op de leukaemie. Daarentegen had behandeling met het Ad-OAP schema een duidelijk effect op reductie van de leukaemische cellen. Ook in dit opzicht blijkt de BNML zich dus als een AML te gedragen. Omdat dit schema naast vincristine en prednison bestaat uit ara-C en adriamycine is

het gunstige effect toegeschreven aan deze laatst genoemde farmaca. Van ara-C is bekend dat het een zeer specifieke toxische werking heeft op een phase van de celcyclus, de DNA synthese fase (S phase). Deze eigenschap leidde tot het bestuderen van de invloed van ara-C op de celcyclus bij de BNML.

In hoofdstuk 4 wordt ingegaan op de theoretische achtergronden van celkinetische begrippen, alsmede op de methodieken die gebruikt kunnen worden om deze grootheden te bepalen.

Flow cytometrische metingen in beenmergcellen na een dosering van ara-C 200 $mg.kg^{-1}$ i.v. injectie in de rat hebben aangetoond dat na de initiële daling van cellen in S phase, er een stijging optreedt die gedurende 8-14 uur na injectie een plateau waarde heeft van $\pm$ 50% cellen in S fase. De hierop volgende stijging in het G_2,M compartiment laat zien dat de cellen synchroon de celcyclus vervolgen. Met behulp van LI en MI studies na ara-C 200 $mg.kg^{-1}$ is zowel kwalitatief als kwantitatief een overeenkomst waargenomen met de flowcytometrische metingen. Dit verschijnsel is als volgt verklaard. De initiële daling van de cellen in S fase wordt veroorzaakt door cytotoxische werking van ara-C op cellen in S fase. Dat de hierna waargenomen stijging van cellen in S fase berust op een verhoogde migratie van cellen uit G_0 in de celcyclus (recruitment) is aangetoond door berekening. Analoge flow cytometrische metingen na oplopende ara-C doses van 50 $mg.kg^{-1}$ -300 $mg.kg^{-1}$ suggereerden dat het recruitment effect het meest uitgesproken is bij de hoogste ara-C doses.

Vervolgens is nagegaan of een tweede en derde injectie ara-C 200 $mg.kg^{-1}$ toegediend op het moment van maximale accumulatie van cellen in S phase opnieuw in staat is recruitment te induceren. Een stijging van het aantal cellen in S phase tot ongeveer 45% werd gezien op 12 uur na het injiceren van de tweede ara-C doses. Echter, na een derde ara-C injectie werd geen stijging van cellen in S fase meer gemeten. Dit wordt verklaard door het feit dat het totaal aantal blasten in het beenmerg door de voorgaande ara-C injecties met ongeveer 1 decade is gereduceerd; hierdoor is met behulp van flow cytometrie geen verandering in het percentage blasten in een celcyclus fase meer aantoonbaar.

In tegenstelling tot de zeer uitgesproken veranderingen in de verdeling van de cellen over de celcyclus compartimenten na ara-C injectie, werden na een i.v. injectie van adriamycin 7.7 $mg.kg^{-1}$ geen veranderingen waargenomen in de DNA histogrammen van leukaemische beenmergcellen. Daarentegen was het totaal aantal blasten per femur na adriamycine injectie gereduceerd met 30%. Hieruit zou de conclusie getrokken kunnen worden dat adriamycine als een cyclus specifiek farmacon beschouwd moet worden; echter uit later onderzoek is gebleken dat

het op leukaemische blasten als een phase-specifiek middel werkt.

In hoofdstuk 5 is eerst nagegaan wat het effect van andere toedieningsroutes van ara-C op de hoeveelheid tumor cellen is. De s.c. en i.m. ara-C toediening resulteert in slechts een geringe reductie van de leukaemische cellen. De i.v. interval behandeling was het meest effectief. Ook vergeleken met een ara-C infuus, is het effect van interval behandeling significant gunstiger. Vervolgens is nagegaan of de waargenomen recruitment en synchronisatie geïnduceerd door een i.v. injectie van ara-C kan resulteren in versterkte leukaemische celafbraak als de tweede injectie van ara-C op het juiste tijdstip van maximale accumulatie in S fase wordt gegeven. Met behulp van de gevoelige LCFU-s methode is het optimale interval voor de 2e ara-C injectie bepaald, dit bleek 12 uur te zijn. Dit komt overeen met het gemeten maximum voor de accumulatie van cellen in S fase. Met behulp van dezelfde techniek zijn de optimale volgorde en het optimale interval voor de combinatie ara-C en adriamycine bepaald. De grootste reductie in the LCFU-s, n.l. 90% of 1 decade, is gevonden als adriamycine 12 uur na ara-C wordt gegeven. Tevens heeft dit onderzoek aangetoond dat adriamycine geen recruterende eigenschap heeft op leukaemische blasten in G_0 fase. Op basis van deze gegevens, i.e. optimale interval voor opeenvolgende ara-C injecties en voor de combinatie ara-C/adriamycine, is het effect op de hoeveelheid leukaemische cellen nagegaan, waarbij de recruterende eigenschap van ara-C optimaal werd gebruikt, i.e., herhaalde ara-C injecties met 12 uur interval. De overleving nam evenredig toe met het aantal ara-C injecties. Met nadruk moet worden gesteld dat de laatste injectie niet uit ara-C mag bestaan, juist vanwege zijn recruterende eigenschap. De theoretische berekening van de verlenging van de overleving wanneer elke ara-C injectie 1 decade leukaemische cellen doodt komt overeen met de waargenomen overleving van 60 ratten behandeld met het schema 6x ara-C/1x adriamycine, alle intervallen tussen de injecties: 12 uur. Hierbij is de hoeveelheid leukaemische cellen gereduceerd van 5×10^9 vóór behandeling tot 10^3. De kort durende periode van aplasie na deze behandeling kon worden overbrugd met 4 bloedtransfusies toegediend om de andere dag.

In hoofdstuk 6 wordt beschreven wat er gebeurt met de normale stamcel (gemeten als CFU-s) tijdens de ontwikkeling van de BNML. Hierbij is geconstateerd dat de stamcellen halverwege het ziekteproces in het beenmerg tot zeer geringe aantallen afnemen, en tegelijkertijd sterk verhoogde CFU-s aantallen worden gemeten in het bloed en de milt. Het totaal aantal stamcellen in beenmerg, milt en bloed is gereduceerd van 400×10^5 tot 150×10^5 in het eindstadium van de leukaemie. Omdat in het stadium van de BNML waarin begonnen wordt met

chemotherapie, de stamcellen juist drastisch verminderen in beenmerg, is het niet mogelijk gedetailleerde effecten van cytostatica te evalueren in leukaemische ratten dag 15 na inoculatie. Het effect van de individuele farmaca op de stamcel is daarom gemeten in normale ratten. Onderzocht is of er verschil is in de reductie van het aantal stamcellen voor de meest gebruikte toedieningsroutes van ara-C: ara-C infuus en i.v. interval behandeling. Het was niet mogelijk de ratten langer dan 24 uur aan het infuus te leggen. De reductie op tijdstip 34 uur na de start van een 24 uurs infuus van 400 mg.kg^{-1} ara-C is 70%, terwijl de reductie na 2x dd 200 mg.kg^{-1}, q = 12 uur op hetzelfde tijdstip na de start van de therapie gemeten, 50% is. Ofschoon het CFU-s verloop gedurende deze periode onderling niet significant verschillend is, is het patroon zeer suggestief voor de verhoogde toxiciteit van ara-C infuus, dat groter wordt als de duur van het infuus langer is.

Omdat het recruterende effect van ara-C op de leukaemische blasten alleen therapeutisch voordeel heeft als de stamcellen niet of in veel mindere mate gerecruteerd worden, is het aantal stamcellen gemeten gedurende oplopende ara-C injecties tot 6x ara-C, q = 12 uur. De uit de lineaire regressielijn berekende reductie van het aantal stamcellen per ara-C injectie is 43%; dit is juist significant meer dan de reductie na 1 injectie ara-C (25%). Echter de reductie van de 2e injectie ara-C (38%) is met beide waarden niet significant verschillend. Geconcludeerd is dat ara-C mogelijk een geringe recruterende werking heeft op de normale stamcellen, maar juist een sterke recruterende werking op leukaemische blasten.

Vervolgens is nagegaan wat de reductie van het aantal stamcellen is in normale en leukaemische ratten die behandeld zijn met het schema 6x ara-C/1x adriamycine, q = 12 uur. Hierbij is gebleken dat in het beenmerg van de normale en de leukaemische ratten ongeveer hetzelfde aantal stamcellen zijn overgebleven, terwijl het aantal stamcellen in bloed en milt in de leukaemische rat bijna verdwenen zijn. Het aantal stamcellen dat over is gebleven na dit schema ($\pm$ 1,2 x 10^5) is een factor 2 a 3 meer dan het aantal dat 100 % bescherming geeft na letha-le lichaamsbestraling (4 x 10^4) voor de BN rat.

In hoofdstuk 7 is de werking van ara-C en adriamycine op de leukaemische populatie en op de normale haemopoietische stamcellen vergeleken. Ara-C werkt zowel op leukaemische cellen als op normale stamcellen als een fase specifiek middel. Adriamycine daarentegen werkt als een fase specifiek middel op leukaemische cellen, terwijl het cyclus specifiek werkt op de normale stamcellen, blijkens de lineaire dose-effect relatie.

Vervolgens is in dit hoofdstuk uitvoerig ingegaan op de klinische relevantie van de BNML studies voor AML. Achtereenvolgens worden een drietal bevindingen besproken die een nadere klinische evaluatie behoeven:

1. Prednison bleek in the BNML een niet effectief agens in het remissie-inductie schema;
2. de s.c. en i.m. toedieningsroutes van ara-C bleken slechts weinig effectief in vergelijking met i.v. toediening van ara-C;
3. ara-C bleek in the BNML recruitment te induceren.

Klinische studies om dit laatste te onderzoeken vereisen herhaalde beenmerg puncties. Uit de literatuur is bekend dat de celcyclustijden bij menselijke AML varieert. Dit resulteert mogelijkerwijs in een individuele therapeutische benadering. Met behulp van de recentelijk ontwikkelde flow cytometrische techniek zou het optimale tijdsinterval voor de volgende ara-C injectie bepaald kunnen worden. In de BNML bleek het optimale tijdsinterval 12 uur te zijn. Dit feit gecombineerd met de uit de literatuur bekende gegevens omtrent de celcyclustijden bij AML maakt de huidige klinische toepassing van het 12 uurs interval suspect. Op grond van voornoemde gegevens lijkt een 24 uurs interval meer op zijn plaats.

ABBREVIATIONS

ALL	acute lymphocytic leukaemia
AML	acute myelocytic or myeloid leukaemia
ara-C	1-β-D-arabinofuranosyl-cytosine
BM	bone marrow
BNML	myelocytic leukaemia in the Brown Norway rat
CFU-c	colony forming units in culture
CFU-s	colony forming units spleen
CML	chronic myelocytic leukaemia
CNS	central nervous system
CR	complete remission
CV	coefficient of variation
GF	growth fraction
h	hour(s)
HSC	haemopoietic stem cell(s)
^{3}H-TdR	tritium labelled thymidine
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
LCFUs	leukaemic colony forming units spleen
LD_{50}	dose of the drug which killed 50% of the animals
LI	labelling index
MI	mitotic index
PLM	per cent labelled mitosis
q	interval
s.c.	subcutaneous
SD	standard deviation
SE	standard error
T_c	cell cycle time

ACKNOWLEDGEMENTS

I would like to express my gratitude to Prof.Dr. D.W. van Bekkum under whose direction the work described in this thesis was carried out.

I am especially indebted to Miss A. Töns for her skilled technical assistance, which was always given with enthusiasm and accuracy.

Special thanks are due to my friend Dr. A. Hagenbeek who introduced me into the field of experimental haematology. This thesis owes much to the stimulating discussions with him and Drs. P. Sonneveld, Dr. M. Aglietta and Drs. E. Prins, all of whom were participants in the experimental leukaemia project.

Without the extensive computer programming composed by Drs. G. Freriks, who also initiated me into the secrets of working with it, many of the calculations could not have been made.

I could always count on the expert advice and assistance of Drs. S.J. Bol, Dr. G.J. van den Engh, Dr. A.F. Hermens, Dr. J.M. Visser, Dr. G. Wagemaker and Mr. N. Vreeken whenever I strayed into their specialities.

I am grateful to Prof.Dr. J. Abels and Prof.Dr. C. Haanen, who critically evaluated the entire manuscript.

I am obliged to Dr. A.C. Ford for careful and painstaking reading of the manuscript and editing the English text.

The realization of this thesis has been greatly dependent on the help of Mrs. M. van der Sman and Miss D. van der Velden for typing the manuscript and that of Mr. J.Ph. de Kler for his expert drawings.

The stimulating activities of my colleagues at the REPGO Institutes are much appreciated.

The pharmaceutical company Upjohn is thanked for its generous gifts of the drug ara-C and Farmitalia for providing the adriamycin.

The work described in this thesis was performed at the Radiobiological Institute of the Organization of Health Research TNO, Rijswijk, The Netherlands. Without the financial support of this organization and the "Koningin Wilhelmina Fonds" of the Dutch National Cancer League, the work described in this thesis could not have been accomplished.

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CURRICULUM VITAE

After working for about two years as a medical technician, she completed secondary school (HBS-B) in 1968. This was followed by studies in medicine at the Vrije Universiteit in Amsterdam from 1968 to 1975. On completion of medical studies, she was awarded a fellowship by the Koningin Wilhelmina Fonds for a period of three and one-half years, during which experimental research was performed at the Radiobiological Institute TNO. In 1976, her paper entitled: "Experimental chemotherapy: a rat model for human Acute Myeloid Leukemia", was awarded a prize for young scientists and was therefore included in the program of Egon Lorentz Memorial Symposia, Fifth Annual Meeting, International Society for Experimental Hematology, Washington, DC. In 1979, she joined the Dutch Childhood Leukaemia Study Group as a co-worker. This thesis represents the investigations carried out at the Radiobiological Institute TNO.