EXTRACORPOREAL IRRADIATION OF THE BLOOD IN A RAT LEUKAEMIA MODEL

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PROEFSCHRIFT

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Aan mijn Zielevriend

...de meest ingrijpende ontdekking aller tijden:
„Ik kan het niet alleen"...

Aan Sonja, Bart en Thijs

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Rationale for the study

At present, the treatment of human haematologic malignancies consists of three major modalities, i.e., (1) radiotherapy, (2) chemotherapy, (3) immunotherapy or combinations of these. For acute leukaemia, great advances in terms of remission induction and survival time have been made during the past decade using various regimens of combination chemotherapy [Till et al., 1973; Beard and Hamilton Fairley, 1974; Penington and Keating, 1974; Vincent et al., 1974; Holland et al., 1975; Thomas et al., 1975; McCredie et al., 1976]. This is particularly true for acute lymphocytic leukaemia in children [Holland and Glidewell, 1972; Simone, 1974; Aur et al., 1974; Zippin et al, 1975; Frei et al., 1976]. In addition, data on the immunotherapy of acute leukaemia – although still largely experimental – seem to be promising, especially when the tumour load has been reduced by preceding chemotherapy [Mathé et al., 1970; Hamilton Fairley et al., 1970; Baker and Taub, 1972; Powles et al., 1973; Vogler and Chan, 1974; Rubens, 1974; Gutterman et al., 1974; Hersh et al., 1976]. Radiotherapy, consisting of low-dose total body irradiation [Johnson et al., 1967; Goswitz et al., 1973; Brace et al., 1974; del Regato, 1974; Johnson, 1976] or local irradiation of tissues such as the central nervous system [Aur et al., 1972; Willoughby, 1976; Dritschilo et al., 1976], the spleen and lymph nodes [Johnson, 1969; De Vita and Canellos, 1972; Peckham, 1974; Revol et al., 1974; Parmentier et al., 1974], has been applied in the treatment of various types of leukaemias and lymphoreticular neoplasms. Patients with acute leukaemia have also been submitted to high-dose whole body irradiation preceding bone marrow transplantation [Alvegard et al., 1975; Herzig et al., 1975; Gale et al., 1976; Thomas et al., 1976].

So far, combination chemotherapy has been proved to be the most effective treatment for human acute leukaemia. However, as known for many years, intensive treatment with cytostatic drugs has several limitations. Firstly, chemotherapy may have to be limited or even withdrawn due to severe toxic side-effects of cytostatics in leukaemic patients. The side-effects include depression of normal haemopoiesis [van Putten, 1974; Marsh, 1976] with subsequent bleeding and infections [Bodey, 1975] and damage to the gastrointestinal tract [Meyler, 1971] and the skin [Dreizen et al., 1975]. These may occur despite optimal supportive care provided by regular blood cell transfusions, antibiotics, barrier nursing, parenteral nutrition, etc. [Levine et al., 1974; McCredie et al., 1974; Bodey et al., 1976]. Secondly, a certain number of patients may show

complete unresponsiveness towards various chemotherapy schedules. Thirdly, chemotherapy is contraindicated in pregnant women. In this, fortunately, small group of patients, treatment with cytostatics should be avoided in order to protect the foetus against irreversible damage.

With these restrictions concerning chemotherapy in mind, extracorporeal irradiation of the blood (ECIB) was introduced as a possible therapeutic measure in the treatment of leukaemia [Lajtha et al., 1962]. This method consists of establishing a blood circuit outside the body in which the continuously flowing blood is irradiated. Physical treatment of the blood flowing through an extracorporeal shunt was actually first published by Heymans in 1921 (see Ch. 1.2). The rationale for this kind of treatment is twofold: (1) leukaemic cells (and normal lymphocytes)* are far more radiosensitive in comparison with the other blood cells, so that the blood can be selectively depleted of leukaemic cells without substantial injury to the normal blood elements; and (2) the treatment takes place completely outside the body, thereby preventing damage to normal tissues. However, in order to reduce the total leukaemic cell population within the body, a certain degree of exchange of leukaemic cells between the various tissues (bone marrow, spleen, etc.) and the blood is required. To achieve this absolute reduction, it is necessary that the growth rate of the leukaemic cell population is lower than its mortality rate under the influence of ECIB. A diminished tumour load thus induced would then be a good starting point for the induction of a remission by further chemo- or immunotherapy. Factors which generally determine the efficacy of ECIB are listed in table 1.1.

Table 1.1 *Factors determining the effectiveness of ECIB*

1. total number of leukaemic cells in the blood
2. total number of leukaemic cells in the tissue depots (bone marrow, spleen, lymphatic tissue, etc.)
3. rate of exchange of leukaemic cells between the tissue depots and the blood
4. radiosensitivity of leukaemic cells
5. rate of leukaemic cell production; and
6. life span of leukaemic cells

Besides its theoretical therapeutical usefulness, the ECIB technique might make a significant contribution to the study of kinetics of proliferation and distribution of leukaemic cells.

Information on cellular kinetics of leukaemia comes from various sources (table 1.2). Proliferation kinetics have been studied in detail using tritiated thymidine (^{3}H-TdR). This DNA precursor is specifically incorporated into

* The radiosensitivity of normal lymphocytes has led to other applications of ECIB – namely, to induce immunosuppression by means of lymphocyte depletion and to study lymphocyte kinetics in normal subjects (see Ch. 1.4.6).

Table 1.2 *Principal methods employed to study kinetics of proliferation and distribution in leukaemia*

1. *Proliferation and differentiation kinetics*
   tritiated thymidine labelling (*in vivo*, *in vitro*)
   pulse cytophotometry
   in vitro cultures of haemopoietic precursor cells in the blood and the bone marrow
2. *Distribution kinetics*
   autotransfusion of isotope-labelled leukaemia cells
   thoracic duct cannulation
   cross circulation
3. *Effects of cell depletion on 1 and 2*
   chemotherapy
   leukapheresis
   extracorporeal irradiation of the blood

cells during the DNA synthesis phase (S phase) of the cell cycle. After pulse labelling (*in vivo* and *in vitro*) or continuous infusion of ^{3}H-TdR (*in vivo*), autoradiography is employed and, from estimates of the labelling index and the per cent labelled mitosis, various parameters of leukaemia growth can be determined. These include the growth fraction, the generation times of leukaemic cells, the duration of the separate phases of the cell cycle (G_1, S, G_2, M), the transit time from the proliferative to the nonproliferative compartment, etc. [Gavosto et al., 1967; Killmann, 1968; Cronkite, 1968; Zimmerman et al., 1968; Clarkson et al., 1970; Theml et al., 1973; Mauer and Lampkin, 1974; Ernst, 1976).

The recently developed fast method of pulse cytophotometry has also provided quantitative information on the proliferation kinetics in leukaemia. By staining the DNA of leukaemic cells with specific fluorescent dyes, a DNA content distribution of the leukaemic cell population can be obtained. Mathematical analysis gives a rough impression of the per cent of cells present in the various phases of the cell cycle (G_0—G_1 : 2n DNA, S : 2n→4n, G_2 : 4n, M : 4n→2n), provided that the leukaemia is of a diploid nature [Büchner, 1974; Quaglino et al., 1974; Hillen et al., 1975].

Furthermore, *in vitro* culture of precursor cells from blood and bone marrow of patients or animals with leukaemia has yielded important information on the proliferation and differentiation capacities of leukaemic (stem) cells and normal haemopoietic (stem) cells at various stages of the leukaemic growth process [Robinson et al., 1971; Tanaka et al., 1973; Metcalf, 1973; Golde et al., 1974; Moore, 1974; Hoelzer et al., 1974a; van Bekkum et al., 1976a; Dicke et al., 1976; Spitzer et al., 1976).

Dynamics of leukaemia cell traffic between the tissues and the blood have been studied by means of autotransfusion of isotope-labelled leukaemia cells and studying their disappearance from the circulating blood and their distribution within the body. These studies have been performed with various isotopes

in acute leukaemia [Killmann et al., 1971; Hoelzer et al., 1972; Boranic et al., 1974], chronic myelocytic leukaemia [Athens et al., 1965; Galbraith, 1966; Chikkappa and Galbraith, 1967; Duvall and Perry, 1968] and chronic lymphocytic leukaemia [Pfisterer et al., 1967; Schiffer, 1968; Strijckmans et al., 1968; Spivak and Perry, 1970; Bremer et al., 1973a; Manaster et al., 1973; Neumann et al., 1974].

Studies involving measurement of leukaemia cell output from the thoracic duct after cannulation of this lymphatic vessel [Binet et al., 1967; Sinha and Goldenberg, 1970; Bremer et al., 1973] and cross-circulation experiments between normal and leukaemic subjects [Bierman et al., 1951; Hollingsworth and Finch, 1957; Eschbach et al., 1965] have also contributed significantly to the knowledge of the kinetics of distribution of leukaemic cells.

Another approach to the study of the kinetics and dynamics of leukaemia (using the parameters mentioned above) is that of inducing a decrease in the number of leukaemic cells present in the organism. Principally, two methods have been employed: general depletion by chemotherapy [Ernst and Killmann, 1969; Klein and Lennartz, 1974; Killmann, 1974] and specific depletion of the blood compartment of leukaemic cells by either cell removal [leukapheresis; Curtis et al., 1972; Vallejos et al., 1973; Hoelzer et al., 1974b; Hadlock et al., 1975] or by cell destruction such as with ECIB (see Ch. 1.4.7). These studies have yielded some valuable information on the presence of feedback mechanisms which operate to compensate for the cell loss. This might be expressed in terms of altering the degree of exchange of leukaemia cells between the tissues and the blood or changing their proliferation characteristics (recruitment of cells into cycle), thereby influencing the population size.

Studies on these parameters will widen our knowledge of the leukaemic growth process and they are therefore of utmost importance for the development of new, more effective therapeutic regimens. Where the parameters mentioned above have been investigated in this study, they will be discussed in more detail.

The effects of ECIB in acute leukaemia from both a kinetic and a therapeutic point of view will be discussed in this thesis. Apart from its radiobiological importance, this study seemed to be justified because of the lack of comprehensive studies in the literature, although several authors have reported on ECIB in various types of human acute (and chronic) leukaemia during the past 15 years (see Ch. 1.4.7). Because of its poorer prognosis in comparison with other types of leukaemia, it was thought to be most profitable to apply this technique in acute myelocytic leukaemia (AML). This view was also strengthened by the facts that: (1) so far, only a limited amount of kinetic information has become available from the rather few ECIB studies in human AML; and (2) there is a complete absence of experimental data on ECIB in AML in the literature. Therefore, the investigations were performed in an animal model

for AML as background support for the clinical situation. Such a model requires the availability of a reproducibly growing, transplantable leukaemia and the feasibility of creating an extracorporeal blood circuit. The easily manageable rat meets both of these prerequisites; it was therefore the animal of choice.

Another important factor in determining the nature of the present study was the availability of so-called "Leukocyte Mobilizing Agents" (LMA). These recently developed synthetic polyanions induce a rapid absolute increase in the number of lymphocytes and leukaemia cells in the peripheral blood after parenteral administration (see Ch. 3). Studies on this cell mobilization phenomenon were considered to be of utmost importance as regards its potential usefulness if combined with ECIB.

The phases of the present study will be outlined in more detail in paragraph 1.5. However, as a background for the present investigations, a short historical review on the development of ECIB will first be given. This will be followed by a description of the various techniques and parameters which are generally used. Subsequently, data on both experimental and clinical ECIB from studies which have appeared in the literature during the past 15 years will be reviewed.

1.2 Historical review on ECIB

As mentioned above, the idea to treat the circulating blood outside the body by physical means was developed by Heymans as early as 1921 [Heymans, 1921]. The main emphasis of his experiments in rabbits was to study the general effects of heating and cooling of blood flowing extracorporeally through a shunt between the carotid artery and the jugular vein. However, he also reported on X-ray and radium treatment of the blood in normal rabbits. After an irradiation period of 2 to 3 hours, no significant haematologic changes were noted. The radiation dose to the blood was not mentioned. He suggested then that ECIB might be a valuable treatment modality for leukaemia.

In view of the technical difficulties involved, only once per decade were data on ECIB published. In 1930, Dall'Acqua and Zopellari [Dall'Acqua and Zopellari, 1930] and, in 1939, Ducuing et al. [Ducuing et al., 1939] reported on ECIB with X-rays in normal experimental animals. They also did not obtain significant biological changes, possibly because of a combination of a limited irradiation time with a consecutive low irradiation dose. In 1957 and 1959, two preliminary reports of O'Brien and co-workers on X-irradiation of circulating blood in dogs and rabbits appeared [O'Brien et al., 1957; O'Brien, 1959]. Here again, no remarkable effects were observed. Arnould et al., applying ECIB with X-rays to anaesthetized rats with carotid-jugular shunts, found a transitory leukopenia and anaemia after 3 hours of ECIB [Arnould et al., 1958]. When this phenomenon was investigated in more detail using

high doses of γ-rays, it was found that the leukopenia was due mainly to the depression of the number of lymphocytes in the peripheral blood [Pellerin et al., 1960]. This initial rapidly induced decrease was soon followed by an increase of up to 2 times the pre-ECIB level within the first 5 days after irradiation. Thereafter, a quick return to normal levels was observed. The rapidly induced anaemia disappeared within 1 to 2 months, depending on the radiation dose.

The early workers in the area of ECIB had to contend with serious methodological difficulties. These prevented them from applying sufficiently high radiation doses to the circulating blood. A systematic approach to the effects of ECIB was initiated by Cronkite and his co-workers, using modern, more effective radiation facilities [Cronkite et al., 1962a; 1962b].

Before discussing the recent developments in experimental and clinical ECIB, it is useful to first summarize the various techniques and parameters used in ECIB.

1.3 Methods and parameters used in ECIB

The main methods and parameters playing a significant role in experimental and clinical ECIB will be summarized in this section; these are:

1. the extracorporeal blood circulation;
2. dosimetry;
3. irradiators; and
4. treatment schedules in ECIB.

1.3.1 The extracorporeal blood circulation

An arteriovenous shunt (carotid artery – jugular vein; femoral artery – femoral vein; radial artery – cephalic vein) is most commonly employed for the creation of an extracorporeal blood circuit. With such an arrangement, ECIB studies have been undertaken in various large animal species such as calves, cows and pigs [Cronkite et al., 1962a; 1962b; Chanana and Cronkite, 1966; Joel et al., 1967a], goats [Binet et al., 1968; Chanana et al., 1971], baboons [Storb et al., 1969], dogs [Wolf and Hume, 1965; Wolf et al., 1966; Pichlmaier and Trepel, 1966; Maginn and Bullimore, 1968; Kriek, 1973] as well as in rabbits [Heymans, 1921, Abramoff et al., 1961; Pompidou et al., 1975] and rats [Cauchi and Field, 1966; Pichlmaier and Trepel, 1966; Pellerin and Remy, 1968]. In calves, the arteriovenous teflon-shunts have been reported to function for up to 50 days [Chanana and Cronkite, 1966]. In clinical ECIB, exteriorized arteriovenous shunts as are used in haemodialysis have been employed [Quinton et al., 1962]. This type of shunt has led to considerable complications in terms of haemorrhages and local inflammation, especially in patients with leukaemia showing disturbances in the blood coagulation and decreased resistance to

infections [Chanana et al., 1968]. However, these risks have been greatly reduced by preparing subcutaneous arteriovenous fistulas (radial artery – cephalic vein). The venous part of the fistula hypertrophies under the influence of the arterial blood pressure and can be punctured repeatedly in order to create an extracorporeal blood circuit [Brescia et al., 1966]. These fistulas can function for several years [Zerbino et al., 1974].

The main factor which may limit the time during which a session of ECIB can be performed is the clotting of the blood within the extracorporeal circuit. To prevent this, regional or systemic heparinization is mostly indicated. However, the risk of blood clotting was greatly diminished when modern siliconized shunt material such as is used for chronic haemodialysis was introduced [Quinton et al., 1962]. Since then, teflon cannulas and silastic rubber coils have been mainly used. An additional advantage of silastic rubber is its high degree of radioresistance. Only after radiation doses in excess of 40 to 60×10^6 rad does the material become brittle and break easily [Cronkite et al., 1968].

Particularly with large extracorporeal volumes, heating of the external blood circuit to 37 °C was found to be necessary. Furthermore, several authors have used specially designed pumps in order to obtain a constant flow rate of the blood through the irradiation field; this is important from a dosimetric point of view.

Besides ECIB, extracorporeal irradiation of the lymph has also been carried out. Lymph flowing from the cannulated thoracic duct is led to a reservoir from which it is pumped through the irradiator back into a vein [Chanana and Cronkite, 1966; Joel et al., 1967b).

Several authors have developed alternative methods to irradiate the circulating blood. Instead of irradiating the blood extra-corporeally, small radiation sources were implanted inside the body close to the blood circulation. In dogs, shields or pellets containing the beta-emitter ^{90}Y were implanted into the abdominal aorta [Oldendorf et al., 1964; Wolf and Hume, 1965]; the insertion of a ^{90}Sr-^{90}Y source into the right atrium of the heart has also been reported [Barnes et al., 1965].

1.3.2 Dosimetry

For a better understanding of the specific dosimetric problems in ECIB, a schematic representation of the method is given in fig. 1.1. This simplified picture shows 3 major compartments which are interacting. The extracorporeal blood circuit contains only a fraction of the total blood volume circulating within the body. A certain fraction of the total number of leukaemic cells present in the circulating blood will be submitted to irradiation per unit time. At the same time, there will be a continuous exchange of leukaemic cells among

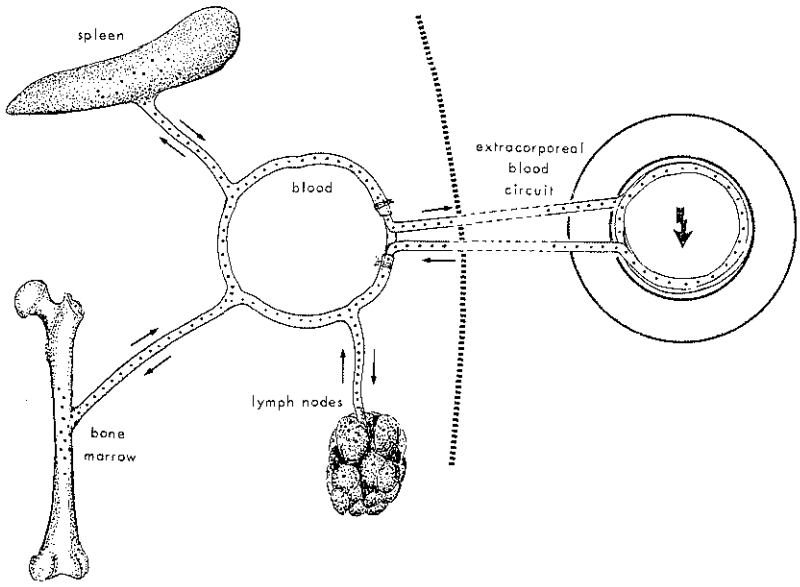

Fig. 1.1 *A schematic representation of extracorporeal irradiation of the blood.*

the various organs affected by leukaemia (e.g. the bone marrow, spleen, lymph nodes, etc.), and the blood, as will be discussed later (see Ch. 2.6.5).

The radiation dose delivered to the total leukaemic cell population during an ECIB session is dependent on 2 main factors: (1) the number of leukaemic cells passing through the irradiation field; and (2) the degree and rate of exchange of leukaemic cells among the various organs and the blood during ECIB.

The most accurate dosimetric unit is the transit dose, which is defined as the radiation dose received by a cell when passing once through the extracorporeal circuit. The transit dose is directly proportional to the volume of the coil which is submitted to irradiation and the dose rate of the radiator and inversely proportional to the flow rate of the blood in the external circuit, i.e.:

$$TD = \frac{V \times R}{F} \tag{1}$$

where TD = the transit dose (rad);
V = the volume of the irradiated coil (ml);
R = the dose rate of the radiator in the shunt tubing (rad/min);
F = the flow rate of the blood in the external circuit (ml/min).

Another useful parameter is the time needed for one blood volume to pass through the shunt: T_b (min.). This can be determined according to:

$$T_b = \frac{V_b}{F} \tag{2}$$

where V_b = the blood volume of the subject (ml).

As regards the amount and distribution of the radiation dose delivered to the total blood volume throughout the ECIB session, several mathematical approaches have been proposed by various authors. Irradiated cells returning from the small irradiated volume to the body will be distributed throughout the total blood compartment. The subsequent sample of blood flowing through the irradiator will therefore consist of both irradiated and nonirradiated cells. Obviously, the distribution of the radiation dose received by the cells present in the blood will change during an ECIB session, as can be calculated by methods of probability theory. Lajtha et al. (1962) discussed the simple exponential character of the process. From the Poisson distribution, it can be calculated that, when one blood volume (V_b) has passed through the radiation field, only 63% of the cells present in the blood volume will have been irradiated one or more times. If the irradiation time is prolonged, this percentage will increase: after 5 blood volumes have passed, 99% of the cells have received at least one transit dose and, after 7 blood volumes, 99.9%. After 14 blood volumes, only a fraction of 10^{-6} has not been irradiated and this is reduced to 10^{-12} after 28 blood volumes.

Extensive mathematical studies on the interrelationship between the volume of the extracorporeal circuit, the flow rate of the blood, the total irradiation time, the dose rate of the irradiator and the dose distribution in the total blood volume have been published. Slatkin et al. developed a computer program which enabled them to calculate the fraction of blood which is irradiated *n* times in each of several total number of passages [Slatkin et al., 1963]. Oliver and Shepstone, using Laplace transforms [Oliver and Shepstone, 1964], and Marsaglia and Thomas, tackling the probability problem with the renewal theory [Marsaglia and Thomas, 1965], also developed mathematical formulae to solve the problem of dosimetry. The "differential equation" model of Slatkin et al. and the "exact probability" model of Marsaglia and Thomas were found to give identical predictions [Karlsson, 1969].

When the irradiation time is sufficiently long (e.g., when more than 7 blood volumes have passed through the irradiator), the distribution of dose becomes symmetrical and a third significant parameter can be easily calculated: the mean dose to the total circulating blood ($D_{\bar{x}}$ in rad). This average is dependent on the volume of the irradiated coil (V in milliliters), the total irradiation time (T_t in min.), the dose rate in the shunt tubing (R in rad/min) and the total blood volume (V_b in milliliters):

$$D_{\bar{x}} = \frac{V}{V_b + V} \times T_t \times R \qquad (3)$$

It is clear that the calculation methods mentioned above are valid only for cells which remain within the circulating blood during the whole period of ECIB, i.e., erythrocytes. Although these cells are rather radioresistant (see Ch. 1.4.2), they still represent the main limiting factor as regards the total irradiation time. Dosimetric calculations concerning lymphocytes and leukaemic cells are considerably more complex, since these cell types are known to exchange continuously between the blood and the various organs (fig. 1.1). With respect to leukaemia, little is now known in quantitative terms about the parameters of this exchange process. Another important factor is the number of newly produced cells during the irradiation period. Information here is also lacking. However, various authors have attempted to simulate the problem mathematically based on speculative assumptions [Lajtha et al., 1962; Oliver and Shepstone, 1964]. Obviously, there is a great need to quantify the rate of exchange of cells between the tissues and the blood, to determine the average time a cell spends in the tissues and in the circulating blood compartment and to obtain information on the change in the number of leukaemic cells due to proliferation during ECIB. Studying these cellular kinetics of leukaemia was one of the major objectives of the investigations to be presented.

The parameters required to determine the radiation dose delivered to the leukaemic cell population during ECIB are summarized in table 1.3.

Table 1.3 *Parameters determining the radiation dose to the leukaemic cell population during ECIB*

1. transit dose
2. dose distribution in the total blood volume
3. mean dose to the blood
4. cellular kinetics of leukaemia

1.3.3 Irradiators

In general, irradiators used in ECIB must allow accurate dosimetry and should deliver a uniform radiation dose along the whole extracorporeal blood circuit. Different types of radiation (β, γ, X-rays, UV) have been used by various authors. The choice of a certain type of radiator depends on the demands of the investigator. The qualities of the different radiation modalities with respect to ECIB will now be briefly discussed.

A. Small β-emitters give only a very low amount of external scattered radiation (Bremsstrahlung). They therefore need very little shielding, which implies reasonable portability of the source because of its rather light weight. A special problem is presented by the fact that the β-radiation is remarkably attenuated

when traversing the shunt material. Furthermore, as a result of the short range of β-particles, there will be an uneven distribution of radiation to cells in different parts of the shunt cross-section. This problem can be overcome for the most part by a special shape of the radiator (e.g. cylindrical) and by winding the extracorporeal shunt to cause maximal turbulence of the flowing blood [for dosimetry: Slatkin and Robertson, 1970]. The most commonly used β-radiation source has been the radionuclide ^{90}Sr-^{90}Y, for which various radiation facilities have been developed [Gilbert and Lajtha, 1966; Libby et al., 1966; Pichlmaier et al., 1967; Holmer et al., 1973]. The applicability of other γ-emitting isotopes such as ^{204}Tl, ^{147}Pm, ^{125}I, and ^{170}Tm is presently being investigated [Griffin and Cross, 1973; Hungate et al., 1974]. Transit doses, as measured by the ferrous sulphate method, are in the range of 1–100 rad for the small portable sources described. If a greater TD is required, one can of course use β-units containing more curies. Heavy metals will then be necessary for adequate shielding, which will make the unit too heavy to be portable. In that case, it is from a dosimetric point of view more advantageous to change to γ- or X-ray sources.

B. Cronkite's group at Brookhaven National Laboratory was the first to develop a powerful ^{60}Co γ-ray source [Kuhl et al., 1964]. By adapting the volume of the extracoporeal blood circuit and the flow rate of the blood (see formula (1), Ch. 1.3.2), they could easily reach a transit dose of as high as 900 rad with a dose rate of 2300 rad/min. Although γ-sources are less easy to handle because of the heavy shielding needed, the dosimetry is most exact. Furthermore, the γ-emitting radionuclides ^{192}Ir and ^{137}Cs have been used to construct powerful γ-radiators [Pellerin and Remy, 1968; Makoski and Hüdepohl, 1973). Both ^{60}Co and ^{137}Cs sources have been applied experimentally and clinically by various investigators.

C. X-ray sources have also been widely used in ECIB. Like γ-rays, they also permit accurate dosimetry. Transit doses ranging from 25 to 900 rad have been obtained by the various authors applying this type of radiation in experimental and clinical ECIB [Cronkite et al., 1962a; 1962b; Thomas et al., 1965; Meuret et al., 1969; Birkeland and Munk, 1972].

For practical reasons, conventional X-rays have been used in this study.

D. Besides ionizing radiation, ECIB has also been carried out with ultraviolet radiation, using cylindrically arranged mercury lamps [Binet et al., 1968; Villeneuve et al., 1968; Vaugier, 1968]. However, the opacity of the blood with respect to ultraviolet rays causes a low degree of uniformity as regards the dose distribution in the blood.

1.3.4 Treatment schedules in ECIB

A substantial number of different treatment schedules has been applied by

the various authors. Effects of continuous ECIB have been compared with effects of relatively short lasting repeated sessions of ECIB. A wide range of transit doses obtained by different dose rates, extracorporeal blood volumes or blood flow rates, has been used [Sipe et al., 1965; Weeke, 1972a; Weeke, 1974]. However, as discussed previously, the main limiting factor in planning a treatment schedule is the accumulation of the radiation dose within the red cell population. Above a certain dose level, haemolysis will commence and further treatment will have to be abolished (Ch. 1.4.2).

In general, repeated short sessions of ECIB spread out over days or weeks have been shown to be equally or more effective in producing cell depletion than is continuous ECIB, and much safer due to the reduced probability of inducing haemolysis. The transit dose should not be too high, since this would increase the radiation injury to the erythrocytes.

1.4 Recent developments in experimental and clinical ECIB

Since 1962, three International Symposia on ECIB have been held [Rijswijk, 1967; Paris, 1968; Tel Aviv, 1969). Together with a substantial number of publications, they form the basis for a summary of results from experimental and clinical ECIB during the past 15 years. This concerns the effects of ECIB on the lymphatic apparatus (studies on lymphocyte kinetics by means of lymphocyte depletion; immunosuppressive action of ECIB) and some other purely clinical applications. Special attention will be paid to the effects of ECIB on the various types of leukaemia. However, side-effects of ECIB will be first discussed. These involve both general effects and specific effects, i.e., injury to the normal elements in the blood, which may limit the applicability of ECIB.

1.4.1 General side effects of ECIB

Patients, especially those with chronic lymphocytic leukaemia (CLL), sometimes develop chills, fever and general discomfort during an ECIB session, which may even necessitate the termination of treatment [Fliedner, 1967; Meuret et al., 1969]. In these cases, exogenous pyrogens (e.g., coming from the irradiated silastic shunt) could be excluded as being the cause. Furthermore, attempts to demonstrate microbial contamination somewhere along the extracorporeal circuit were all negative. However, a positive correlation was found between the onset of the general symptoms and the degree of lymphocyte destruction in patients with CLL. When the decrease in the number of lymphocytes exceeds 20,000 cells per mm^3 blood during one session of ECIB, there is a high risk of developing a febrile reaction [Meuret et al., 1969]. This indicates that breakdown products of destroyed lymphocytes might act like

endogenous pyrogens. When ECIB is halted, the general symptoms disappear quickly.

1.4.2 Effects of ECIB on erythrocytes

As stated earlier, radiation induced injury to erythrocytes is the main limiting factor in determining the tolerable radiation dose. In contrast with other cells such as thrombocytes and granulocytes, which spend only part of their life span in the blood, erythrocytes reside solely in the blood. Since the total dose delivered to erythrocytes during one or more sessions of ECIB can be calculated (Ch. 1.3.2), the possibility of developing haemolysis can be predicted if the radioresistance of the red cells is known.

The radioresistance of human erythrocytes was investigated by submitting them to γ-irradiation (^{60}Co) *in vitro*, labelling them with ^{51}Chromium, and transfusing them into the original normal donor and determining their life span in the blood [Schiffer et al., 1966]. In the dose range studied (35,000–200,000 rad), the erythrocyte survival times were found to be functions of the radiation dose. Shortening of the life span was observed with a dose of only 35,000 rad, which indicates increased haemolysis. The *in vitro* irradiated erythrocytes showed no morphological abnormalities. It should be noted that, in contrast to the acute high dose *in vitro*, frequent lower doses received during ECIB would permit repair mechanisms to become operative. In 10 patients with various types of leukaemia undergoing ECIB, Schiffer et al. correlated the need for blood transfusions with the mean dose accumulation within the red cell population. Although individual patients behave in a different way (more or less active erythropoiesis; haemolysis due to the leukaemic process itself), transfusion requirements apparently increased with increasing radiation dose.

In a later study on haemolysis in patients with CLL [Andersen et al., 1970], a considerable reticulocytosis indicating haemolysis developed and a decreased life span of erythrocytes was observed after mean cumulative doses between 18,500 and 34,000 rad were reached. The radiosensitivity of young erythrocytes was also investigated. This was done by injecting the patient with ^{59}Fe a few days before the commencement of ECIB. By determining the ^{59}Fe-activity in the blood, representing young erythrocytes which had incorporated the isotope while still located in the bone-marrow, the radiosensitivity of this particular class of cells could be evaluated. It was concluded that young red cells are less radiosensitive than the erythrocyte population as a whole. This is in accordance with observations on bovine erythrocytes [Schnappauf et al., 1965].

With respect to other animal species, data on red cell injury during ECIB have been published for the rat [Pellerin et al., 1960; Pellerin and Remy, 1968] and the baboon [Storb et al., 1969]. In rat erythrocytes, a mean total dose of 2 to 3×10^5 rad during one ECIB session induced a moderate haemolytic

anaemia, which was restored within 10 days. Doses ranging from 4 to 7×10^5 rad caused a more severe haemolysis. However, the rats survived and normal erythrocyte levels were again reached 3 weeks after the radiation injury. One rat even survived 1.2×10^6 rad [Pellerin and Remy, 1968]. This rather high degree of radioresistance of rat erythrocytes is of importance in view of the application of ECIB to rats in this thesis.

1.4.3 Effects of ECIB on platelets

Mature thrombocytes have been shown to be extremely radioresistant. When rabbit platelets were irradiated *in vitro* with 50,000 rad γ-rays, their life span *in vivo* did not differ from nonirradiated ones [von Sebestik et al., 1967]. The radioresistance of human platelets was investigated in leukaemic patients treated by ECIB [Greenberg et al., 1968]. It was demonstrated that thrombocytes which accumulated a mean dose of up to 5000 rad had the same survival curves *in vivo* (^{51}Cr-labelling) as before irradiation. In fact, the radioresistance was much higher, as was studied in a haematologically normal patient. Autologous platelets irradiated *in vitro* with γ-rays in a dose range of 10×10^3 to 75×10^3 rad exhibited no change in *in vivo* life span as compared with nonirradiated thrombocytes. Furthermore, clinical observations of platelet levels in 41 patients (34 with leukaemia and 7 with renal disease) undergoing ECIB revealed no thrombocytopenias due to the irradiation.

A decrease in the platelet count can sometimes be observed during ECIB; this can be explained by a certain tendency for platelets to sequestrate in the extracorporeal coil [Field and Dawson, 1967].

It can be concluded that platelets (in contrast to erythrocytes) are not a limiting factor in the application of ECIB because of: (1) their high degree of radioresistance; and (2) their relatively short life span in the blood (10–12 days). During repeated sessions of ECIB, the accumulation of radiation dose is certainly lower for the platelet as compared with the rather long-lived erythrocyte (life span: 120 days). Radiation doses in excess of 75×10^3 rad, which might damage circulating platelets when delivered during their life span, are not relevant, since injury to erythrocytes would have already become intolerable after such doses.

1.4.4 Effects of ECIB on granulocytes

To date, no direct radiation injury to normal granulocytes due to ECIB has been demonstrated in man [Meuret et al., 1969]. This is not to be expected because of the short life span of mature granulocytes in the blood ($T/_2 =$ 5.5—10.4 h; see table 2.11) and their relatively high radioresistance.

1.4.5 Effects of ECIB on blood chemistry

In general, ECIB does not induce significant changes in the chemical composition of the blood. However, various authors have reported deviations from normal which can sometimes be observed in individual cases. No clear-cut dose-effect relationships could be established up to now.

Particularly in leukaemic patients with a large number of cells in the peripheral blood, an elevation of serum uric acid can be observed when ECIB is initiated. This is due to excessive killing of leukaemic cells. It is then sometimes necessary to give allopurinol to prevent the formation of urate stones, which especially threaten patients with impaired kidney function [Lajtha et al., 1969].

The serum proteins have a high degree of radioresistance; however, a temporary increase in α- and β-globulins [rabbit: Abramoff et al., 1961] and γ-globulin and fibrinogen [calf: Hollard et al., 1969; man: Meuret et al., 1969] has been observed. A transitory decrease in serum albumin has also been reported [rabbit: Abramoff et al., 1961; man: Lajtha et al., 1969]. No underlying cause could be established for these phenomena.

Kidney function (urea), liver function (transaminases, alkaline phosphatase) and electrolyte levels were unaltered by ECIB, as studied in dogs and man [Maginn and Bullimore, 1968].

1.4.6 Effects of ECIB on the lymphatic apparatus

Lymphocytes represent the most radiosensitive cell population in the peripheral blood [Trowell, 1952; Schrek and Stefani, 1964]. Their D_0 is approximately 70 rad [van Bekkum, 1974]. Based on this knowledge, various workers have applied ECIB both experimentally and clinically for two main purposes: (1) to induce immunosuppression; and (2) to study lymphocyte kinetics in conjunction with lymphocyte depletion.

Most of the experimental work has been performed in the calf by Cronkite's group at Brookhaven National Laboratory. Prolonged continuous ECIB with a transit dose of 900 rad produces a marked lymphopenia [Cronkite et al., 1962a; 1962b]. During ECIB, the number of lymphocytes in the blood decreases exponentially until a low constant plateau is reached. If the irradiation is continued, no further reduction is observed. Apparently, an equilibrium is established between the destruction and removal of lymphocytes and the replenishment from tissue pools. This is confirmed by histological examination of the various lymphoreticular organs. If ECIB is continued after the state of equilibrium is reached, a significant depletion of lymphocytes from these organs becomes apparent, i.e., cells are being mobilized [Cottier et al., 1964]. The degree of depletion with time followed an exponential function with at least two

components [Ruchti et al., 1970]. The first, corresponding to a rapid fall, would be related to the elimination of an easily mobilizable pool of lymphocytes. The second slow component would correspond to a more sessile pool of lymphocytes, which exchanges only very slowly with lymphocytes in the blood compartment. By applying planimetry, the cell content of the various tissues involved (spleen, lymph nodes and thymus) could be measured and estimates could be made of the ratio between the sizes of the easily mobilizable and the more sessile tissue pools of lymphocytes.

Histologically, the following changes have been noted. In the spleen, the lymph follicles show a marked decrease in size, which is mainly due to a loss of small lymphocytes in the cuffs surrounding the germinal centers. The germinal centers themselves remain intact. Loss of lymphocytes was also observed in the peripheral zones of the white pulp and in the red pulp. Lymphocyte depletion occurred preferentially at the inner border of the cortex ("paracortical zone") in the lymph nodes. In the thymus, the thickness of the cortex decreased in most cases. The bone marrow shows no remarkable changes. Disintegrating cells are found in the spleen, in the lung capillaries and in the liver sinusoids.

The histological picture after prolonged ECIB is similar to that produced by neonatal thymectomy [Waksman et al., 1962]. ECIB seems to preferentially destroy lymphocytes from thymus-dependent areas ("T lymphocytes"). In the peripheral blood, it was observed that the number of small lymphocytes is particularly reduced [Sipe et al., 1965]. These lymphocytes have been described as being long-lived recirculating immunocompetent cells, capable of rejecting tissue homografts [Gowans, 1965; Ford and Gowans, 1969]. Thus, ECIB has important implications with regard to immunosuppression as will be discussed later.

Various transit doses have been used to induce a lymphopenia. With doses ranging from 30 to 900 rad, identical degrees of lymphopenia are produced after 24 hours, although the condition develops more rapidly with higher transit doses [Sipe et al., 1965]. The time needed for the blood lymphocyte level to return to the preirradiation value varies from days to many months. This depends on the degree of cell depletion as well as the rate of new cell production. In view of the specific destruction of the population of T lymphocytes, the thymus is of utmost importance with respect to the recovery process. Evidence for this was obtained from the observation that ECIB-treated thymectomized calves require much more time for complete recovery as compared with intact animals [Cronkite et al., 1968].

ECIB-induced lymphopenia has also been described for other animal species such as goats [Chanana et al., 1971), baboons [Storb et al., 1969], dogs [Wolf et al., 1966; Pichlmaier and Trepel, 1966; Maginn and Bullimore, 1968; Kriek, 1973], rabbits [Pompidou et al., 1975] and rats [Pichlmaier and Trepel, 1966; Pellerin and Remy, 1968].

ECIB (in combination with thoracic duct cannulation, isotope-labelling and/or extracorporeal irradiation of the lymph) has opened up a means to study various facets of lymphocyte behaviour such as: 1) the size of the intravascular and extravascular (mobile or sessile) pools of lymphocytes; 2) the average time spent by lymphocytes in the blood; 3) the rate and degree of lymphocyte mobilization from tissue stores; 4) pathways of lymphocyte migration; 5) lymphocyte production rates; 6) the function of lymphocytes; and 7) the factors controlling lymphopoiesis, etc. [Cronkite et al., 1964; Cottier et al., 1964; Wagner et al., 1967; Cunningham et al., 1967; Cronkite et al., 1968; Field et al., 1972].

In considering the mechanism by which lymphopenia develops during ECIB, it is presumed that intact irradiated lymphocytes or fragments of these are rapidly removed from the circulation by the reticuloendothelial system. This presumption is based on the following observations: 1) after *in vitro* incubation of lymphocytes which have passed one time through the irradiation field (TD 900 rad), 87% of the cells disintegrate within a six-hour period [Cronkite et al., 1962a]; 2) ^{51}Cr-labelled lymphocytes irradiated *in vitro* with 1000 rad disappear quickly when infused into the circulation and do not recirculate [Jansen et al., 1969]; and 3) direct damage to lymphocytes during ECIB was established by the observation of dicentric chromosomal aberrations [Field et al., 1972]. The rapid development of lymphopenia indicates the occurrence of interphase death. Stress with associated increases in adrenocortical activity contributing to lymphocyte depletion by ECIB does not seem to play a major role, as was concluded from ECIB studies in adrenalectomized calves [Joel et al., 1976].

Ionizing radiation has been widely applied to induce immunosuppression in organ transplantation [van Bekkum, 1974]. ECIB offers the advantage of inducing a lymphopenia without damage to other cells. This technique has therefore been employed both experimentally and clinically in attempts to promote the survival of tissue allografts.

Data have been published on prolongation of skin allografts in ECIB-treated calves [Chanana et al., 1966; Chanana et al., 1969], rats [Pichlmaier and Trepel, 1966] and baboons [Storb et al., 1969]. To evaluate possible clinical applications, ECIB was also applied in renal transplantation. Survival of allografted kidneys was found to be prolonged when ECIB was performed in dogs [Maginn and Bullimore, 1968] and goats [Chanana et al., 1971].

It has substantially less effect on humoral immunological activity. In primates [Storb et al., 1969] and calves [Stoner et al., 1969], the antibody responses to sheep erythrocytes and tetanus toxoid, respectively, were only slightly or not at all depressed. This may be explained by the fact that, in contrast to cellular immunity (migrating lymphocytes), the humoral response

involves a relatively large contribution of regional lymph nodes which are easily reached by circulating antigens.

ECIB has been clinically applied to patients before and/or after kidney transplantation. A favourable circumstance in these cases is that these patients, because of renal disease, have already been equipped with an arterio-venous shunt for regular haemodialysis. Under these circumstances, ECIB can be easily performed. In 1966, two publications appeared on reversal of graft rejection when ECIB treatment was started after renal transplantation [Wolf et al., 1966; Merril et al., 1966]. Later systematic studies indicate that acute rejection episodes can be avoided [Maginn and Bullimore, 1967; Rosengren et al., 1968; Persson et al., 1969; Weeke et al., 1970; Birkeland and Munk, 1972; Schmidt et al., 1974]. Furthermore, it was established by means of lymphocyte transformation assays that the mitogenic reactivity of lymphocytes present in the blood and the lymph nodes after ECIB showed large individual variations without a distinct pattern [Weeke and Sørensen, 1971; Weeke, 1972b; Birkeland, 1976; Birkeland et al., 1976].

ECIB has not been of clinical benefit when used for immunosuppressive purposes in subacute glomerulonephritis [Andersen et al., 1968].

1.4.7 Effects of ECIB on leukaemia

As mentioned earlier (Ch. 1.1), Lajtha and co-workers were the first to introduce ECIB as a possible therapeutic measure in the treatment of leukaemia [Lajtha et al., 1962]. Since that time, several groups of investigators have studied the effects of ECIB on various forms of human leukaemia. In general, the clinical indications to apply ECIB treatment have been:

(1) resistance to chemotherapy;
(2) contraindications for chemotherapy, e.g., toxicity, pregnancy; and
(3) severe leukocytosis with the risk of developing leukostasis.

For the theoretical evaluation of the possibilities of using ECIB in leukaemia therapy, it is very important to know the factors inherent in the leukaemic process which determine its efficacy (table 1.1). The first of these is the number of leukaemic cells in the blood. Patients with high white cell counts may be the best candidates for ECIB; those with aleukaemic leukaemia may benefit little unless there is a high rate of exchange between blood and tissue stores. The second consideration is the number of leukaemic cells in tissue stores such as the bone marrow, the spleen, the lymph nodes and elsewhere. In early leukaemia, this number may be in the order of 10^{11} [Lajtha et al., 1962]. In advanced disease with hyperplastic bone marrow and enlarged spleen and lymph nodes, the number may be in the order of $10^{12}-10^{13}$. The third factor is the rate of exchange of leukaemic cells between the tissue depots and the blood. So far, no exact data are available on this point. Fourthly, the radiosensitivity of leukaemic

cells is of importance. *In vivo* assay methods in experimental leukaemia models have shown that the D_0 value ranges from 60 to 160 rad [Hewitt and Wilson, 1959; Whitmore and Till, 1964; Bush and Bruce, 1965]. Using *in vitro* techniques, data have been obtained on the radiosensitivity of cells derived from human leukaemias. In chronic lymphocytic leukaemia (CLL), some cases have been reported with cells sensitive to as low a dose as 2 rad [Schrek and Stefani, 1964]. In view of the fact that other cases are resistant to as much as 1000 rad, it can be concluded that there is a large range of sensitivities among the various classes of leukaemia. By studying the blood disappearance of reinfused isotope-labelled irradiated leukaemia cells, information was also obtained on the radiosensitivity when this was compared with the fate of nonirradiated reinfused cells [Andersen et al., 1974]. Furthermore, by following the fate of ^{3}H-cytidine labelled lymphocytes in CLL during ECIB, a correlation was established between the radiosensitivity and the lymphocyte RNA turnover. Increased radioresistance and thus a reduced effectiveness of ECIB was found to be related to more rapid RNA turnover [Strijckmans et al., 1969]. The fifth concern is the rate of leukaemic cell production; this will be discussed later. However, the general concept at present is that acute leukaemia is characterized by a rather slow growth rate as compared with normal haemopoiesis [Killmann, 1968; 1972]. Finally, the lifespan of leukaemic cells must be taken into account. Several studies suggest that, to some extent, leukaemia may be a disease of overaccumulation of cells with a long lifespan (see Ch. 1.4.7c). If this is true, ECIB might be quite effective in reducing the mass of leukaemic tissue. It is obvious that precise data on the factors mentioned above have not been completely obtained in human leukaemia.

Systematic experimental ECIB studies have not been performed because of the lack of suitable animal models. The most commonly used animal species for leukaemia studies, i.e., the mouse, would obviously give rise to insurmountable technical difficulties. As regards experimental ECIB, only the group of Cronkite has described the effects of ECIB on spontaneous malignant lymphoma and lymphocytic leukaemia in 5 cattle [Cronkite et al., 1965; Joel et al., 1967a]. Repeated sessions of ECIB, with transit doses ranging from 300 to 2000 rad, reduced the lymphocyte count in the peripheral blood of all of these animals. The daily induction of lymphopenia with return to higher lymphocyte levels the next day indicates that the blood lymphocytes were exchanging rather rapidly with lymphatic tissue stores. This was also supported by the observation that the size of enlarged lymph nodes neither increased nor decreased during the period of ECIB treatment. This might mean that production of malignant cells was keeping pace with destruction of mobilized cells. The cows showed different rates of peripheral recovery after the cessation of ECIB. This might be explained in terms of different rates of mobilization from the leukaemic tissue mass to the blood. No complete remissions were observed in these studies.

As regards human leukaemia, the evaluation of ECIB is rather complex because of different stages of the process in different patients, concomitant therapy regimens, ethical considerations, etc. An attempt will be made to review data on the effects of ECIB in various types of human leukaemia.

a. Chronic lymphocytic leukaemia (CLL)

Of all the forms of leukaemia, ECIB treatment in CLL has been studied in the greatest detail. A considerable proportion of lymphocytes in CLL are long-lived recirculating cells [Schiffer, 1968; Zimmerman et al., 1968]. However, the rate of recirculation of leukaemic cells is rather slow in comparison with that of normal lymphocytes [Binet et al., 1967; Bremer et al., 1973b; Flad et al., 1973]. By both isotope studies and ECIB-induced depletion patterns, several functional cell compartments could be distinguished in CLL. Cells in the circulating blood pool (CBP) exchange continuously with a rapid exchangeable pool (REP) which is built up by an intravascular noncirculating compartment, like the marginal pool (MP) for granulocytes and an extravascular compartment (the rapidly exchangeable tissue pool: RETP), which is not defined anatomically at present. The REP may be 1.5 to 60 times larger than the CBP [Strijckmans et al., 1968; Schiffer et al., 1969; Spivak and Perry, 1970; Bremer et al., 1973a; Scott et al., 1973; Neumann et al., 1974]. The third compartment is the slowly exchanging tissue pool (SETP), which contains the bulk of malignant lymphocytes (spleen, lymph nodes, bone marrow).

From these observations, it is clear that, in principle, ECIB might deplete the CBP and REP to a large extent, while the SETP would be affected to a much lesser degree. With transit doses ranging from 25 to 720 rad, ECIB has indeed reduced the number of lymphocytes in the peripheral blood to varying degrees in most cases, whereas the decrease in the size of the spleen and the lymph nodes (indicating the size of the RETP and SETP) has been variable: some patients show a clear-cut size reduction, others do not. Although bone marrow infiltration persists in all cases (apparently due to a slow exchange), improved normal haemopoiesis has been reported after ECIB treatment [Thomas et al., 1965; Wolf and Hume, 1967; Storb et al., 1968; Begemann et al., 1968; Garret and Lajtha, 1968; Andersen et al., 1968; Evers, 1969; Meuret et al., 1971; Cronkite, 1971; Girard, 1974]. Another important observation was that ECIB does not promote selection of more radioresistant leukaemic cells: later courses of ECIB had the same cytolytic effect as did the initial ones.

Three distinctive patterns of response to ECIB have been described [Field et al., 1970]:

1) a rapid decrease in the peripheral blood cell count during the course of treatment, the count remaining low for a prolonged period afterwards. With

this pattern, extravascular exchanging pools are small in relation to the circulating pool; the rate of exchange is relatively rapid;

2) a less rapid decrease in the lymphocyte count, followed by a rather rapid return to pretreatment values when ECIB is discontinued. This pattern was correlated with a large extravascular pool of cells which exchanged rather slowly with the blood;

3) a slow or negligible decrease in the blood cell count during ECIB. However, the count continues to fall for a variable period after treatment has ceased and remains at a low level for some time before it rises again. This phenomenon is observed in patients who have both proliferating precursor cells and nonproliferating end cells in their blood [Theml et al., 1973]. Because ECIB would preferentially deplete the small proliferating fraction, the population of nonproliferating cells naturally dies out in the time period after ECIB.

From these data, the conclusion would be that, although ECIB can deplete the body of a significant number of cells in patients with CLL [up to 12×10^{12} cells being destroyed in one series of treatment; Field et al., 1970], only partial remissions are achieved, due to the rather poor exchange of cells between the tissues and the blood. The limited number of patients does not permit definite conclusions concerning the survival time after ECIB treatment. It has been suggested that ECIB should be started in an early stage of CLL before there is extensive organomegaly and its sequelae [Cronkite, 1967]. It might then be possible to prevent organomegaly by intermittent depletion of the blood and the rapidly exchanging pool of lymphocytes.

b. Chronic myelocytic leukaemia (CML)

By means of isotope studies in CML, it has been observed that leukaemic cells are able to recirculate between blood, bone marrow and spleen [Perry et al., 1966; Galbraith, 1966; Chikkapa and Galbraith, 1967; Duvall and Perry, 1968]. Since leukaemic cells, which have a prolonged blood disappearance time in CML as compared with normal granulocytes [Athens et al., 1965], are freely exchanging with tissue stores, ECIB might theoretically progressively deplete all of the leukaemic cell compartments such as the circulating blood pool, the noncirculating marginal pool [Athens et al., 1965] and the tissue pools.

Seven cases of CML treated by ECIB have been described in the literature at the time of this writing [Cronkite, 1967; Lajtha et al., 1969]. With transit doses ranging from 150 to 500 rad, ECIB reduced the peripheral cell count for varying periods of time in all patients. In the early stage of treatment, the spleen usually became larger before the blood counts started to decrease. A concomitant increase in uric acid excretion has lead to the conclusion that radiation-injured cells accumulate in the spleen. The size of the spleen was

later substantially decreased in most cases. Although immature granulocytic cells sometimes completely disappeared from the blood during a treatment course, complete remissions have never been obtained. After cessation of ECIB, relapse of the leukaemic process occurred in a matter of days to several weeks. Several conclusions can be drawn from these studies in a rather small group of patients. Firstly, the transit doses used can cause considerable damage to granulocytic leukaemic cells. Secondly, the size reduction in the spleen during ECIB indicates the existence of a rapid exchangeable tissue pool in CML. The low efficiency of ECIB treatment in CML might be due to inadequate cellular exchange, an insufficient transit dose or an inadequate treatment time. Further studies in a larger group of patients to evaluate the possible usefulness of ECIB in CML are clearly indicated.

c. Adult acute leukaemia (AL)

Acute leukaemia is characterized by an accumulation of immature haemopoietic cells (maturation defect) in combination with a deficient production of mature functioning cells, i.e., erythrocytes, granulocytes and thrombocytes [Killmann, 1968]. These two phenomena seem to be related, as is indicated by the fact that restoration of the production of mature functioning cells is preceded by spontaneous or therapeutically induced disappearance of the immature leukaemic blast cells (LBC). However, no definitive answers can be given as to the causative factors underlying these related phenomena at the present time, although some insight into the pathogenesis of the disease has been gained during the past decades. Observations relevant to the present study will be summarized briefly below.

For a long time, it has been thought that acute leukaemia is a disease process resulting from rapid, uncontrolled cellular proliferation [Wintrobe, 1961]. However, during the past 10 years, it has been shown that LBC are quite heterogenous with respect to their kinetic behaviour [Killmann, 1968; 1972]. At the present time, it is proposed to distinguish between relatively actively proliferating LBC, which proliferate less rapidly than normal neutrophilic precursors and "quiescent" cells, which may constitute cells in a long G_1 phase, G_0 cells (out of cycle) and end cells [Cronkite, 1968; Clarkson, 1969; Gavosto et al., 1969; Mauer and Lampkin, 1974]. Nonproliferating G_0 cells are believed to originate from the proliferating LBC compartment [Gavosto et al., 1964] and to maintain the capacity to proliferate again after a suitable stimulus [Saunders and Mauer, 1969; Strijckmans et al., 1970; Killmann, 1972]. The LBC found in the peripheral blood belong mainly to the nonproliferating compartment [Killmann, 1968; 1972]. By reinfusion of autologous labelled leukaemia cells in acute myelocytic leukaemia (AML), it has been demonstrated that LBC exchange rather rapidly with a marginal pool which is 1.2 to

3.5 times larger than the intravascular circulating pool [Hoelzer et al., 1972]. The reported blood transit times of LBC (32–46 h; see table 2.11) are long when compared to those of normal granulocytes (5.5–10.4 h; Killmann et al., 1963; Clarkson et al., 1969; 1970]. From other studies, it has been suggested that leukaemic cells leaving the blood can return to the bone marrow and the spleen [Killmann et al., 1971; Rosen et al., 1975]. Whether or not these cells can start to proliferate after lodging in these sites is still a matter of debate, although positive evidence has been obtained for other anatomical sites, e.g., the development of subcutaneous leukaemic nodules. Another possibility is that cells are released into the circulation again ("recycling") after lodging in spleen and bone marrow [Tarocco et al., 1972].

Based on this knowledge, it seems justified to apply ECIB to patients with acute leukaemia according to the indications mentioned above. Theoretically, there may be a chance to induce at least a partial remission. Several reports on the effects of ECIB on various types of human acute leukaemia have appeared. In 1962, Lajtha and co-workers performed the first pilot study in 2 patients, one with acute myelomonoblastic leukaemia and another with acute monocytic leukaemia [Lajtha et al., 1962]. These patients were submitted to only 2 and 1 short lasting ECIB sessions, respectively. No apparent effect on the course of the disease was noted. However, of some importance was the observation that the whole procedure of ECIB was well tolerated and without any harmful side-effects. Another early attempt was made with 2 children with acute myelomonocytic leukaemia. However, these patients died very shortly after ECIB was applied [Field and Dawson, 1967]. From this and other early studies, it was concluded that the treatment should not be started too late in the course of the disease [Evers and Haanen, 1967; Meindersma and Barendsen 1967].

The group of Cronkite has reported on the effects of ECIB in 14 adults with AML [Schiffer et al., 1968]. They received repeated 4-hour sessions (transit dose: 236 to 535 rad) up to a total irradiation time of 57 to 880 hours. The median survival time of 8.0 months for patients treated with ECIB was as good as other published survival data from patients treated with chemotherapy at that time. Some cases with a rather long survival time have been reported (16.5 and 18 months). From this study, there appeared to be a correlation between increased survival time and a transit dose exceeding 340 rad. In 75 per cent of these patients, there was a marked decrease in the number of leukaemic cells in the peripheral blood. However, no complete remissions were achieved in any case. Some patients showed an increase in the number of platelets, whereas in some patients the size of the spleen also decreased, as measured by spleen scanning. One pregnant patient was carried through to delivery by ECIB and thus avoided possible fetal injury due to chemotherapy.

Lajtha and co-workers described the results of ECIB treatment using continuous irradiation (1 to 5 days) in 8 patients with AML [Lajtha et al.,

1969]. Their findings can be summarized as follows: 5 patients treated early after diagnosis (5 to 8 weeks) all showed a decrease in the peripheral number of LBC which was maintained for some weeks. In some of these patients, the response to subsequent chemotherapy seemed to be more pronounced after a series of ECIB. In 3 patients where ECIB was started in a later stage of the disease (7 months after diagnosis), only a short-lasting reduction in the white cell count was observed. In none of the cases were significant changes in the bone marrow noted. This may indicate that there was virtually no exchange of LBC between the marrow and the blood. The conclusion of Lajtha and co-workers was that ECIB might be especially beneficial if applied to patients who have not responded to the usual chemotherapeutic measures within 1 or 2

Table 1.4 *Changes in proliferation parameters in acute myelocytic leukaemia measured before and after ECIB*

patient no.	duration of ECIB (h)	LI-BM (%)		MI-BM (%)	
		pre	post	pre	post
1	18	3.4	19.6	0.2	3.2
2	6	17.0	52.2	0.1	5.5
3	20	3.1	3.6	0.2	0.2
4	23	6.1	5.5	0.2	0.2
5	12	18.4	29.5	2.1	2.4
6	24	8.2	18.1	1.1	1.4
7	16	9.4	12.2	0.6	1.2

LI-BM: labelling index of leukaemic bone marrow cells after *in vitro* incubation with tritiated thymidine
MI-BM: mitotic index of leukaemic bone marrow cells
pre/post: before/after ECIB
Patients no. 1–6: Chan and Hayhoe (1971); patient no. 7: Ernst et al. (1971)

Table 1.5 *Changes in proliferation parameters in acute myelocytic leukaemia measured during ECIB*

hours after start of ECIB	LI-BM (%) patient no.			MI-BM (%) patient no.			LI-blood (%) patient no.
	5	6	7	5	6	7	7
0	18.4	8.2	9.4	2.1	1.1	0.6	0.8
2			9.2			0.3	0.6
4	17.6	18.4		1.8	0.9		
8	19.8		11.9	1.6		1.5	0.7
12	29.5	19.7		2.4	1.2		
16			12.2			1.2	0.6
24		18.1			1.4		

LI-BM/blood: labelling index of leukaemic bone marrow/blood cells after *in vitro* incubation with tritiated thymidine
MI-BM: mitotic index of leukaemic bone marrow cells
Patients no. 5 and 6: Chan and Hayhoe (1971); patient no. 7: Ernst et al. (1971); see table 1.4

months after diagnosis. Essentially similar findings were reported by 3 other groups of investigators treating a total of 10 AML patients [Souhami et al., 1969; Chan and Hayhoe, 1971; Andersen et al., 1974]. Temporary clinical improvement was sometimes seen. In some patients, however, the dissemination of the disease seemed to be accelerated by the ECIB procedure; e.g., extensive leukaemic infiltration of the skin was observed some days after irradiation was discontinued. A rapid increase in peripheral LBC was also noted in these patients after cessation of ECIB. This could possibly be accounted for by increased proliferation of LBC in the marrow or by a stimulation of their release from the marrow.

Only 4 cases of acute lymphocytic leukaemia (ALL) treated by ECIB have been reported [Thomas et al., 1965; Garret, 1967; Souhami et al. 1969]. These patients were in the terminal stage of the disease. Generally, ECIB induced a reduction in the peripheral LBC count; however, no complete remissions were observed. After cessation of ECIB, chemotherapy with 6-mercaptopurine appeared to be much more effective than usual in 2 patients, suggesting that the irradiation resulted in an increase in sensitivity to this drug.

From the observation that several patients after ECIB show a rapid rise in the number of peripheral LBC, an accelerated dissemination of the disease and an increased sensitivity to certain drugs, the possibility was considered that destruction of LBC by ECIB might stimulate the bone marrow to increase its proliferative activity, possibly through a feedback mechanism. This hypothesis was tested by measuring the mitotic index (MI) and the *in vitro* tritiated thymidine labelling index (LI) of marrow LBC before, during and after ECIB [Chan et al., 1969; Chan and Hayhoe, 1971; Ernst et al., 1971]. A total number of 7 patients with AML were studied (table 1.4). The duration of the ECIB session varied from 6 to 24 hours. In 5 of the 7 studies, a clear-cut increase in the LI was observed, occurring in some patients as early as 4 to 6 hours after the initiation of ECIB (patient no. 2, table 1.4; patients no. 5, 6 and 7, table 1.5). In the patients with an increased LI, the MI was also increased, although these changes were less consistent than the changes in the LI. However, since the duration of mitosis may be as short as 1/20 of that of the synthesis of DNA, the MI is a less reliable indicator of cellular proliferation than the LI. On the other hand, an increased LI might also be due to synchronization of the LBC in the marrow by ECIB. This seems to be less probable, because the same effect was observed in different patients, in different stages of the leukaemia, at different times following ECIB performed with different dose rates. Of the 2 patients who did not show changes in the LI or MI, one had no demonstrable circulating LBC at the time ECIB was performed. In the second patient, who had a high peripheral LBC count, the kinetic behaviour of the leukaemic cell population appeared to have been different, although a transient increase in the LI and MI might have been missed in this study.

The major conclusion resulting from these studies is that at least part of the quiescent, nonproliferating LBC population does not consist of end cells but of cells which may be recalled into the proliferative cycle (recruitment) by an appropriate stimulus. In this instance, the acute depletion of peripheral LBC by ECIB seems to be the causative factor. In AML, an analogous stimulus may be provided when LBC are merely removed from the peripheral blood (leukapheresis). However, the increase in LBC proliferation measured by means of the LI takes a much longer time with this procedure, i.e., in the order of several weeks after repeated sessions of leukapheresis are started [Reich et al., 1971; Hoelzer et al., 1974b]. This difference between ECIB and leukapheresis might be explained by the fact that, in ECIB, destroyed cells remain within the body and may release some factors which stimulate cell proliferation in a more effective way, whereas, in leukapheresis, the cells are merely removed. These studies support the hypothesis that a feedback mechanism exists between the blood and the bone marrow in acute leukaemia.

The observations mentioned above might have important implications as regards more effective chemotherapy with cycle-active drugs subsequent to ECIB. The small group of patients studied all showed a decrease in the number of LBC in the blood, which seemed to be more rapid and more sustained than if chemotherapy was used without preceding ECIB. The chemotherapy employed consisted of cytosine arabinoside (Ara-C) or a combination of Ara-C, methotrexate (MTX), 6-mercaptopurine and prednisone [Chan and Hayhoe, 1971] or MTX and vincristine [Ernst et al., 1971]. However, no complete remissions could be induced.

d. Lymphoreticular neoplasms

Only a few cases of lymphoreticular neoplasms treated by ECIB have been reported. These include 2 patients with widespread lymphosarcoma [Dawson et al., 1967], one patient with a reticulum cell sarcoma [Sharpe et al., 1967] and one patient suffering from Hodgkin's disease [Sharpe et al., 1968]. In the 2 lymphosarcoma patients, a small, but sustained, decrease in the elevated peripheral lymphocyte count together with a reduction in size of enlarged lymph nodes was observed. The lymph nodes, however, returned to their original size within a few days after ECIB was discontinued. It was suggested that this was due to redistribution of surviving lymphoid cells rather than to an accelerated rate of cellular proliferation. In the 2 other patients, who had a normal number of cells in the blood, the objectives of the ECIB treatment were to gain insight into the dosimetry of the ECIB procedure and lymphocyte kinetics, e.g., the mean residence time of lymphocytes in the blood and the size of the extravascular pool of lymphocytes in the body. Both of these parameters

were studied by analysing chromosomal aberrations within lymphocytes induced by ECIB.

1.5 Contents of the present study

The study to be presented will be divided into the following parts. In Chapter 2, the rat leukaemia model will be described in detail in terms of morphological and functional characteristics.

Chapter 3 will deal with leukocyte mobilizing agents (LMA). Cell mobilization studies in both normal and leukaemic animals will be discussed. Results of the clinical application of LMA to patients with leukaemia will also be reported.

In Chapter 4, the model which was developed in order to apply repeated sessions of ECIB to rats will be discussed, including the cannulation and ECIB procedures as well as physical entities such as radiation equipment and dosimetry. The effects of ECIB on the rat leukaemia will be evaluated by means of haematologic and kinetic parameters. Long-lasting sessions of ECIB will be compared with repeated short-lasting ones. In addition, the efficacy of cell mobilization in combination with ECIB will be discussed.

Finally, in Chapter 5, a general discussion and conclusions together with recommendations for application of ECIB in the future will be presented.

CHAPTER 2

THE RAT LEUKAEMIA MODEL

2.1 Introduction

As mentioned earlier (see Ch. 1.1), the studies were performed in a rat model for acute myelocytic leukaemia. The leukaemia originated in a female rat of the inbred Brown-Norway strain at Rijswijk (BN/Rij) in 1971 following 3 intravenous injections of 2 mg 9, 10-dimethyl 1,2-benzanthracene (DMBA) 100 days earlier. In general, such a model should: (1) be transplantable within the given animal strain; and (2) have a reproducible growth pattern throughout the subsequent transplantation generations. The model to be described meets both prerequistites (van Bekkum, personal communication). It proved to be transplantable in the BN rat strain by means of cellular transfer. Because of its myelocytic nature (see Ch. 2.4), it has been named the BN myelocytic leukaemia (BNML).

In this chapter, the model, which resembles human acute myelocytic leukaemia in many aspects, will be analysed in detail with respect to morphological and functional characteristics. The materials and methods which were generally used will be first described.

2.2 General experimental procedures

2.2.1 Experimental animals

The experiments were carried out with the inbred Brown-Norway (BN) rat strain produced in the Rijswijk colony. Male rats between 15 and 20 weeks of age were used. Water and food were always supplied *ad libitum* during the experiments.

2.2.2 Preparation of cell suspensions

Spleen

After excision and cleaning, spleens were cut into small pieces with scissors. The pieces were subsequently squeezed through nylon gauze. In this way, a monocellular suspension using Simms' solution was obtained.

Bone marrow

Femurs were removed and cleaned of adherent muscle tissue. Then the proximal ends were cut with scissors and the femoral shafts were emptied by

repeated flushing with Tyrode's solution using a bent needle placed on a syringe. The collected bone marrow was filtered through nylon gauze.

Blood

To obtain large amounts of blood, the abdominal aorta was punctured under ether anaesthesia. Seventy to eighty percent of the total circulating blood volume can be collected in this manner. To prevent coagulation of the blood, sodium citrate (Natrii Citras 3.1%, ACF Chemiefarma, Holland) was added up to a final concentration of 20%.

Cell counting

The number of nucleated cells present in cell suspensions was counted in a Bürker type haemocytometer using Türck's solution (0.01% crystal violet and 1% acetic acid in saline). The number of dead cells was determined by means of eosin (0.2%) uptake.

2.2.3 Leukaemia transfer

In most of the experiments, the leukaemic spleen was used as a source of leukaemic cells for transplantation. The required number of cells, obtained through dilution of a monocellular suspension, was then injected intravenously into a tail vein in a volume of 1 ml.

In order to maintain a reproducible growth pattern of the leukaemia, no more than two subsequent transplant generations were allowed. After each second transplantation, leukaemia transfer was performed with cells from a large frozen stock of identical batches. Leukaemic cells were frozen in sterile plastic ampoules of 5 ml volume ($4–5 \times 10^7$ cells/ml). Cells were suspended in Simms' solution supplemented with the cryoprotective agent dimethylsulphoxide (DMSO) (10%) and with calf serum (20%). Cells were cooled at a rate of freezing of 1 °C/min which was controlled by a thermocouple placed into an identical ampoule situated in the freezing chamber. As soon as the cooling temperature reached −50 °C, the ampoules were stored in a liquid nitrogen container. To thaw the frozen suspensions, the ampoules were placed in a 40 °C waterbath. The cells were then diluted with Simms' solution at an ultraslow stepwise rate described by Schaefer et al. (1972). The cells were washed and counts were performed. Viability of the leukaemic cells as checked by both eosin uptake and bio-assays was not altered by the freezing and thawing procedure.

In this way, all experiments could be carried out within 5 transplantation generations.

2.2.4 *Haematological parameters*

Blood sampling

For haematological follow-up during the experiments, blood samples were obtained either from the tail by cutting off its tip or from an extracorporeal aorta by-pass to be described later (see Ch. 4.2). The blood was collected in small plastic vials containing some grains of dipotassium ethane-diamino-tetra-acetic-acid (EDTA; British Drug Houses Ltd., England) to prevent coagulation.

Blood cell counting

Total leukocytes and erythrocytes were counted with a coulter counter model B (Coulter Electronics Inc., USA) after appropriate dilution of the blood sample with Isoton (0.1% sodium azide, Coulter Electronics Ltd., England). Zaponin (2.5% acetic acid, Coulter Electronics Ltd., England) was added to lyse the red cells before counting leukocytes. Platelets were counted in a haemocytometer using Feissly's stain (3% cocaine HCl, 0.1% sodium azide; Apotheek Haagse Ziekenhuizen, Holland). After vital staining with brillant cresyl blue (3.3% in absolute alcohol), the number of reticulocytes per 1000 erythrocytes was determined.

Haematocrit

Microcapillaries filled with blood were centrifuged for 5 min at 11,500 rpm (13.5 g). Haematocrits were then determined by means of a microcapillary reader (International Equipment Co., Boston, Mass., USA).

Cytology

Blood smears were prepared according to standard methods. After drying in air, the cells were stained with May-Grünwald stain for 3 min and, after 1 min in a pure phosphate buffer solution (pH 6.9), stained for 15 min with Giemsa solution (diluted 1:26 with the same buffer). One min washing with glass-distilled water and drying in air completed the procedure.

The cytological composition of the bone marrow was studied after precipitation of femoral bone marrow cell suspensions according to the method described by Sayk [Sayk, 1960]: 20 µl of Tyrode's solution containing 10^5 cells were mixed with 4 drops of Hanks' Balanced Salt Solution supplemented with 20% fetal calf serum; the cells were then sedimented onto glass slides for 25 min at room temperature. After drying in air, the slides were fixed in absolute methanol for 3 min and stained as described above.

2.3 *Cytogenetics and DNA content*

Cytogenetic analysis of leukaemic cells indicated the presence of a specific chromosome marker in the BNML, i.e., a fusion between chromosomes 2 and 7 (Drs. J. C. Klein and A. Th. van der Velden, Rijswijk). All BNML cells have a diploid number of chromosomes (n = 42).

The DNA content of BNML cells was determined in single Feulgen-stained smears with the Barr and Stroud integrating microdensitometer (fig. 2.1; Dr. G. Haemmerli, Zürich). The histogram revealed that the majority of BNML cells contained a slightly increased amount of DNA (2.3 n) as compared with normal rat lymphocytes (2.0 n). Because of the diploidy of the leukaemic cells, this might be due to either an increased amount of DNA per chromosome or the presence of a large proportion of BNML cells in early DNA synthesis phase.

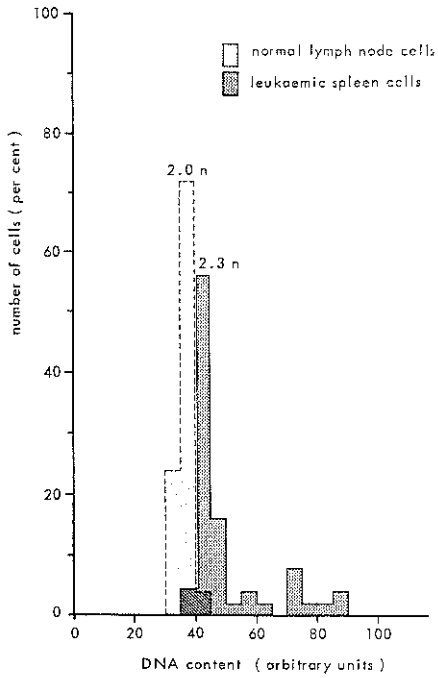

Fig. 2.1 *DNA content of BN myelocytic leukaemia cells as measured by the integrating microdensitometer* (Haemmerli, 1974).

2.4 *Cytological and cytochemical characterization*

2.4.1 Cytology

With light microscopy, the leukaemic cells in the blood can be described as follows (fig. 2.2). They are large cells (diameter 11–16 μ) with a relatively

large oval or kidney-shaped nucleus showing a coarse chromatin pattern. The nucleus often contains a large irregular nucleolus and is surrounded by a rim of dark blue cytoplasm containing many coarse purple granules. Auer rods are not observed. It was noted that many leukaemic cells in a blood smear were damaged and showed extensive karyorrhexis or even complete loss of cell structure. This may indicate a greater fragility of the BNML cells as compared to normal leukocytes. Leukaemic cells in the bone marrow have the same morphological features.

Electron microscopy (Dr. H. Felix, Zürich) reveals the following characterisistics (fig. 2.3). Besides a normal number of lysosomes and mitochondria, an abundance of endoplasmic reticulum is observed in the cytoplasm; it shows dilated cisternae. The Golgi apparatus, which is larger than normal, also shows dilated cisternae and vacuoles. In addition, a great number of large granules is present in the cytoplasm, most of them being extracted as artifacts. An appreciable number of cytoplasmic microtubules and only a few microfilaments are also evident.

According to the morphologists, the nucleus, the granules and the large

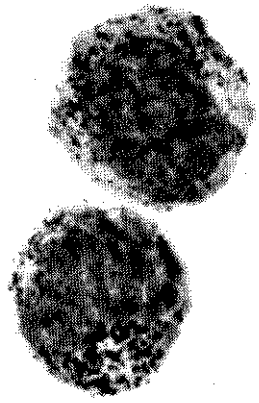

Fig. 2.2 *Light microscopic preparation of leukaemic promyelocytes in the peripheral blood* (×2100).

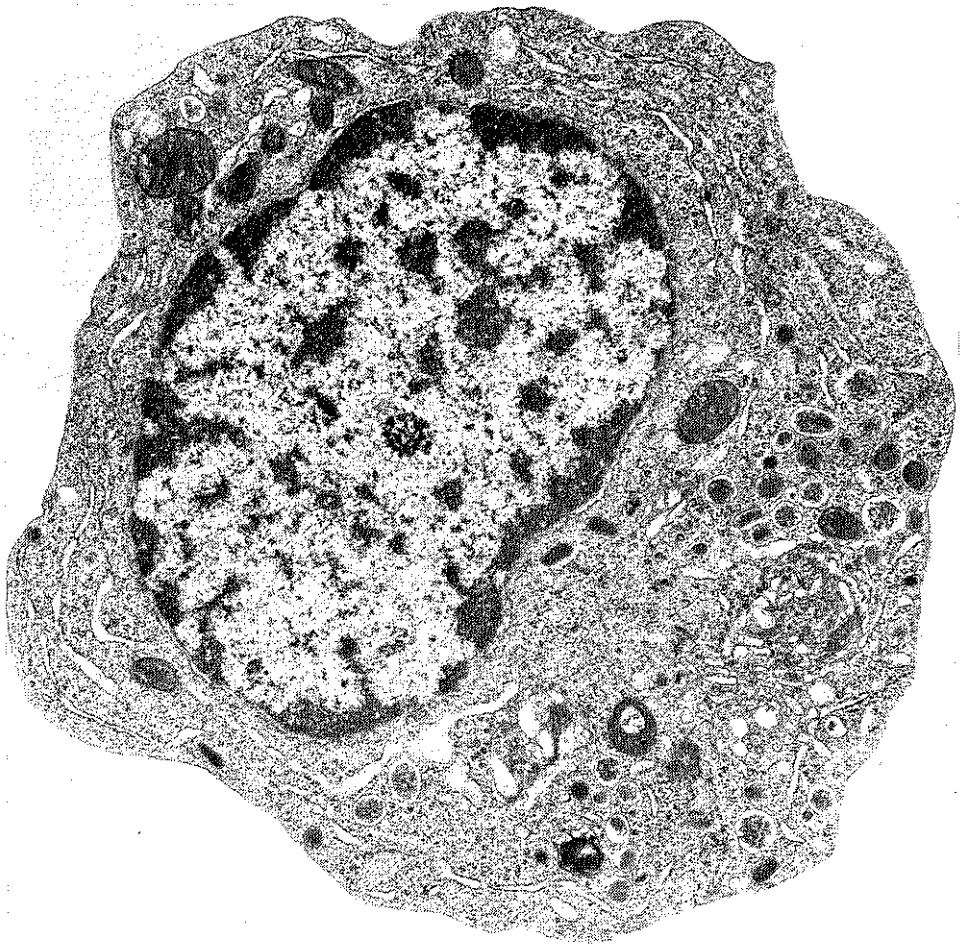

Fig. 2.3 *Electron microscopic preparation of a leukaemic promyelocyte in the peripheral blood* (×18000; Dr. H. Felix, Zürich).

quantity of endoplasmic reticulum are typical for cells of a promyelocytic leukaemia [Bernard et al., 1963; Tan et al., 1972; Valdivieso et al., 1975; Spremolla et al., 1976].

2.4.2 Cytochemistry

The cytochemical characterization of the BNML is summarized in table 2.1. The cells studied were obtained from terminal stage leukaemic blood. According to a generally accepted classification [Hayhoe, 1969; Flandrin and

Table 2.1 *Cytochemical characterization of the BN acute myelocytic leukaemia*

stain	reaction
peroxidase (Graham-Knoll)	++
esterases	
naphthol-AS-D-chloroacetate	+
α-naphthyl acetate	+
Sudan Black	+++
PAS	+*
β-glucuronidase	–
phosphatases	
acid	++
alkaline	–

* PAS: periodic acid Schiff: light diffuse discoloration.

Daniel, 1973], the observed reactions confirm the promyelocytic nature of the leukaemic cells.

2.5 Histological pattern of spread

Rats were inoculated with 10^7 BNML cells and sacrificed at various times until spontaneous death occurred at the 28th day. Organs and tissues were fixed in 4% buffered formaldehyde. Microscopic preparations stained with haematoxylin-phloxin-safran were produced by standard procedures in the Department of Pathology of the Institute for Experimental Gerontology TNO. The major histological findings during leukaemia development are summarized below.

2.5.1 Bone marrow

During the first week after inoculation, no apparent changes from normal are observed in the femoral bone marrow. At day 8, myeloid hyperplasia is noted. Some small foci of leukemic blast cells also appear at this time. Between days 8 and 18, the tumour cell deposits grow out as large clusters at the expense of normal haemopoiesis (fig. 2.4). The number of megakaryocytes decreases steadily and erythropoiesis and myelopoiesis are also disappearing. After day 18, only a very few small foci of normal haemopoiesis are left. During the last 7 days before death, the entire bone marrow cavity consists of leukaemic cells. In addition, there is also periosteal and intermuscular infiltration with leukaemia (fig. 2.5). An increasing amount of necrotic debris is observed in the bone marrow cavity.

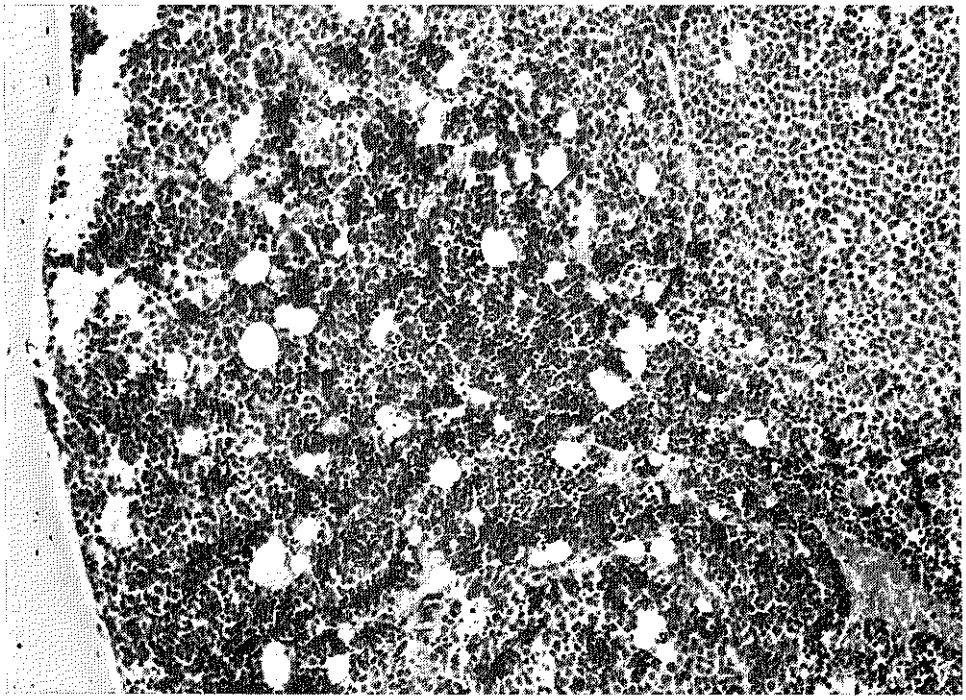

Fig. 2.4 *A large cluster of leukaemic cells (right) with remaining normal haemopoiesis (left) in the femoral bone marrow* ($\times$ 122,5; day 10 after inoculation).

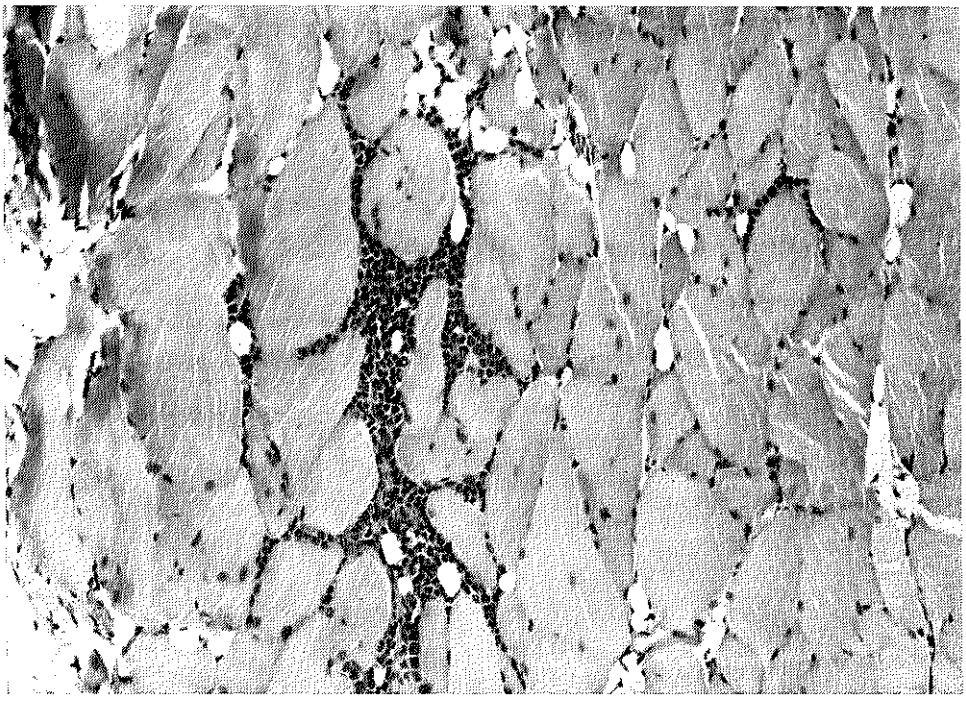

Fig. 2.5 *Intermuscular infiltration with leukaemic cells* ($\times$ 122,5; day 23 after inoculation).

2.5.2 Spleen, lymph nodes and thymus

Spleen

Although a very few small foci of normal haemopoiesis exist until day 8, in particular, the number of megakaryocytes and erythropoietic cells in the red pulp increases after this day. At the same time, the first clusters of tumor cells appear in the red pulp. The white pulp shows normal active lymph follicles. At the 18th day, most of the red pulp has been replaced by leukaemic cells. Foci of

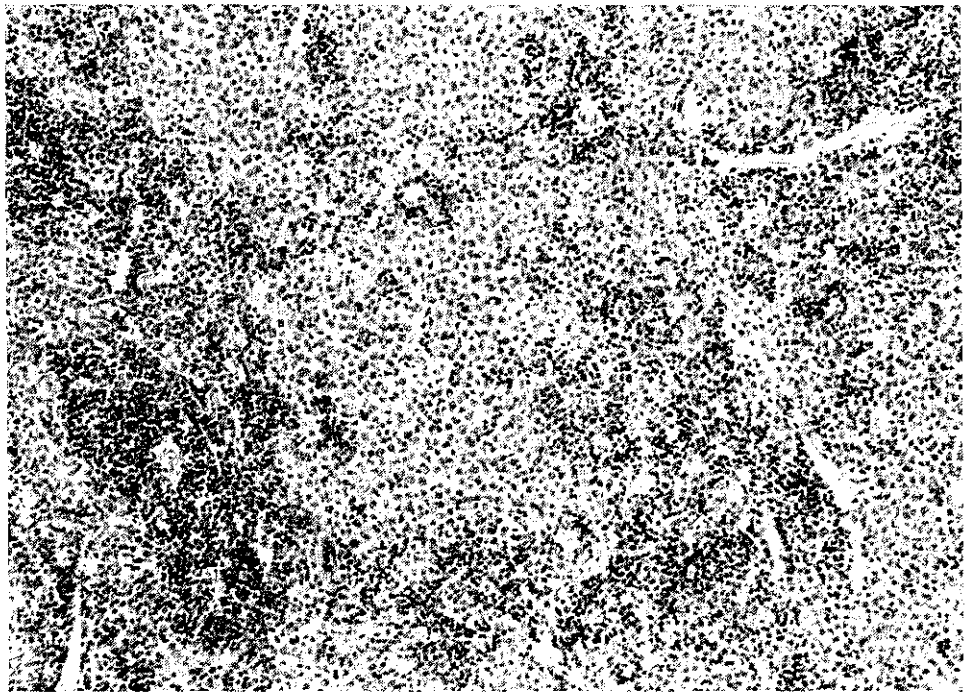

Fig. 2.6 *Replacement of the red- and the white pulp in the spleen by leukaemic cells.* Remnants of a normal lymph follicle are observed at the lower left part of the picture (× 122,5; day 18 after inoculation).

active normal haemopoiesis are observed within the tumour mass. The white pulp also shows considerable changes from normal at this time. The number of follicles has decreased, which is explained by the fact that many of them have been partly or completely replaced by the leukaemia (fig. 2.6). However some follicles are still intact. From day 18 until death, virtually the whole spleen is replaced by leukaemic tissue. Only some remnants of normal follicles and a few small clusters of haemopoietic cells are left.

Lymph nodes

Up to day 14, no apparent changes from normal are visible. Thereafter, a preferential pattern of infiltration is observed; i.e., some nodes, such as the para-aortic and liver hilus nodes, become heavily infiltrated with leukaemia cells, whereas others remain intact and active without any signs of leukaemia, e.g., the mesenteric, axillary and inguinal lymph nodes and the Peyers' patches. When a node is affected, the first clusters of tumour cells are observed in the sinusoids. From there, a diffuse infiltration occurs throughout the entire lymph node, ending up in a complete loss of normal architecture by total replacement with leukaemic cells.

Thymus

In the terminal stage of the disease, some diffuse infiltration with BNML cells is noted in the cortex and in the medulla. At the same time, the cortex shows complete atrophy.

2.5.3 Liver

The first individual leukaemic cells can be recognized in the sinusoids at day 4. At the 8th day, randomly distributed aggregates of about 10 tumour cells are observed (fig. 2.7). Signs of normal haemopoiesis, in particular erythropoiesis, although not excessive, are also noted. From day 14 onwards, a second wave of invasion occurs with the portal triads as the preferential sites (fig. 2.8). From there, sheets of leukaemic cells diffusely infiltrate the sinusoids. During the last week of the disease, the whole organ is heavily infiltrated and there is degeneration and death of many individual hepatocytes. Normal haemopoiesis completely disappears in this terminal period.

2.5.4 Lungs

The first perivascular aggregates of leukaemic cells become apparent between days 8 and 14 (fig. 2.9). At day 14, these deposits of tumour cells have started to infiltrate into the interstitial lung tissue, thereby causing obliteration and destruction of alveoli (fig. 2.10). Some subpleural leukaemic infiltrates are also noted at this time (fig. 2.11). During the last 2 weeks of the disease, the process of infiltration continues. Peribronchial lymphatic tissue is partly or completely infiltrated by BNML cells. The interalveolar septa are markedly thickened; both emphysema and atelektasis occur. Intra-alveolar haemorrhages are frequently observed. The pattern of tumour growth takes the form of large nodular aggregates (fig. 2.12).

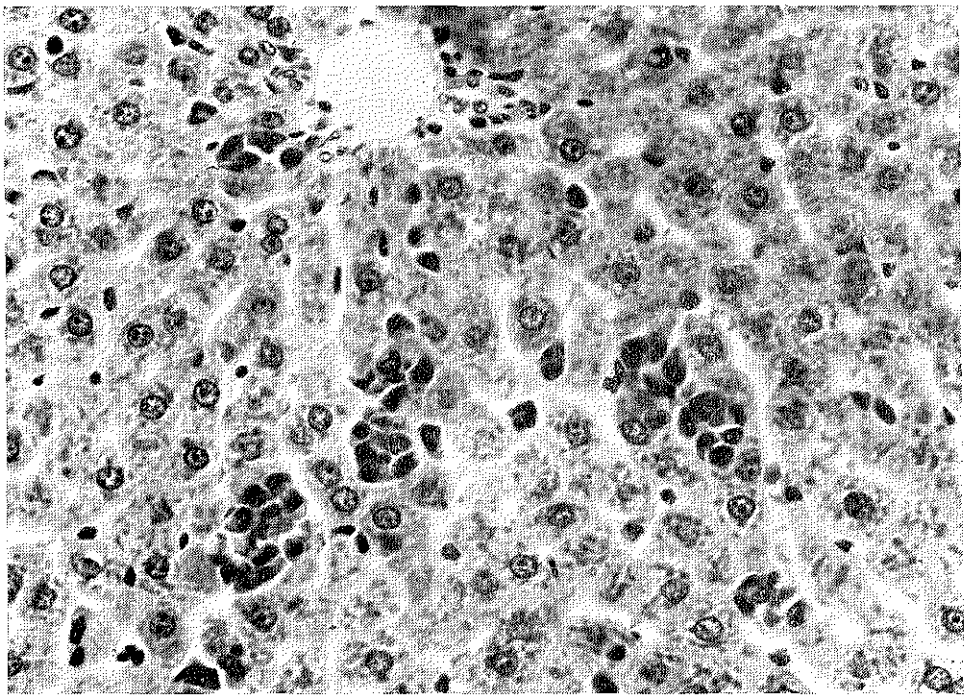

Fig. 2.7 *Small aggregates of leukaemic cells in the sinusoids of the liver* (×292; day 8 after inoculation).

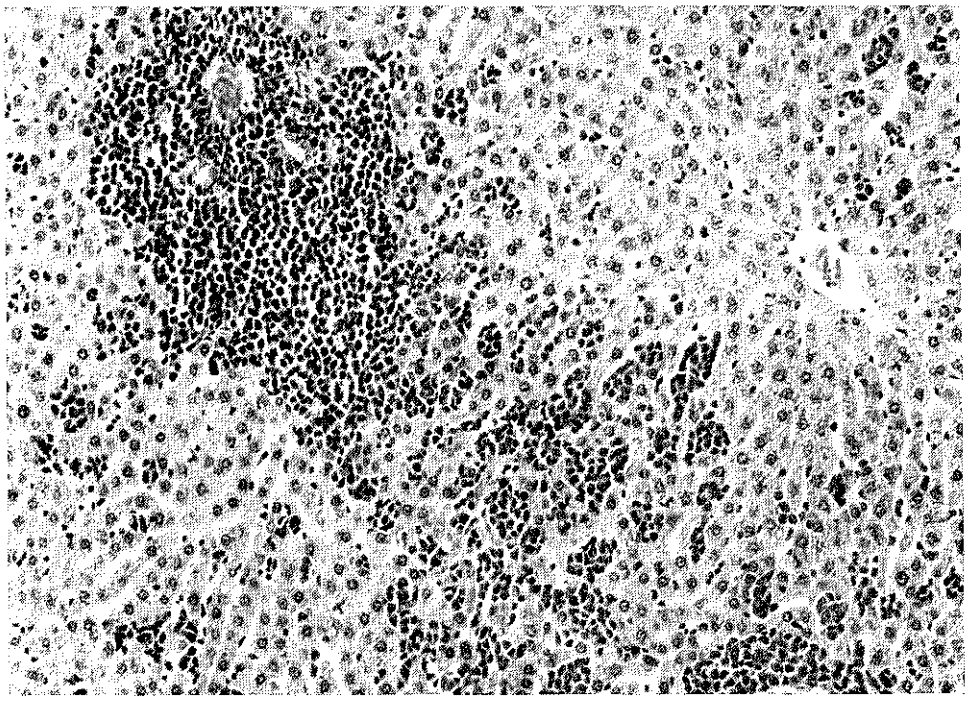

Fig. 2.8 *Massive infiltration with leukaemic cells of the liver parenchyma starting at the portal triads* (×122,5; day 18 after inoculation).

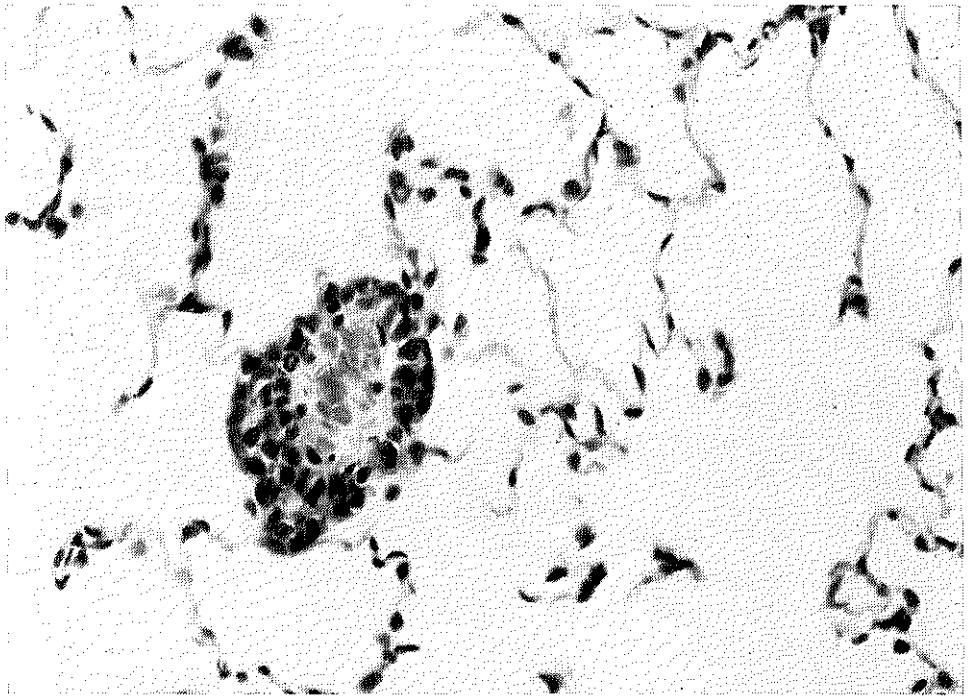

Fig. 2.9 *Aggregates of leukaemic cells around a small venule in the lungs* (× 292; day 8 after inoculation).

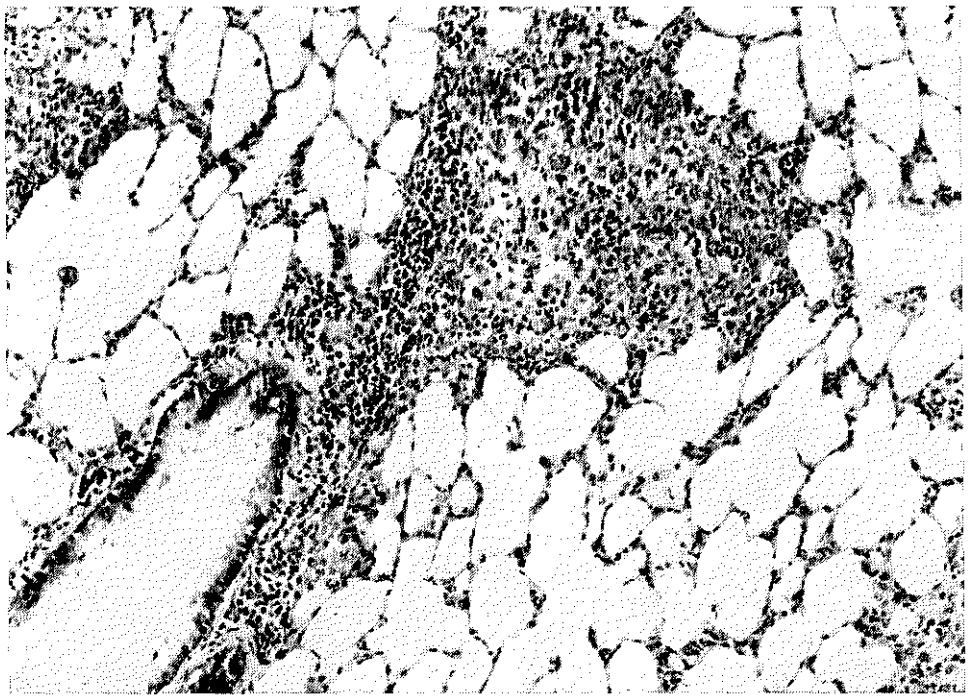

Fig. 2.10 *Infiltration with leukaemic cells of the lung parenchyma causing obliteration and destruction of alveoli* (× 122,5; day 18 after inoculation).

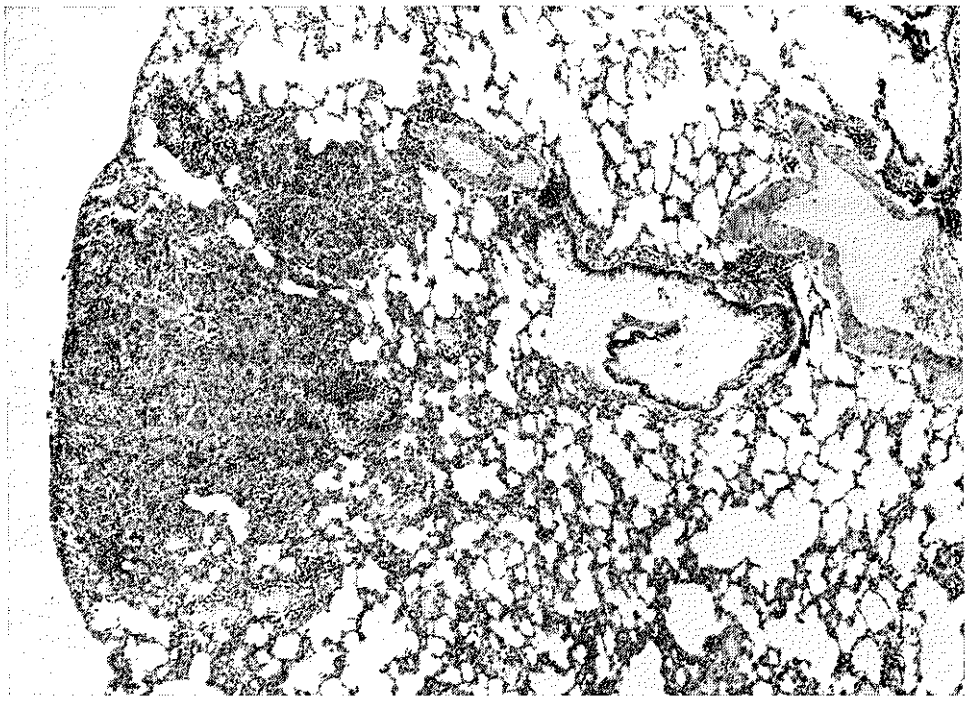

Fig. 2.11 *Subpleural infiltrate with leukaemic cells* (×46,5; day 18 after inoculation).

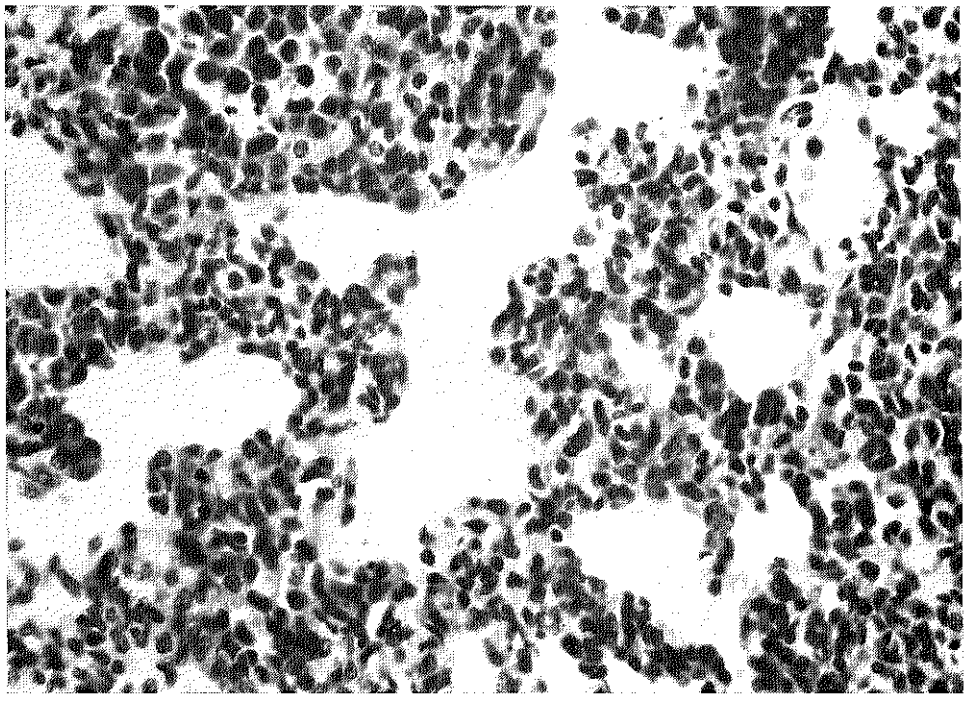

Fig. 2.12 *Nodular aggregates of leukaemic cells in the lung parenchyma* (×292; day 23 after inoculation).

2.5.5 Kidneys

No apparent signs of kidney involvement are present up to the 18th day. However, tumour cell deposits are observed in the adipose tissue surrounding the pelvis (fig. 2.13). From day 18 onwards, leukaemic cells are observed around vessels in the cortex, from where they infiltrate into the parenchyma leading to local destruction of tubules (fig. 2.14). This process continues as the leukaemia progresses. In the terminal stage, many foci of tumour cells are observed in the cortex and solid sheets of BNML cells are also present in the hilar region. However, the glomeruli remain rather well preserved. The medulla shows no signs of leukaemia throughout the entire disease period.

2.5.6 Miscellaneous

All other organs which were examined histologically during leukaemia development, such as the skin, the skeletal muscles, the heart, the intestines, the pancreas, endocrine and exocrine glands, the genitals and the adrenals, remained free of leukaemia infiltration. The central nervous system (CNS) generally also remains unaffected. In earlier transplantation generations, nodular aggregates of leukaemic cells compressing nerve roots develop along the spinal cord. This may result in paralysis of the upper and lower limbs. No clear signs of meningeal involvement are present. However, haemorrhages are occasionally observed in the spinal cord.

2.5.7 Discussion

Essentially, the infiltration pattern of leukaemic cells into the various organs runs the same course as in human AML [Robbins, 1968; Gunz and Baikie, 1974]. The following phenomena, however, deserve some more attention.

After inoculation, the first microscopic signs of leukaemia are found in the bone marrow, the spleen, the liver and the lungs. In fact, as will be described later (see Ch. 2.6.3), the majority of injected BNML cells initially lodges in these organs. The bone marrow and the spleen are the first organs to be completely replaced by leukaemic tissue. After the number of leukaemic cells in these organs have exceeded a certain critical level, around day 14 a release to the circulating blood occurs (see Ch. 2.6.2) followed by a second wave of invasion into the various organs. This release may be enhanced by changes in the vascular structure induced by leukaemic cells as has been described for the L5222 leukaemia [Hoelzer et al., 1974c]. The secondary phenomenon is clearly demonstrated by the lymph nodes and the kidneys, which show signs of leukaemia only after days 14–18, and by the liver where leukaemic cells appear around the portal triads after day 14.

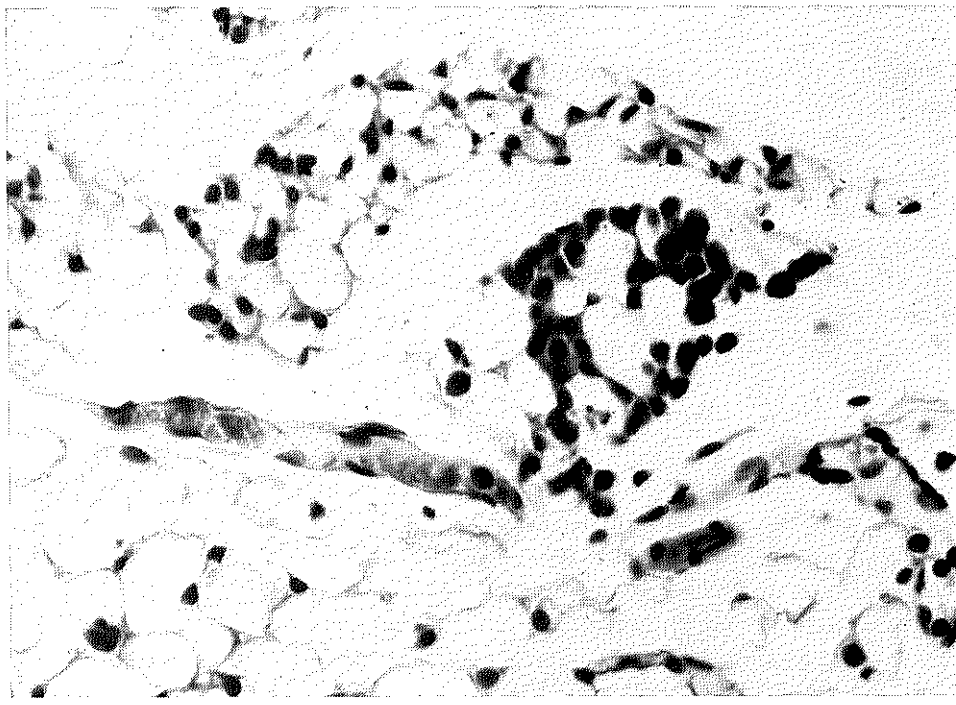

Fig. 2.13 *Deposits of leukaemic cells in the adipose tissue surrounding the renal pelvis* (×292; day 18 after inoculation).

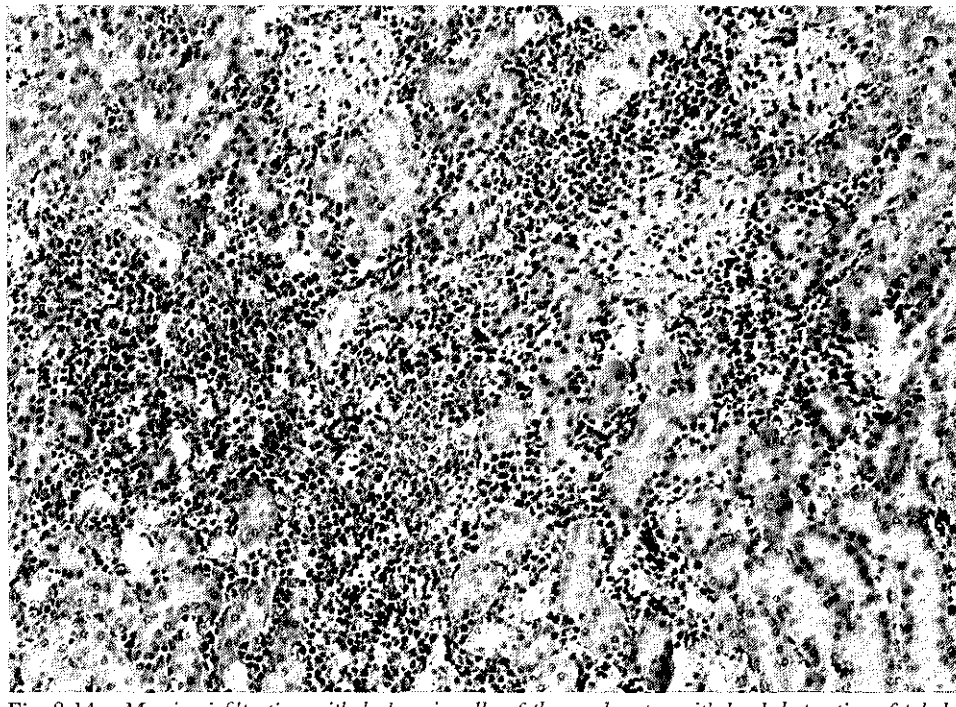

Fig. 2.14 *Massive infiltration with leukaemic cells of the renal cortex with local destruction of tubules* (×122,5; day 28 after inoculation).

Another striking phenomenon is the gradual disappearance of normal haemopoiesis. This will be discussed in more detail (see Ch. 2.6.5). Histology shows that the number of normal haemopoietic elements in the bone marrow decreases after day 8. This might be partly due to an increased release to the peripheral blood, as indicated in fig. 2.19, where the number of mature granulocytes in the blood rises after day 8. At the same time, an increasing number of foci of normal haemopoiesis is observed in the liver and especially in the spleen. In the late stage of the disease, haemopoiesis is maintained to a certain degree by these two organs, as the bone marrow is completely replaced by leukaemic cells. Finally, normal haemopoiesis in the liver and the spleen also decreases because of massive infiltration with leukaemic cells.

The heavy infiltration of specific sites of the lymphatic apparatus by leukaemic cells needs some more discussion. Especially the spleen, the para-aortic and liver hilus lymph nodes and the bronchus-associated lymphatic tissue in the lungs are completely replaced by leukaemia. This probably reduces the cellular and humoral immune reactivity of the leukaemic rat. However, the T cell mitogenic reactivity, measured by means of PHA or Con-A stimulation, was unchanged in the mesenteric lymph nodes (Dr. A. M. Kruisbeek, Rijswijk). These lymph nodes are free of leukaemia during the entire disease period. In the thymus, enhanced mitogenic responses were noted in the terminal stage. This might be explained by the fact that the number of reactive T lymphocytes in the medulla shows a relative increase as the number of nonresponsive cortical lymphocytes decreases. The same phenomenon is observed in corticosteroid treated animals which show a considerable immunosuppression [Andersson et al., 1972]. Thus, an enhanced thymocyte mitogenic response might well correlate with an overall impaired immunoreactivity.

The selectivity of lymph node involvement can be explained by the individual drainage areas, e.g., the para-aortic nodes drain the lower limbs which contain many leukaemic cells in the bone marrow; the liver hilus nodes receive afferent lymph from the leukaemic liver and possibly also from the leukaemic spleen. Moreover, direct evidence for transport of leukaemic cells via lymphatics has been obtained by the observation that the thoracic duct fluid contains increasing numbers of BNML cells during leukaemia development. As compared with other organs, the thymus shows a remarkable resistance to invasion by leukaemic cells. This has also been described for other rat leukaemia models such as the Shay myelocytic leukaemia [Harris et al., 1958; Kelenyi, 1959], the L5222 leukaemia [Hoelzer et al., 1973a] and the SAL myelocytic leukaemia [Wrathmell, 1976]. Cortex atrophy may be due to an increased production of corticosteroids by tumour bearing animals [Fachet et al., 1975], caused either by stress or leukaemia-induced toxicity.

The fact that the CNS remains free of leukaemia seems to be specific for the

BNML; CNS involvement is a prominent feature in other rat leukaemia models [Rosin and Zajicek, 1961; Calvo and Hoelzer, 1976].

Finally, death is due to a combination of several factors. Severely depressed normal haemopoiesis which quickly leads to thrombocytopenia (see Ch. 2.6.2) causes bleeding at various sites (gastrointestinal tract, respiratory tract, spinal cord, etc.). The infiltrated lungs with often superimposed infections also present serious problems with respect to cardiovascular function. The heavy infiltration of the liver and the kidneys is quite likely to lead to impaired function of these organs. In summary, bleeding, infections and impaired organ functions are the responsible factors in the death of the animal.

2.6 Kinetics of leukaemic growth

Various *in vivo* assays were performed to quantify the leukaemic growth process. These included:

1) cell titrations;
2) growth rate of whole organs;
3) distribution kinetics;
4) proliferation kinetics.

2.6.1 Cell titrations

Dose-survival time

Rats were inoculated with varying numbers of BNML cells (10^3–10^7) obtained from either the blood, the spleen or the bone marrow. Average survival times were calculated and dose-survival curves were plotted after linear regression analysis by computer.

From fig. 2.15, it is clear that BNML cells harvested either from the spleen, the blood or the bone marrow show an identical dose-survival relationship.

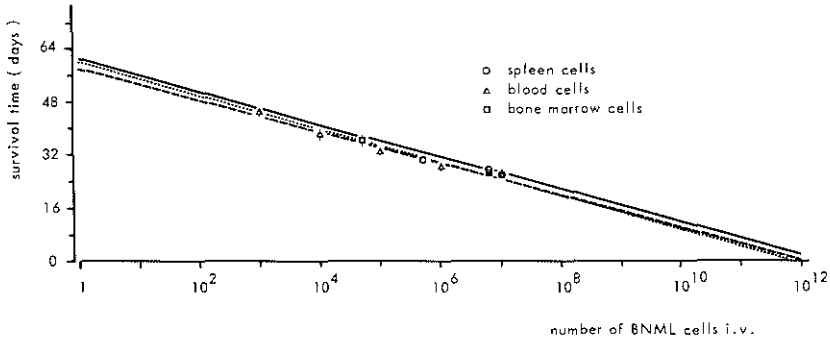

Fig. 2.15 *Dose-survival time curves for BNML cells from different sources.* Each point represents means (±SE) of 5–11 rats.

After extrapolation, one single cell would be expected to cause death after about 60 days. A total tumour load of 10^{12} cells would be incompatible with life, as is deduced from extrapolation towards the X-axis. This would correspond to approximately 1 kg of tumour tissue, which is apparently unreal. The actual tumour cell mass will be dealt with later (Ch. 2.6.5).

The linear relationship between log cell dose and survival time provides a means to determine the number of BNML cells in a given organ.

Dose-spleen weight

Graded numbers of BNML cells (10^4–10^8) were inoculated into various groups of rats. The animals were sacrificed twenty-five days later. The spleens were removed an their fresh weights determined.

The relationship between the spleen weight and varying doses of BNML cells can be characterized as follows (fig. 2.16). For each tenfold increase in the number of inoculated cells, the spleen weight increases by a factor of about 2 (1.7–2.2). However, this holds only for doses up to 10^7 cells. For doses below this, the curve can be used to determine the number of BNML cells in a given cell suspension. A higher cell dose (10^8) does not cause a further increase in the spleen weight; a plateau has apparently been reached. The implications of these findings will be discussed below (Ch. 2.6.5).

TD_{50}

The effective dose of BNML cells which kills 50% of the animals (TD_{50}) was

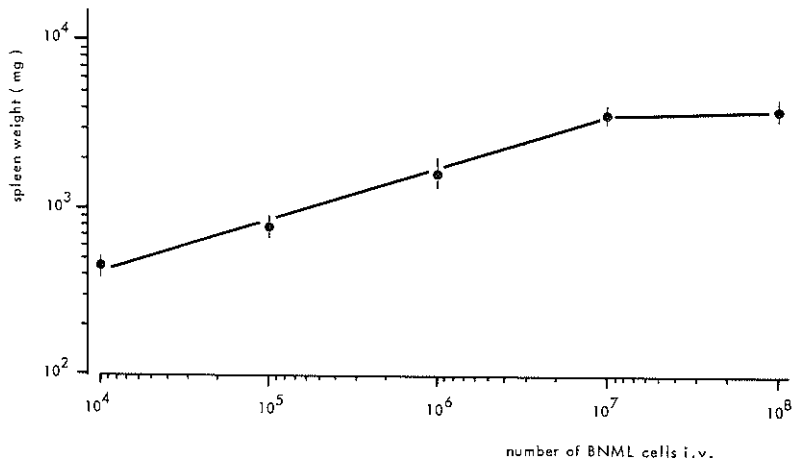

Fig. 2.16 *Spleen weight at day 25 after inoculation with graded numbers of BNML spleen cells.* Each point represents means (±SD) of 5 rats.

determined by injecting rats with low numbers of cells obtained from terminal stage femoral bone marrow (84% leukaemic cells; 17% dead cells) and scoring the number of deaths within 150 days after inoculation. All remaining rats survived beyond this period. Each cell dose was injected into a group of 6 rats. The data were evaluated by means of probit analysis. In these experiments, siliconized glassware (Siliclad, Clay Adams, Parsippany, USA) was used to prevent cells from sticking to the wall of the bottles.

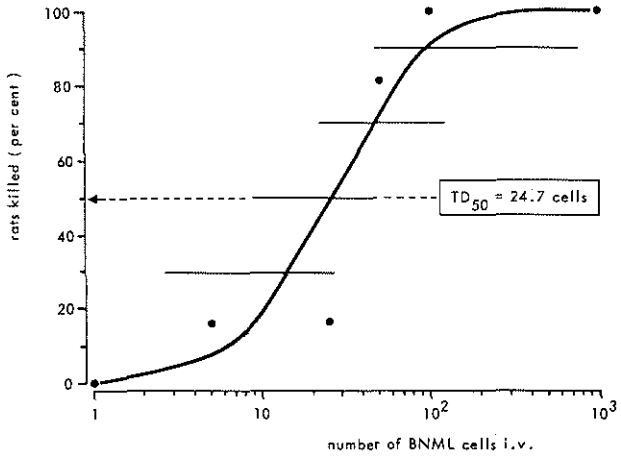

Fig. 2.17 *Per cent of rats killed by low doses of BNML bone marrow cells.*
Each point was obtained from 6 rats receiving the same cell dose.
Horizontal bars represent 95% confidence limits.

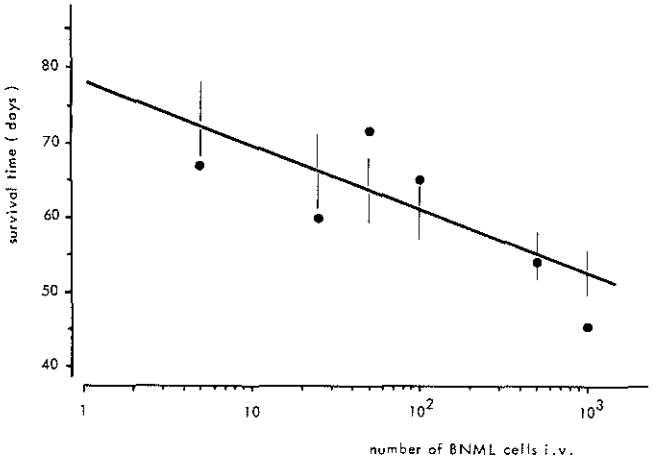

Fig. 2.18 *Survival after injection with low doses of BNML bone marrow cells.*
Each point represents means of 1–6 rats.
Vertical bars represent 95% confidence limits.

From fig. 2.17, a TD_{50} value of 24.7 cells is obtained within 95% confidence limits ranging from 9.0 to 48.6 cells. The dose-survival curve was plotted by linear regression analysis (fig. 2.18). Although none of the rats which received 1 BNML cell died, the mean survival time calculated after extrapolation of this curve would be 78.3 days if one leukaemogenic cell was inoculated. This figure is somewhat larger than the one obtained from the dose-survival study using larger inocula (fig. 2.15). All rats that died in the TD_{50} experiment showed the classical BNML picture.

2.6.2 Growth rate of whole organs

Peripheral blood

Rats were inoculated with 10^7 BNML cells and killed at regular intervals up to day 28. The following parameters were determined: total and differential

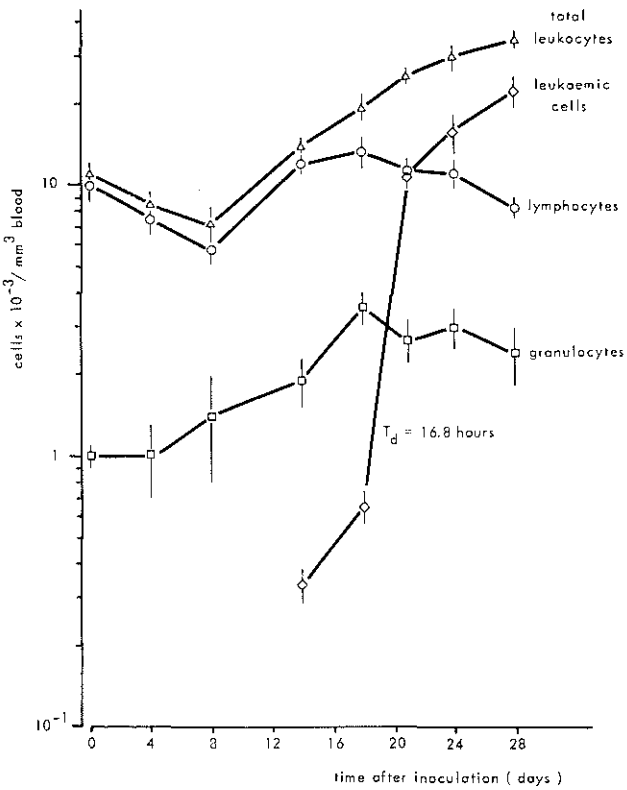

Fig. 2.19 *Changes in the number of cells in the peripheral blood after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 5–7 rats.
Day 0: non-leukaemic controls.

counts of the peripheral blood, number of erythrocytes, reticulocytes, platelets and haematocrit values.

Changes in the absolute number of total leukocytes, leukaemic cells, lymphocytes and granulocytes during the development of the leukaemia are plotted in fig. 2.19. Granulocytes show a moderate increase from the beginning. At the terminal stage, a two to threefold increase in the initial number is noted. After an initial drop, the number of lymphocytes again increases to remain at a fairly constant normal level. Leukaemic cells are detected only at the end of the second week. They show a rapid exponential increase (T_d = 16.8 h) up to day 21. Thereafter, the curve begins to level off. Besides granulocytes, leukaemic cells are mainly responsible for the observed elevated total leukocyte counts during the last 2 weeks of the disease.

Table 2.2 *Differential blood cell counts* after inoculation with 10^7 BNML spleen cells*

	days after inoculation						
	0	4	8	14	18	23	28
myeloblasts	–	–	0.3	1.7	1.7	2.7	1.7
promyelocytes	–	–	–	0.7	1.7	49.0	63.7
myelocytes	–	0.3	1.0	1.3	3.3	4.3	2.7
metamyelocytes	–	0.3	2.7	1.3	3.3	2.0	2.0
J^1 neutrophils	0.7	2.3	0.3	8.3	5.3	2.0	1.0
PM^2 neutrophils	3.3	5.3	9.0	3.0	6.0	1.7	1.3
eosinophils	1.0	2.0	4.0	1.3	1.3	0.3	–
lymphocytes	93.7	89.3	80.3	80.3	70.3	37.7	24.3
monocytes	1.0	0.7	1.0	–	–	–	–
normoblasts	–	–	–	0.3	0.3	0.3	2.3

* Expressed as % of the total number of nucleated cells (see fig. 2.19).
Each value represents means of 3 rats.
J^1 = juvenile.
PM^2 = polymorphonuclear.
Day 0 = non-leukaemic controls.

Differential counts for the peripheral blood are presented in more detail in table 2.2. The most striking event is the rapid increase in leukaemic promyelocytes from day 14 onwards. Another interesting phenomenon is the appearance of immature myelocytic cells such as myeloblasts, myelocytes and metamyelocytes. On the other hand, lymphocytes show a relative decrease during leukaemia development. Normoblasts appear in the blood during the last 2 weeks of the disease.

Fig. 2.20 shows the changes in the number of erythrocytes and reticulocytes and haematocrit values during leukaemia development. Erythrocytes and

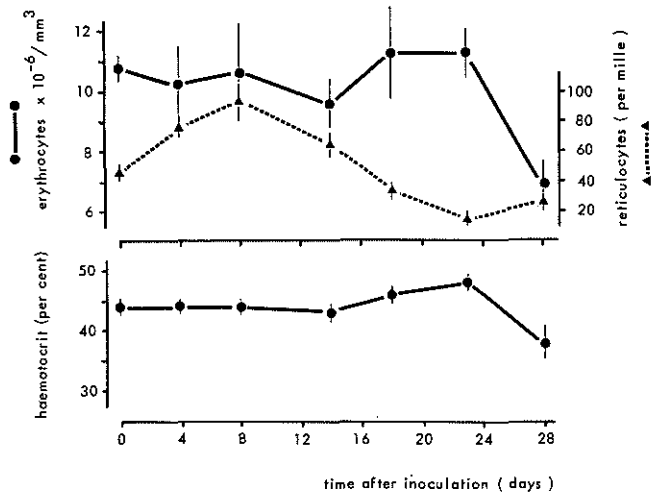

Fig. 2.20 *Changes in the number of erythrocytes and reticulocytes and haematocrit values after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 6 rats.
Day 0: non-leukaemic controls.

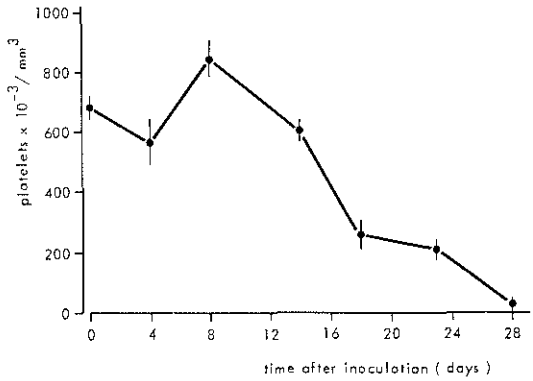

Fig. 2.21 *Changes in the number of platelets in the peripheral blood after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 6 rats.
Day 0: non-leukaemic controls.

haematocrit values show a decrease towards the later stage of the disease. A decrease in the number of reticulocytes is apparent from day 8 onwards.

Platelets show a rapid decline beginning at day 8 after inoculation (fig. 2.21).

Bone marrow

Fig. 2.22a shows the changes in total cellularity in the femoral bone marrow

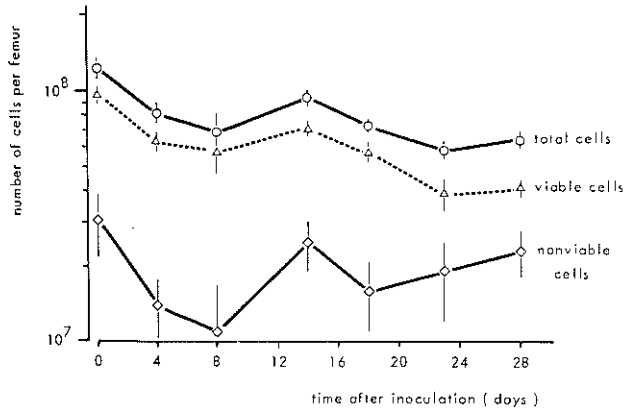

Fig. 2.22a *Changes in femoral bone marrow cellularity after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 3 femurs.
Day 0: non-leukaemic femurs.

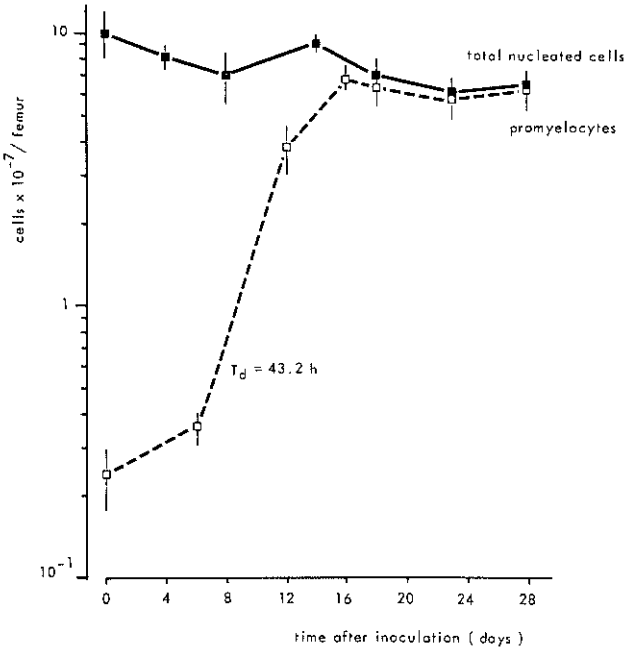

Fig. 2.22b *Changes in the absolute number of leukaemic promyelocytes in the femoral bone marrow after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 3 femurs.
Day 0: non-leukaemic controls.

during leukaemia development expressed in terms of viable and nonviable nucleated cells. After an initial decrease in total cellularity, a small increase is observed around day 14; this is followed by a decrease again during the last 2 weeks of the disease. The proportion of nonviable cells relative to the total number of cells tends to increase as the leukaemia progresses.

Table 2.3 *Differential bone marrow cell counts* after inoculation with 10^7 BNML spleen cells*

	days after inoculation						
	0	6	12	16	18	23	28
myeloblasts	2.0	1.0	0.7	0.3	0.1	0.3	0.4
promyelocytes	2.4	4.8	44.8	84.7	90.1	96.7	98.0
myelocytes	5.0	4.6	1.6	0.6	0.3	0.1	0.1
metamyelocytes	9.6	11.3	4.0	1.9	0.7	0.3	0.1
J^1 neutrophils	22.6	21.3	10.5	4.5	5.9	0.6	0.2
PM^2 neutrophils	2.5	2.2	1.1	0.7	0.6	0.3	0.1
eosinophils	3.6	10.1	12.1	1.9	1.4	0.3	0.2
erythroblasts	4.8	2.6	0.9	0.2	–	–	–
normoblasts	21.6	17.2	6.4	1.3	0.8	0.3	0.1
megakaryocytes	1.0	1.0	0.6	–	–	–	–
lymphocytes	23.2	22.8	17.4	3.6	1.2	0.5	–
monocytes	0.2	0.3	0.3	–	–	–	–
plasma cells	1.2	0.4	0.7	0.3	–	–	–

* Expressed as % of the total number of nucleated cells (see fig. 2.22a).
Each value represents means of 3 rats.
J^1 = juvenile.
PM^2 = polymorphonuclear.
Day 0 = non-leukaemic controls.

Table 2.3 shows the differential bone marrow cell counts. The most striking events can be summarized as follows. Normal haemopoiesis for all cell lines is severely depressed during leukaemia development. Lymphocytes, monocytes and plasma cells completely disappear. Leukaemic promyelocytes increase steadily from day 12 onwards and finally almost completely replace the normal bone marrow elements. The absolute numbers of leukaemic promyelocytes were calculated from total and differential cell counts. These are plotted in fig. 2.22b. Their doubling time between days 6 and 12 is 43.2 h.

Apart from the morphological analysis, bioassays were performed to quantify the number of leukaemic cells in the bone marrow during the early stage after tumour inoculation. At 3 h, 24 h, 72 h and 144 h after injection with 10^7 BNML cells, rats were killed and 10^7 femoral bone marrow cells were injected into new recipients. The number of leukaemic cells in these inocula, which cannot be quantified on a morphological basis, could be derived by scoring survival times

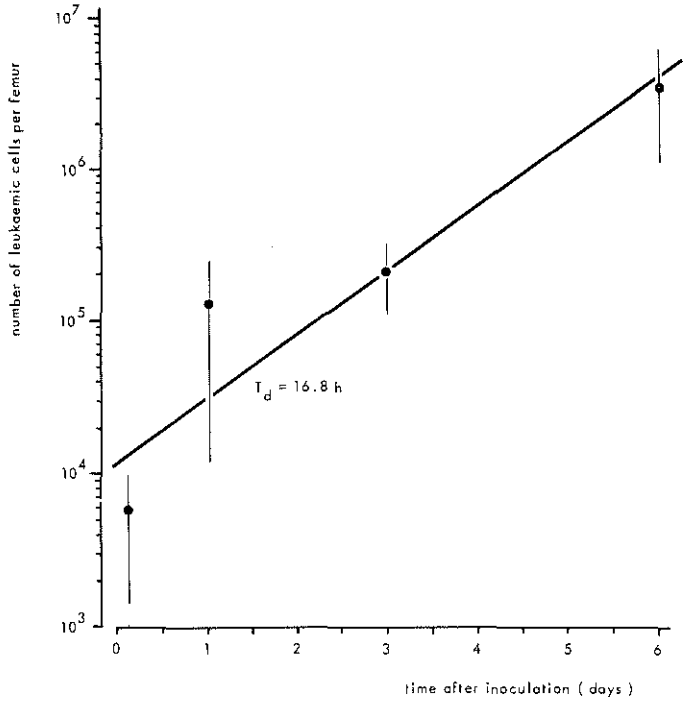

Fig. 2.23 *Changes in the number of leukaemic cells in the femoral bone marrow after inoculation with 10^7 BNML spleen cells (bio-assay).*
Each point represents means (±SE) of 6 rats.

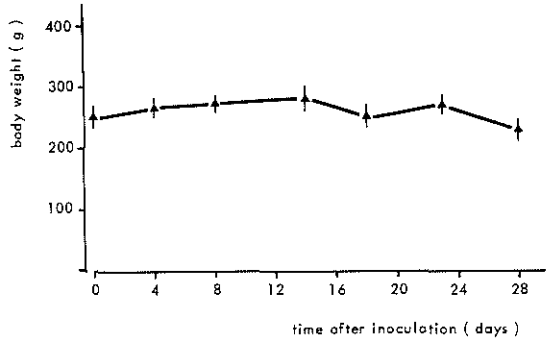

Fig. 2.24 *Changes in body weight after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SD) of 6 rats.
Day 0: non-leukaemic controls.

and comparing these with standard dose-survival curves produced with the initial leukaemic cell suspension consisting of more than 90% leukaemic cells. From the known initial total number of cells per femur, the absolute number of leukaemic cells could be calculated. These are given in fig. 2.23. From the curve which was plotted after linear regression analysis, the doubling time (T_d) of the leukaemic cell population was derived: $T_d = 16.8$ h.

Body weight

From fig. 2.24, it is clear that no significant changes in body weight occur during the entire disease period. Therefore, changes in individual organ weights can be directly compared without relating them to changing body weights.

Liver, spleen and thymus

Changes in the fresh weights of the liver, the spleen and the thymus during leukaemia development are presented in fig. 2.25.

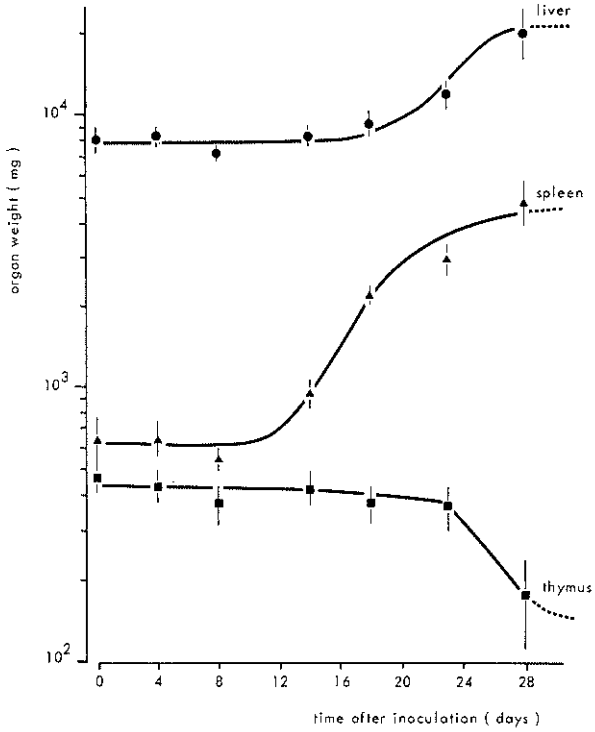

Fig. 2.25 *Changes in the weight of the liver, the spleen and the thymus after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SD) of 6 rats.
Day 0: non-leukaemic controls.

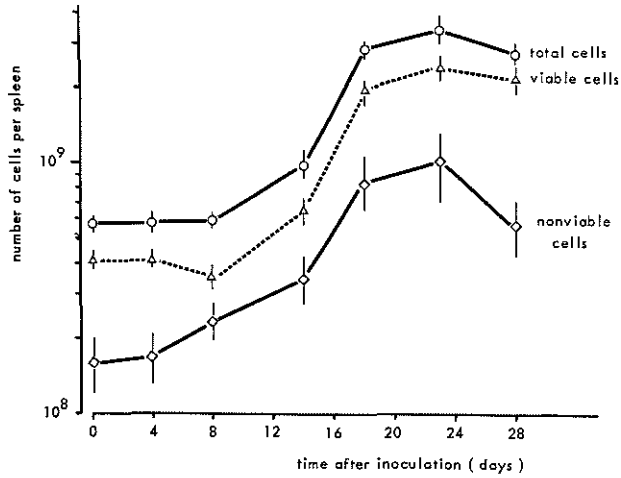

Fig. 2.26 *Changes in spleen cellularity after inoculation with 10^7 BNML spleen cells.* Each point represents means (±SE) of 3 spleens. Day 0: non-leukaemic spleens.

After an initial lag period of 14 and 8 days, respectively, both the liver (day 14–28) and the spleen (day 8–28) show a phase of exponential growth after inoculation with BNML cells. At death, the liver weight is 2 to 3 times normal and the spleen weight has increased by a factor of 8. The weight of the thymus decreases significantly in the terminal stage.

Changes in spleen cellularity during the development of the BNML are shown in fig. 2.26. A rapid increase in the total number of nucleated spleen cells is observed after day 8. From day 18 until death, a plateau is noted. Although the absolute number of nonviable cells increases with increasing cellularity, the same percentage level is maintained, i.e., about 30% of the total number of cells are dead. In table 2.4, splenic cellularity as a function of time after leukaemia inoculation is expressed as the number of cells per gram of spleen. One gram of splenic tissue contains approximately 10^9 nucleated cells at all stages of the disease.

Table 2.4 *Changes in splenic cellularity after inoculation with 10^7 BNML spleen cells*

days after inoculation	spleen weight (g)	number of cells ($\times 10^9$)	number of cells ($\times 10^9$) per gram spleen
8	0.555±0.024	0.59±0.04	1.06±0.11
14	0.942±0.048	0.99±0.13	1.05±0.15
18	2.212±0.065	2.65±0.18	1.20±0.07
23	3.012±0.302	3.19±0.44	1.06±0.01
28	4.160±0.382	3.90±0.26	0.94±0.08

Values represent means (±SE) of 5 measurements.

By means of a bioassay (dose-survival), the absolute number of leukaemic cells was determined in the spleen during the first week after leukaemia transfer by the same method as described for the femoral bone marrow. From fig. 2.27, a doubling time (T_d) of 37.2 h was derived.

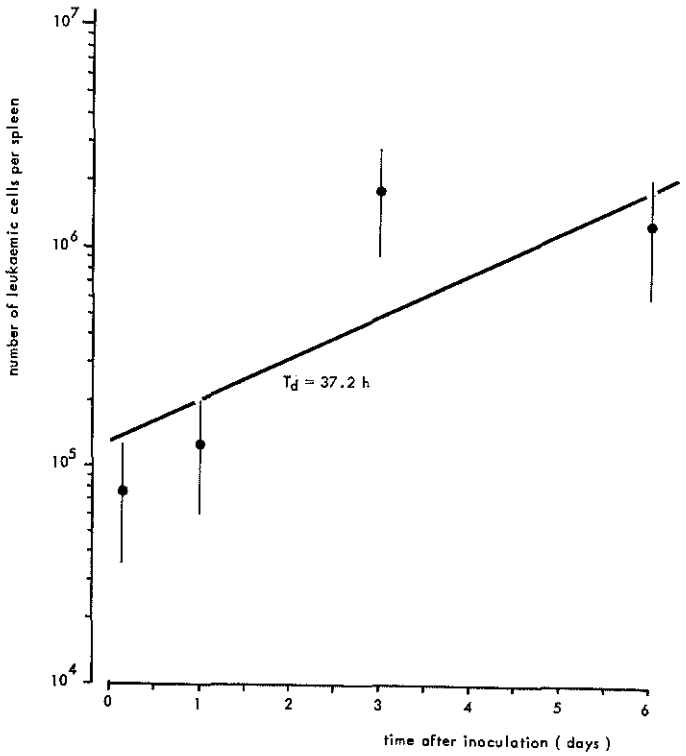

Fig. 2.27 *Changes in the number of leukaemic cells in the spleen after inoculation with 10^7 BNML spleen cells (bio-assay).*
Each point represents means (±SE) of 6 rats.

2.6.3 Distribution kinetics

The distribution of injected leukaemic cells within the organism was performed with BNML cells obtained from the blood after *in vitro* separation and labelling with ^{51}Chromium. The reasons for choosing this particular isotope will be discussed later (see Ch. 2.6.5). Various functional compartments can be quantified by determination of patterns of blood disappearance and organ uptake, as will also be described (see Ch. 2.6.5). Moreover, quantitative information can be obtained on cell traffic, e.g., transit times and turnover rates in the various compartments.

Technical aspects of cell separation, labelling and *in vivo* follow-up will be first described.

Cell separation

To obtain leukaemic cells from the blood of terminal stage rats, a two-step separation procedure was used. The objective was to remove the erythrocytes, which also bind ^{51}Chromium. The cells were first allowed to sediment spontaneously after mixing with Haemaccel (Behring Pharma, Amsterdam, Holland) in a concentration of 1 volume of cell suspension per 2 volumes of Haemaccel. The calcium ions present in the Haemaccel solution were neutralized by adding 0.5 g EDTA/100 ml Haemaccel to prevent blood coagulation after mixing. Sedimentation was allowed to take place for 15–30 min at room temperature in centrifuge tubes placed at an angle of 30 °C to the horizontal surface. The supernatant which contained the leukocytes was removed and Haemaccel was again added to the remaining sediment. After this second sedimentation (which was meant to increase the total cell yield), both supernatants were pooled. With this procedure, the total recovery was between 90 and 100% of the original number of cells present. After the cells were washed once with 0.9% NaCl (10 min, 700 g), the suspension was carefully layered onto a mixture of Ficoll 400 (MW 400,000; Pharmacia Fine Chemicals, Uppsala, Sweden) and Isopaque (Nyegaard and Co, Oslo, Norway), with a density of 1.077 g/cm^3. One volume of cell suspension containing $0.5-1.5\times10^8$ leukocytes per ml plus one volume of Ficoll-Isopaque was then centrifuged (20 min, 700 g). The leukocyte layer at the interface was removed and washed twice with 0.9% NaCl. The total cell yield after this second separation step was between 60 and 70%. The leukocytes, of which 5–15% were scored as dead cells, consisted mainly of leukaemic cells (60–80%); however, contamination with granulocytes and lymphocytes seemed to be inevitable. Erythrocytes were efficiently removed. In the final suspension, they represented about 2–8% of the total number of blood cells present. Platelets were virtually absent.

^{51}Chromium-labelling

Ten ml of the cell suspension containing $3–10\times10^7$ cells/ml were placed in a water bath at 37 °C. ^{51}Chromium (Na_2CrO_4; specific activity 50–400 mCi/mg Cr; The Radiochemical Centre, Amersham, England) was added in a concentration of 40 μCi/10^8 cells. In no case did the Na_2CrO_4 concentration exceed 0.8 μg/ml. After 45 min incubation with intermittent mixing, 30 mg ascorbic acid (Lamers and Idemans, 's-Hertogenbosch, Holland) per 200 μCi ^{51}Cr was added. Ascorbic acid reduces the remaining free hexavalent chromium to the trivalent state which is incapable of further cell binding [McMillan and Scott,

1968]. The cell suspension was then washed 3 times with 0.9% NaCl (10 min, 700 g). This resulted in the effective removal of about 98–99% of the excess ^{51}Cr in the supernatant. A final cell count was made and the cell suspension was diluted to an appropriate concentration. The percentage of dead cells at the end of the procedure was between 10 and 20%. The total cell yield calculated from the initial number before separation was betweeen 50 and 60%. Separation and labelling take about 3 hr.

Determination of cell distribution *in vivo*.

Before the suspension was injected, several samples were taken for calculating the total amount of cell-bound ^{51}Cr to be injected per rat. In a given sample, the γ-radioactivity emitted by the isotope, expressed as counts per min (cpm), was measured in a well-type scintillation counter (Model 4227, Nuclear-Chicago Corp., Des Plaines, Ill., USA). The background radioactivity was subtraced from each counted sample. Each rat received a fixed volume of the suspension (usually 1 ml injected within 10 sec) either intravenously or intra-arterially through an exteriorized cannula which was inserted into the abdominal aorta (see Ch. 4.2). Depending on the type of experiment, arterial blood samples (0.2 ml) were then taken at regular time intervals from the canula or directly from the abdominal aorta by puncture after general anaesthesia with ether. The ^{51}Cr activity of the samples was then determined. To determine the organ uptake of labelled leukaemic cells at a given time after injection, rats were killed by an ether overdose and the organs were removed and their fresh weights determined. The whole organ or a weighed part of it was then measured and recalculated for ^{51}Cr γ-activity. To establish bone marrow uptake, the count for one femur was determined after it had been carefully cleaned of adherent muscles.

The total number of ^{51}Cr-labelled leukaemic cells per circulating blood volume (1) and per organ (2) as a percentage of the injected radioactivity was calculated as follows.

(1) Blood:

$$\text{Recovery} = \frac{V_b \times cpm_s}{V_s \times cpm_{inj.}} \times 100\%,$$

where V_b is the circulating blood volume (ml), V_s is the volume of the blood sample (ml) and cpm_s and $cpm_{inj.}$ represent the radioactivity in the blood sample and the total amount of injected radioactivity, respectively.

(2) Organs:

$$\text{Uptake} = \frac{W_t \times cpm_s}{W_s \times cpm_{inj.}} \times 100\%,$$

where W_t stands for total organ weight (mg) and W_s for the weight (mg) of the organ sample on which the counts (cpm_s) were made.

Experiments

a. Kinetics of organ uptake of ^{51}Cr-labelled BNML blood cells during 24 h after infusion.

^{51}Cr-labelled BNML blood cells were injected i.v. into 30 rats at day 23 after inoculation with 10^7 BNML cells. Groups of 3 rats were sacrificed at regular intervals (1, 5, 15 and 30 min, 1, 2, 4, 8, 19 and 24 h after infusion) to determine the amount of labelled cells present in the circulating blood and in the various organs at that particular time.

Blood disappearance and organ uptake of labelled leukaemic blood cells are plotted in figs. 2.28, 2.29 and 2.30. After a rapid initial cell loss from the circulating blood, the blood disappearance curve shows 2 main components (fig. 2.28). The migration of cells to the spleen, the kidney, the femoral bone marrow (fig. 2.29) and the liver (fig. 2.30) is characterized by a rapid uptake within the first 2 h. A plateau is reached between 2 and 4 h. From 4 h onwards, a very slow decrease in radioactivity is noted in these organs.

A different picture is seen in the lungs (fig. 2.30). Shortly after infusion, about 40% of the infused cells are trapped in this organ. This is followed by a rapid

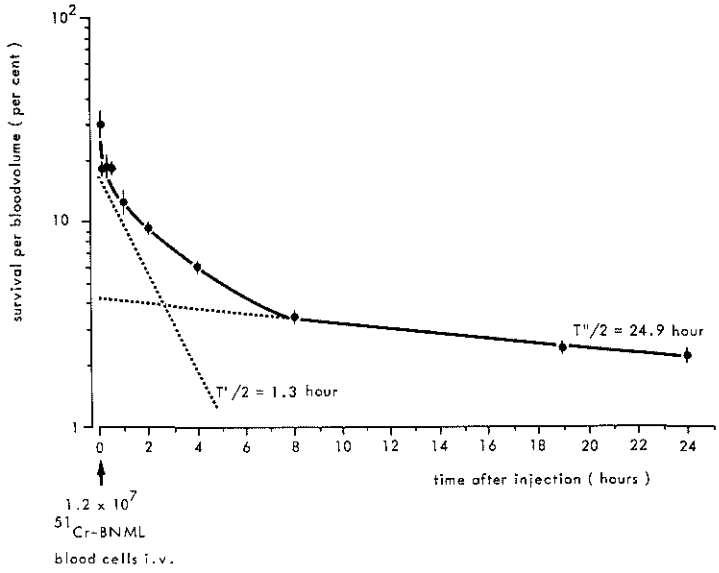

Fig. 2.28 *Blood disappearance of ^{51}Cr-labelled BNML cells after i.v. infusion into rats at day 23 after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 3 rats.

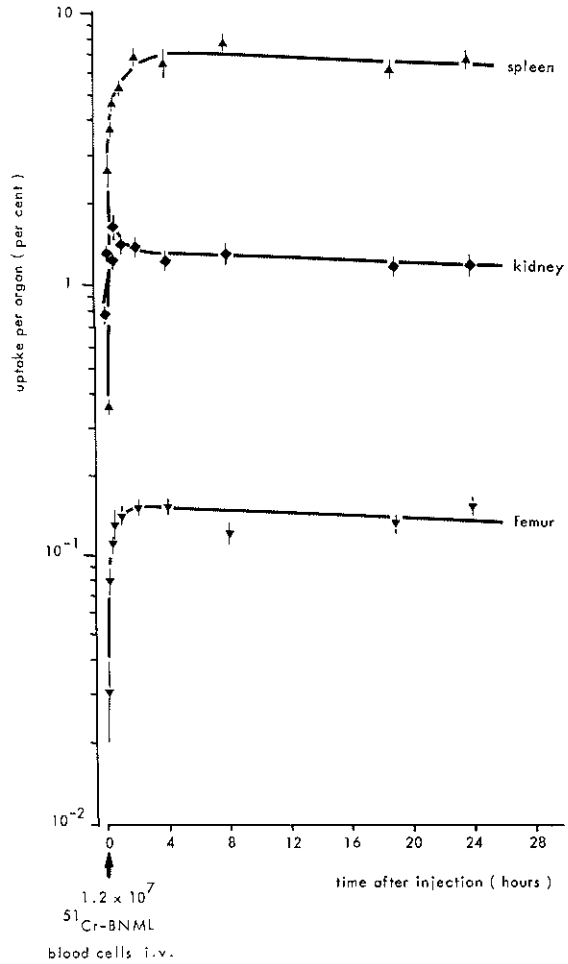

Fig. 2.29 *Organ uptake of ^{51}Cr-labelled BNML cells after i.v. infusion into rats at day 23 after inoculation with 10^7 BNML spleen cells.*
Each point represents means (±SE) of 3 rats.

disappearance characterized by 2 main components: up to 2–4 h, a rather rapid release occurs; from 4 h up to the end of the observation period, a plateau is established.

b. Distribution of ^{51}Cr-labelled BNML blood cells infused at various stages after leukaemia inoculation.

Three groups of 5 rats each received labelled BNML blood cells i.a.: group 1 without previous inoculation with leukaemic cells (day 0, controls), and groups 2 and 3 at the 10th and 28th day (terminal stage) respectively, after 10^7 BNML

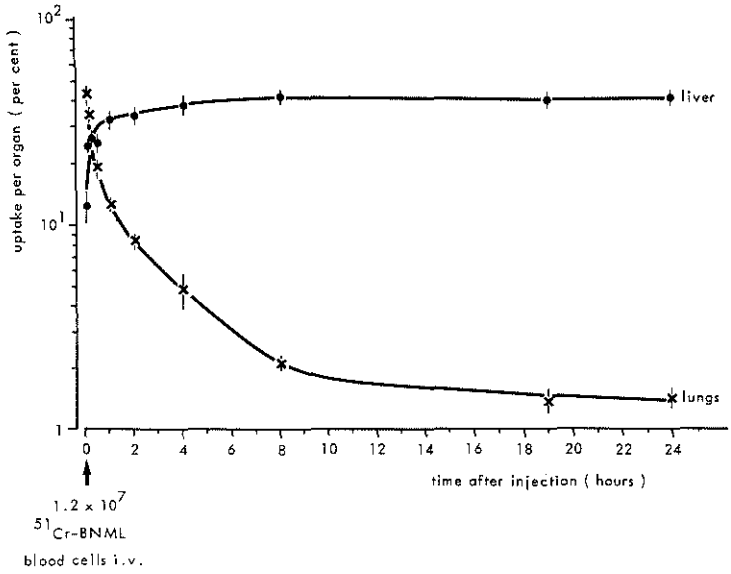

Fig. 2.30 *Organ uptake of* ^{51}Cr-*labelled BNML cells after i.v. infusion into rats at day 23 after inoculation with* 10^7 *BNML spleen cells.*
Each point represents means (±SE) of 3 rats.

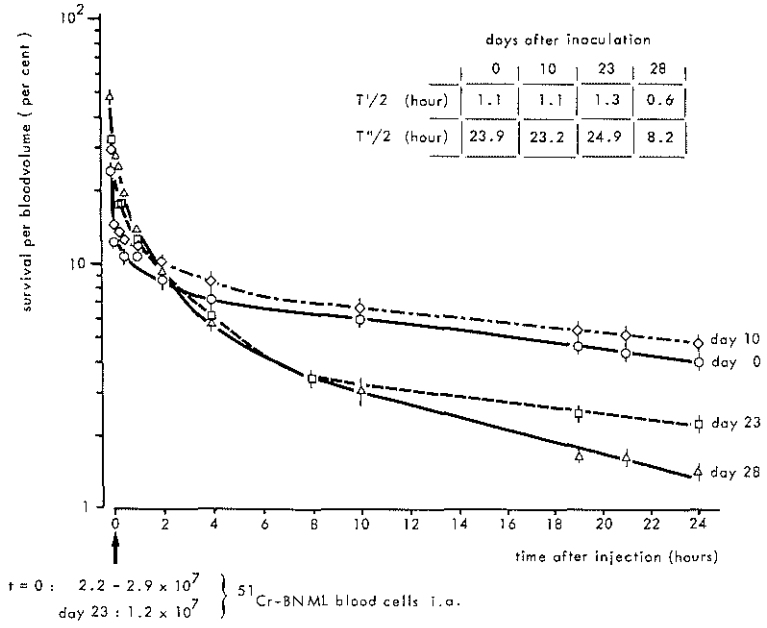

Fig. 2.31 *Blood disappearance of* ^{51}*Cr-labelled BNML cells after i.a. infusion into rats at various stages after inoculation with* 10^7 *BNML spleen cells.*
Each point represents means (±SE) of 3–5 rats.
Day 0: non-leukaemic controls. For further explanation: see text.

cells. An aorta cannula was inserted 5 days earlier. In each experiment, the radioactivity in the blood was determined at 1, 5, 15 and 30 min and 1, 2, 4, 10, 19, 21 and 24 h after infusion. Immediately after the last blood sample, the rats were killed to establish the organ uptake.

The blood disappearance curves are plotted in fig. 2.31. Note that the results from the previous experiment (day 23) are also inserted. The initial recovery from the circulating blood pool (CBP) increases and the recovery at 24 h decreases as the leukaemia progresses. The half value times of the first (T'/2) and second (T''/2) component of the exponential blood disappearance curve are in the same range at days 0, 10, and 23, as computed by linear regression

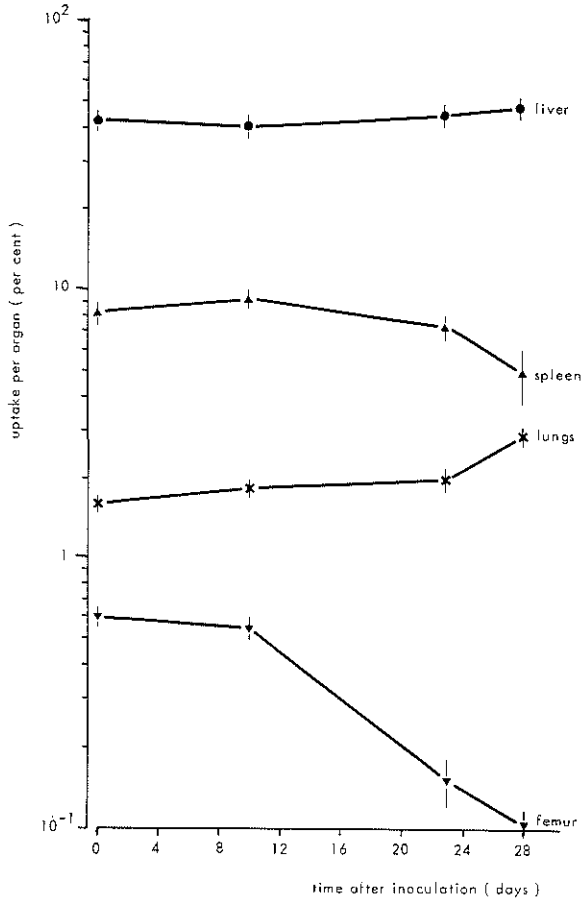

Fig. 2.32 *Organ uptake of ^{51}Cr-labelled BNML blood cells after i.a. infusion into rats at various stages after inoculation with 10^7 BNML spleen cells.*
The uptake was measured at 24 h after infusion of labelled cells.
Each point represents means (±SE) of 3–5 rats.
Day 0: non-leukaemic controls.

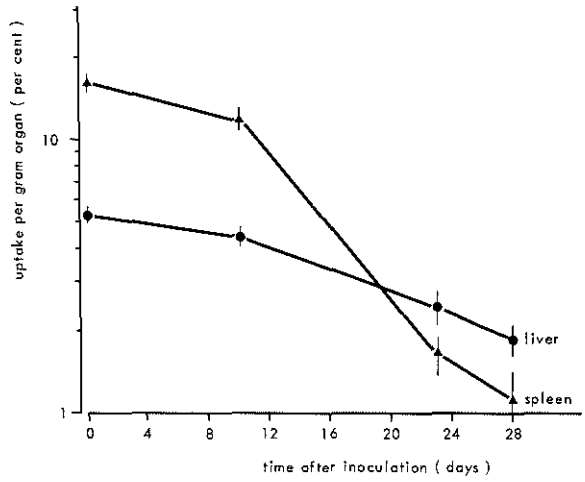

Fig. 2.33 *Uptake of ^{51}Cr-labelled BNML blood cells per gram organ after i.a. infusion into rats at various stages after inoculation with 10^7 BNML spleen cells.*
The uptake was measured at 24 h after infusion of labelled cells.
Each point represents means (±SE) of 3–5 rats.
Day 0: non-leukaemic controls.

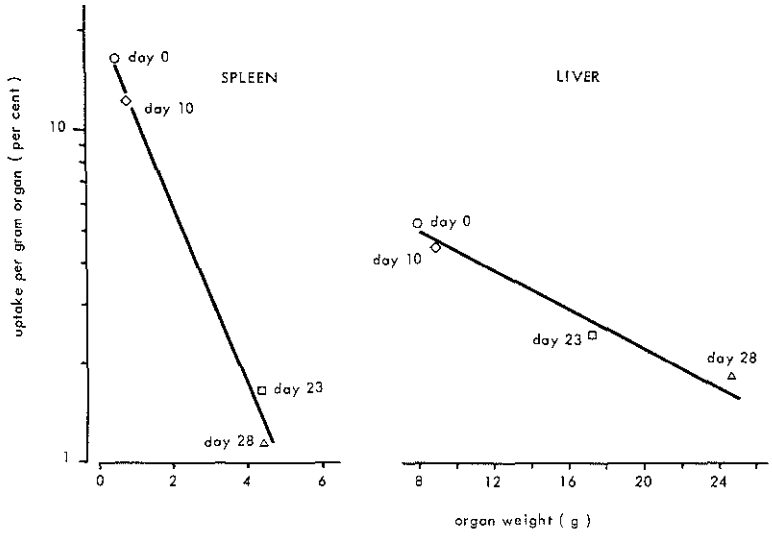

Fig. 2.34 *Relationship between the uptake of ^{51}Cr-labelled BNML blood cells per gram organ and the total organ weight at various stages after inoculation with 10^7 BNML spleen cells.*
The uptake was measured at 24 h after infusion of labelled cells.
Each point was derived from the means of 3–5 rats.
Day 0: non-leukaemic controls.

analysis. However, both values have decreased significantly in the terminal stage (day 28).

In fig. 2.32, the uptake of cells by various organs at 24 h after infusion during the various stages is presented. The liver and the lung take up more cells in the terminal stage of the leukaemia, whereas the spleen and particularly the femoral bone marrow show a significantly decreasing uptake starting after day 10. However, as the weights of various organs increase during leukaemia growth, it is useful to express the uptake per gram organ. This relationship is shown for the spleen and the liver in fig. 2.33. In both organs, the per cent uptake of labelled cells per gram decreases as the leukaemia develops. The greatest reduction is found in the spleen: from 16.5%/g at day 0 to 1.1 %/g at day 20. As is shown in fig. 2.34, the uptake of cells per gram organ decreases exponentially as a function of the organ weight.

2.6.4 Proliferation kinetics

Two major approaches for gaining insight into the cellular kinetics of proliferation will be described:

a) the tritiated thymidine labelling index at various stages of the disease, which gives a rough indication of the growth fraction of leukaemic cells;
b) the per cent labelled mitoses curve, from which the cell cycle parameters can be derived.

a. Tritiated thymidine labelling index (LI).

Eighteen BN rats were inoculated with 10^7 BNML cells. At days 4, 8, 14, 18, 23 and 28, a group of 3 rats received 1 μCi tritiated thymidine (^{3}H-TdR) per gram body weight i.p. (specific activity 2 Ci/mmol; The Radiochemical Centre, Amersham, England). Three normal rats served as controls. Thirty minutes after ^{3}H-TdR injection, the three rats were sacrificed and impression slides of the leukaemic spleen were made on coated glass slides (K_2 nuclear research emulsion, Ilford, Essex, England) for direct autoradiography. After an exposure time of 5 weeks, the preparations were developed according to standard procedures [Hermens, 1973] and stained with haematoxylin-eosin. The number of labelled leukaemic cells in each preparation was counted and divided by the total number of leukaemic cells. A total number of 500 to 600 cells was counted per slide. In this way, the labelling index (LI) was obtained, which is a direct measure of the proportion of leukaemic cells in S phase, i.e., which have incorporated ^{3}H-TdR relative to the total number of cells present at the time of labelling, or:

$$\mathrm{LI} = \frac{\text{number of labelled cells}}{\text{total number of cells}}$$

Table 2.5 *Tritiated thymidine labelling index (LI) of BNML cells in the spleen*

days after inoculation with 10^7 BNML spleen cells	4	8	14	18	23	28
fraction of labelled BNML cells (LI)	*	*	0.42	0.39	0.43	0.30

* LI not measurable.
Each value represents means of 3 rats.

The LI of BNML cells in the spleen at various stages of the disease are presented in table 2.5. Each value represents the average of 3 rats. No values for the LI at days 4 and 8 can be given with any certainty because of the low concentration of BNML cells at that time. A rather constant LI is observed between days 14 and 23. A significantly lower value is obtained at day 28.

b. Per cent labelled mitoses (PLM) curve.

At the 18th day after inoculation with 10^7 BNML cells, 44 rats received 1 μCi ^{3}H-TdR per gram body weight i.p. (specific activity 2 Ci/mmol; The Radiochemical Centre, Amersham, England). Every hour thereafter, up to 44 h, one rat was sacrificed, with the exception of the first animal, which was sacrificed 0.5 h after injection with ^{3}H-TdR. Unfortunately, no rat was killed at 24 h. Impression slides of the leukaemic spleen were made on coated glass slides and processed for direct autoradiography as described above (a). The preparations were screened for labelled and unlabelled mitoses, i.e., cells in the metaphase or early anaphase. Up to a total of 100 mitoses were counted per slide. Cells containing more than 4 grains were considered to be labelled. The per cent labelled mitoses was calculated as follows:

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

Except for the impression slides of the spleen, the other internal organs were fixed in Bouin. No further autoradiographic analysis of these specimens could be performed due to the lack of facilities. Besides, it appeared to be very difficult to label leukaemic cells in the bone marrow, because of the very poor isotope uptake in that tissue.

Fig. 2.35 shows the PLM values plotted against the time after ^{3}H-TdR injection. Two peaks can be clearly distinguished: the first appears between $t = 5$ h and $t = 8$ h; the second, without a clear cut plateau, is observed at $t = 20$ h. From the graph, the cell cycle parameters can easily be derived [Howard and Pelc, 1951; 1953]. They are summarized in table 2.6. Grain count analysis showed that, at $t = 20$ h, labelled mitoses contained approximately 40% fewer grains than at $t = 5$ h.

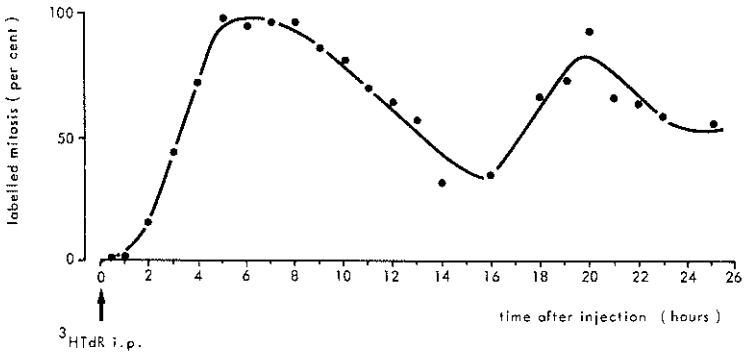

Fig. 2.35 *Per cent labelled mitoses (PLM) curve for BNML cells in the spleen at day 18 after inoculation with 10^7 BNML spleen cells.*
^{3}H-TdR = tritiated thymidine. For further explanation: see text.

Table 2.6 *Cell kinetic parameters of the BN acute myelocytic leukaemia at the 18th day after inoculation with 10^7 BNML spleen cells*

	hours
G_1 duration	0.8
S duration (T_s)	10.0
$G_2 + \frac{1}{2}$ M duration	3.2
	+
cycle time (T_c)	14.0

The data were derived from a percent labelled mitoses curve prepared from impression slides of the spleen (see fig. 2.35).

2.6.5 Discussion

The BNML may be regarded as one of the most realistic animal models presently available for human acute (pro)myelocytic leukaemia. In summary, this statement is based on the following facts:

1. there is cytological and cytochemical evidence for the promyelocytic nature of the disease (see Ch. 2.4);
2. in contrast with many other animal leukaemia models, the BNML has a rather slow growth pattern showing central and peripheral spread comparable with the human disease; and
3. normal haemopoiesis gradually disappears during the development of the leukaemia, which also holds for human AML.

The third argument deserves some more attention at this point.

An impression of the state of normal haemopoiesis can be obtained by determining the number of erythrocytes, leukocytes and platelets in the peripheral blood. However, research during the past few years has provided means to study the blood cell precursor compartment in the haemopoietic

organs. Pluripotent haemopoietic stem cells (HSC) can be assayed by their capacity to form colonies in the spleen (CFUs) after injection into lethally irradiated animals. This spleen colony assay can be applied to mice [Till and McCulloch, 1961] and rats [Comas and Byrd, 1967]. *In vitro* tissue culture techniques have provided another possibility to study the precursor compartment (CFUc). With these methods, which are also applicable to man, myeloid precursor cells or perhaps even HSC can be assayed [van Bekkum and Dicke, 1972; Robinson, 1974].

In the BNML model, the *in vitro* colony formation of bone marrow cells was studied during leukaemia growth [fig. 2.36; van Bekkum et al., 1976a]. As the BNML cells are unable to form colonies, the observed *in vitro* growth is derived from normal primitive precursor cells (CFUc). Fig. 2.36 shows a decline in the number of colonies starting at day 6–8 after inoculation, i.e., from the time of the first microscopic appearance of leukaemic cells (see Ch. 2.5.1, table 2.3). Hardly any colonies could be detected in the last 14 days of the disease.

Based on the fact that BNML cells have a much slower growth rate *in vivo* as compared with normal HSC, *in vivo* CFUs assays could be developed to selectively score normal HSC. Spleen colony assays from the femoral bone marrow showed a similar disappearance pattern for HSC (CFUs) during leukaemia development [fig. 2.37; Colly and Hagenbeek, 1976]. After day 16, less than 1% of the original number of CFUs were recovered. This absolute numerical decrease in HSC in the bone marrow was further supported by

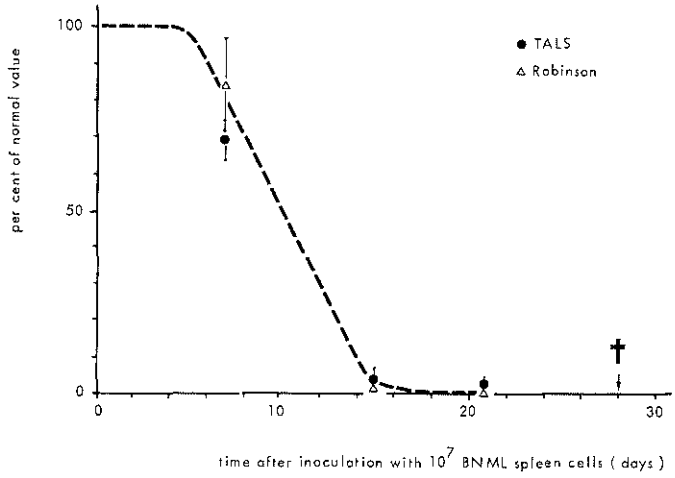

Fig. 2.36 *Characteristic pattern of in vitro colony formation by bone marrow cells during progression of the BN leukaemia.*
● thin agar layer culture (TALS); △ Robinson culture system. Assays were performed on pooled total bone marrow from 2 rats, 2–3 culture plates per point. Vertical bars indicate SE. †: death of leukaemic rats (van Bekkum et al., 1976a).

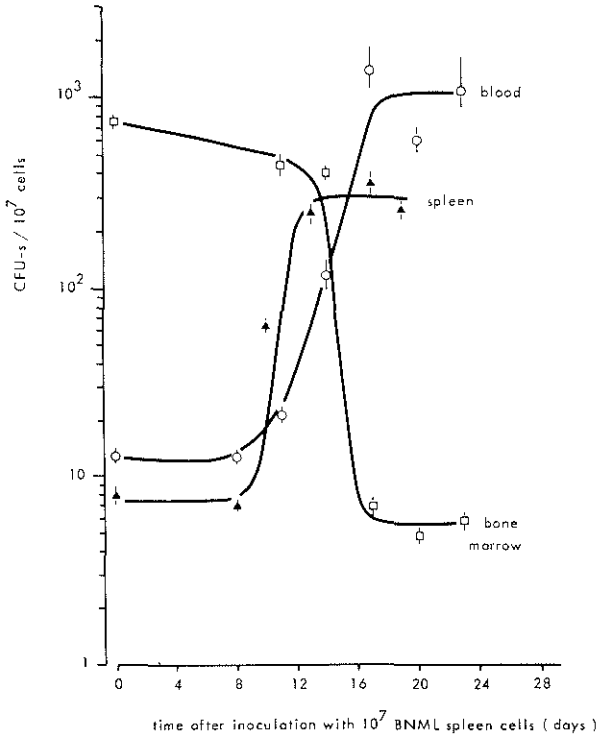

Fig. 2.37 *Changes in the number of colony forming units spleen (CFUs) in the bone marrow, the blood and the spleen during progression of the BN leukaemia.*
Each point represents means (±SE) of 2–3 experiments with 8–10 recipient mice per spleen colony assay.
Day 0: non-leukaemic controls (Colly and Hagenbeek, 1976).

electron microscopical preparations of bone marrow fractions which contained the highest concentrations of HSC (density gradient centrifugation technique). Stem cell counts based on morphological identification were considerably lower in these fractions than those of normal marrow fractions, i.e., less than 1–10% of the normal number of HSC could be recovered [van Bekkum et al., 1976b]. However, at the time of the sudden decrease in HSC in the bone marrow, a dramatic increase is observed in the blood and in the spleen (fig. 2.37).

The explanation might be that normal HSC are forced to emigrate from the bone marrow to the blood as the marrow is being replaced by leukaemic cells. They subsequently lodge at sites suitable for further proliferation, i.e., the spleen and also the liver which histologically actually show signs of extramedullary haemopoiesis during the second half of the disease (see Ch. 2.5). In table 2.7, the absolute number of normal haemopoietic stem cells (CFUs)

Table 2.7 *Quantitation of the total number of normal haemopoietic stem cells (CFUs) in the bone marrow, the blood and the spleen in normal and leukaemic rats*

	total number of cells ($\times 10^7$)	CFUs/10^7	total number of CFUs/organ ($\times 10^3$)
day 0			
bone marrow	1000	750	187,500
blood	13	13	42
spleen	60	8	120
			187,662
day 23			
bone marrow	500	5.5	688
blood	38	1000	9,500
spleen	400	300	30,000
			40,188

Day 0: non-leukaemic controls; day 23: after inoculation with 10^7 BNML cells. CFUs: colony forming unit spleen. The total bone marrow cellularity was derived from data on 1 femur (see fig. 2.22a), representing 1.2% of the total bone marrow. The total number of leukocytes in the blood was obtained by multiplying the number of leukocytes/ml (see fig. 2.19) with the total blood volume (12.5 ml). Splenic cellularity was derived from spleen weight (see fig. 2.25), i.e. 1 g = 10^9 cells (see table 2.4). CFUs/10^7: see fig. 2.37. To calculate the absolute number of CFUs per organ a measured f-factor of 0.004 was used (Colly and Hagenbeek, 1976).

present in the various organs is roughly quantified. There is apparently a redistribution of stem cells. However, at day 23 after inoculation, their total number has decreased to 21% of the original value at day 0. This is due to the excessive absolute numerical decrease in CFUs in the bone marrow. In this tissue, only 0.4% of the initial stem cell pool is left at day 23. Normal haemopoiesis is almost completely replaced by the leukaemic cell population. However, because no measurements were performed on other organs where extramedullary haemopoiesis takes place (such as e.g. the liver), the total number of stem cells at day 23 might be significantly greater than the one given in table 2.7. This would certainly reduce the difference between days 0 and 23 to some extent. Despite the remaining population of normal stem cells, normal haemopoiesis still becomes defective (figs. 2.20 and 2.21). Theoretically, this might be due to:

a. direct cell to cell interaction between leukaemic cells and normal cells. However, mixed *in vitro* culture studies of leukaemic and normal bone marrow cells did not reveal a depression of normal colony formation [van Bekkum et al., 1976a];
b. humoral factors produced by leukaemic cells which would inhibit proliferation of normal HSC. The observation that colony formation by normal

bone marrow in diffusion chambers implanted into leukaemic hosts was not affected did not support this hypothesis [van Bekkum et al., 1976a];

c. normal precursor – c.q. stem cells being transformed into leukaemic cells; whether or not this plays a significant role is not clear at the moment;
d. altered kinetics of proliferation of HSC at extramedullary sites, e.g., a decreased growth fraction, a prolonged cell cycle time and/or an increased cell loss rate possibly because of a suboptimal microenvironment.

In summary, the BNML is characterized by decreasing normal haemopoiesis as the leukaemia progresses. The major mechanism responsible for this could be a forced disappearance of normal blood cell precursors from the bone marrow because of the piling up of leukaemic cells. Although extramedullary haemopoiesis temporarily takes over part of the bone marrow production, it finally falls short in meeting the demands. This pattern is very similar to the one which has been described for many cases of human AML [van Bekkum and Dicke, 1972; Moore, 1974; van Bekkum et al., 1976a]. Other authors studying transplantable animal leukaemias have also described decreasing normal haemopoiesis in the bone marrow, although not to the zero level, during leukaemia development [L5222 myelomonocytic leukaemia in the BD IX rat, Hoelzer et al., 1973b and c; 1974d; RF mouse myelomonocytic leukaemia, Tanaka et al., 1973].

As regards bone marrow cellularity, the total number of cells shows a slow and slight decrease during leukaemia development with an increased proportion of nonviable cells at the terminal stage (fig. 2.22a).

The rapidly decreasing numbers of HSC in the bone marrow between days 8 and 14 (fig. 2.37) is followed by a decline in the number of normal erythropoietic and myelopoietic cells and megakaryocytes in that organ (table 2.3). Erythroblasts and normoblasts show a significant decline from day 6 onwards. The bone marrow transit time for erythropoietic cells of 2 days fits in with these observations [Bond et al., 1965]; i.e., if haemopoiesis at the stem cell level significantly decreases, this will be rapidly followed by a numerical decrease in committed precursor cells, proliferating, differentiating and mature cells. After day 16, erythropoietic cells have almost completely disappeared from the bone marrow. Normoblasts are observed in the blood from day 14 until death (table 2.2). This indicates that their disappearance from the bone marrow is probably caused by a combination of defective production and apparent spontaneous or forced release. In the peripheral blood, the first sign of defective erythropoiesis is the decreasing number of reticulocytes (fig. 2.20). However, they do not reach the zero level; some normal extramedullary erythropoiesis is preserved. Erythrocytes (and haematocrit values) remain at a fairly constant level up to day 23 (fig. 2.20). Thereafter, a rapid decline to 65% of the original number is noted within a period of 5 days. This decrease cannot be solely explained by diminished production. With an erythrocyte life span of about

50 days [Belcher and Harriss, 1959], a complete cessation of erythropoiesis would result in a fall in red cell count of only 2% per day. Thus, other factors must be taken into account. A decreased life span of erythrocytes due to the leukaemic state (intrinsic defects, haemolysis) has been ruled out by ^{51}Chromium-labelling studies. The blood disappearance curves of labelled red cells from leukaemic donors infused into normal or leukaemic rats were identical with those of erythrocytes obtained from normal rats. Therefore, besides defective production, loss of erythrocytes by bleeding, which is a prominent feature in the terminal stage, seems to be the major factor responsible for the developing anaemia.

Decreasing thrombopoiesis is also an early sign of defective haemopoiesis. Normally, 40 per cent of the rat platelets are lost and replaced per day [Ebbe and Stohlman, 1965]. This means that a change in marrow megakaryocytes is quickly reflected in the platelet count in the blood, since the bone marrow transit time for megakaryocytopoiesis in the rat is only 2–3 days [Ebbe et al., 1966]. Indeed, megakaryocytes are no longer observed in the bone marrow after day 12 (table 2.3). Between days 12 and 18, the number of platelets in the blood is rapidly decreasing (fig. 2.21). However, this decrease is not as rapid as would be expected from a complete cessation of thrombopoiesis. Therefore, it can be concluded that extramedullary production of platelets in the spleen and the liver (see Ch. 2.5) will partly compensate for the lack of thrombopoiesis in the bone marrow during this period. From day 23 until death, extramedullary production fails completely; the platelet count in the blood then drops by approximately 30–40% per day. The severe thrombocytopenia together with the probably decreased production of clotting factors by the heavily infiltrated liver may well explain the haemorrhagic diathesis which is observed in the terminal stage of the leukaemia. In addition, Hilgard (1977) found signs of disseminated intravascular coagulation and thrombotic microangiopathy in the terminal stage of the disease. Both phenomena are well-known to occur in human acute promyelocytic leukaemia [Nagai et al., 1973; Gralnick and Sultan, 1975; Sakuragawa et al., 1976].

In the bone marrow, lymphocytes, monocytes and plasma cells decrease gradually during leukaemia development (table 2.3). This may be due to a decreased production and an increased release. Evidence for an increased release of lymphocytes is obtained from fig. 2.19, where an elevated blood lymphocyte count is observed after day 8. It may be noted that, in the terminal stage, the thymus, which is one of the major lymphatic organs, regresses. Despite the diffuse infiltration with leukaemic cells (see Ch. 2.5.2), the thymus shows a significant reduction in weight (fig. 2.25), which is due to the excessive atrophy of the thymic cortex (see Ch. 2.5.2).

Similar to normal erythropoiesis, granulopoiesis in the bone marrow shows a significant decline starting after day 6 (table 2.3). There is a marked reduction

in the number of all white cell precursors. Apart from diminished production, increased release to the blood will be responsible for this phenomenon (see fig. 2.19). After day 8, the number of granulocytes in the blood starts to increase to end up in a two to threefold increased number. In the late stage of the disease when myelopoiesis in the bone marrow is virtually absent, the elevated number of peripheral granulocytes will be due to increased production in extramedullary sites, since their blood transit time is rather short (6.1–11.4 h; see table 2.11). It might be that the remaining normal haemopoietic stem cells preferentially produce cells of the myelocytic series. In addition, immature myelocytic elements such as myeloblasts, myelocytes and metamyelocytes are also appearing in the blood (table 2.2). In theory, these cells might also be differentiated leukaemic cells. This, however, remains to be established.

In the bone marrow, eosinophils show an increase between the time of inoculation and day 12 (table 2.3), which is also reflected in the blood (table 2.2). Although an explanation is lacking, it is well-known that a transitory episode of eosinophilia can occur during the course of human or animal leukaemia or lymphoma [Hayhoe, 1960; Spry, 1972; Rizzo et al., 1976].

Leukaemic promyelocytes rapidly increase in the bone marrow and in the blood after days 6 and 14, respectively (tables 2.2 and 2.3; figs. 2.22b and 2.19). After day 18, the number of promyelocytes in the bone marrow does not change (fig. 2.22b). At the same time, they rapidly increase in the blood (T_d = 16.8 h; fig. 2.19). This indicates that a significant proportion of newly produced leukaemic promyelocytes in the bone marrow is released to the blood. In addition, proliferation of blood promyelocytes might also contibute to the small doubling time. Cell kinetic considerations will be discussed in more detail later.

From fig. 2.15 it is clear that leukaemic cells taken either from the spleen, the blood or the bone marrow in the terminal stage of the disease produce similar dose-survival curves, i.e., there is no functional difference between the cells from the different sources. The concentration of clonogenic leukaemic cells in the various cell populations will therefore be the same. In most of the experiments, the easily available spleen was used for leukaemia transfer. When the inoculated cell dose is increased by a factor 10, i.e. 3 cell doublings, the survival time is reduced by approximately 5 days. From these data, the average T_c value can be derived as being 40 h, assuming that all cells are in cycle (growth fraction = 1) and that there is no cell loss during proliferation. This value, however, is much higher than the one calculated from the PLM curve (T_c = 14 h; see Ch. 2.6.4). The discrepancy will be explained later.

Besides the dose-survival curves, the dose-spleen weight curve (fig. 2.16) might also serve as a means to determine the number of leukaemic cells present in a given suspension, provided that this does not exceed 10^7 cells. When doses between 10^7 and 10^8 leukaemic cells are inoculated, no further significant

increase in spleen weight at day 25 is observed. Apparently, an equilibrium has been reached where cell production is keeping pace with cell loss. A similar plateau phenomenon can be expected for the dose-survival curves (fig. 2.15): simple linear extrapolation towards the X-axis leads to a tumour load of 10^{12} cells which would be incompatible with life. However, the actual tumour load is much lower, as will be discussed in the context of the ^{51}Cr-studies.

^{51}Chromium has been widely used as a radioactive label in haematology since 1950. In contrast to other cell labels (e.g., ^{3}H-TdR), ^{51}Cr labels cells irrespective of their proliferative status. Furthermore, the isotope decays by both electron capture and gamma emission with 9% of its disintegrations in the form of 320 KeV gamma rays; the physical half life is 27.8 days. It can be used for both external scanning and autoradiography [Ronai, 1969], which is not possible with the other label that is frequently used for studies on distribution kinetics, ^{32}P-diisopropylfluorophosphate (DF^{32}P). The ^{51}Cr method has provided useful information on the lifespan and organ distribution of erythrocytes and platelets [Ebaugh et al., 1953; Aster and Jandl, 1964]. In addition, leukokinetic studies have been reported on normal granulocytes [Dresch et al., 1975] and lymphocytes [Torelli et al., 1971; Scott et al., 1972] and in patients with acute lymphocytic leukaemia [McCall et al., 1955], acute myelocytic leukaemia [Boranic et al., 1974; Rosen et al., 1975], chronic myelocytic leukaemia [Duvall and Perry, 1968; Scott et al., 1971] and chronic lymphocytic leukaemia [Pfisterer et al., 1967; Coco and Merritt, 1970; Spivak and Perry, 1970; Scott et al., 1973; Neumann et al., 1974].

With our labelling procedure, autoradiography showed that more than 90% of the leukaemic cells were labelled. This procedure (incubation for 45 min at 37 °C) seems to be optimal according to various authors [McCall et al., 1955; McMillan and Scott, 1968]. Intracellular Cr, probably bound to cytoplasmic proteins [Scaife and Vittorio, 1964], is known to be reduced from a hexavalent to a trivalent state. The trivalent state cannot be reutilized by cells [Rajam and Jackson, 1958; Bunting et al., 1963]. Moreover, no significant elution of ^{51}Cr from leukocytes could be demonstrated by various authors during the first 24 h after labelling [McCall et al., 1955; Dresch and Najean, 1967; Duvall and Perry, 1968; McMillan and Scott, 1968; Lilien et al., 1970]. Just as described by Hersey (1971), the ^{51}Cr excretion in 24 hr collections of urine was found to be very low in BNML rats, i.e., between 1.5 and 3% of the injected ^{51}Cr per day. The ^{51}Cr dose used in our studies (40 μCi/10^{8} cells) is known to be in the optimal range for cell labelling [Rajam et al., 1958; McMillan and Scott, 1968].

The Na_2CrO_4 concentration never exceeded 0.8 μg/ml. This concentration is far below the cytotoxic dose. Various authors have reported on unimpaired leukocyte viability and function after labelling [McMillan and Scott, 1968; Spivak and Perry, 1970; Hersey 1971] as well as unaffected tumour growth

after injection of labelled tumour cells with concentrations below 2.5–10 μg/ml [Rajam et al., 1958; Vincent, 1962; Fisher and Fisher, 1967]. Our findings with the BNML are in accordance with this. Neither an increased number of dead cells nor a significant prolongation of the survival time was observed after separation, labelling and injection of BNML cells as compared with non-treated cells.

In summary, ^{51}Cr seems to be suitable as a tracer isotope for leukaemic cells *in vivo* because of its firm intracellular binding with no remarkable short-term elution, its low toxicity and the technical feasibility of performing external scanning. The major limitation is its lack of labelling specificity. ^{51}Cr binds to erythrocytes, platelets, normal leukocytes and leukaemic cells. Therefore, careful separation techniques are required before cell labelling is applied. However, autoradiographic studies showed that the affinity of ^{51}Cr differs from one cell type to the other [Eyre et al., 1970]. AML cells showed by far the highest labelling intensity, whereas the small lymphocytes label with an affinity twice that of polymorphonuclear leukocytes, 15–30 times that of the erythrocyte and 70–100 times that of the platelet. Based on these findings and on the fact that we are using cell preparations containing up to 80% leukaemic cells (see Ch. 2.6.3), it can be roughly calculated that the ^{51}Cr radioactivity caused by the contamination with labelled nonleukaemic blood cells was less than 5% in our studies and was therefore neglected.

The interpretation of the blood disappearance curves is schematically presented in fig. 2.38. The theoretical curve represents the average picture obtained in many experiments (see fig. 2.28 and 2.31). Essentially, 3 main components are recognized. Only 20% of the infused labelled cells are recovered from the circulating blood pool (CBP) directly after infusion. This means that the cells are distributed in a pool which is – in this case – 5 times larger than the CBP. As has been described for normal granulocytes [Athens et al., 1961; Meuret et al., 1973; Dresch et al., 1975], where it was shown that this effect is not due to the rapid removal of damaged cells, this pool is referred to as the total blood pool (TBP) of leukaemic cells. It consists of the circulating blood pool (CBP) and the noncirculating or marginal pool (MP). By microscopic examination, the marginal position of white blood cells along the walls of venules was demonstrated long ago [Vejlens, 1938]. It might be assumed that the infused leukaemic cells also exchange with a marginal pool. The same events have been described after reinfusion of labelled cells in human AML [Hoelzer et al., 1972; Rosen et al., 1975] and in another rat myelocytic leukaemia [Sadler and Alexander, 1976]. This temporary margination is substantiated by the finding that the radioactivity in the lungs is rather high during the first few hours after infusion of labelled cells (fig. 2.30). Thereafter, a rapid decline to low values is observed. Again, the same phenomena have been described for human AML

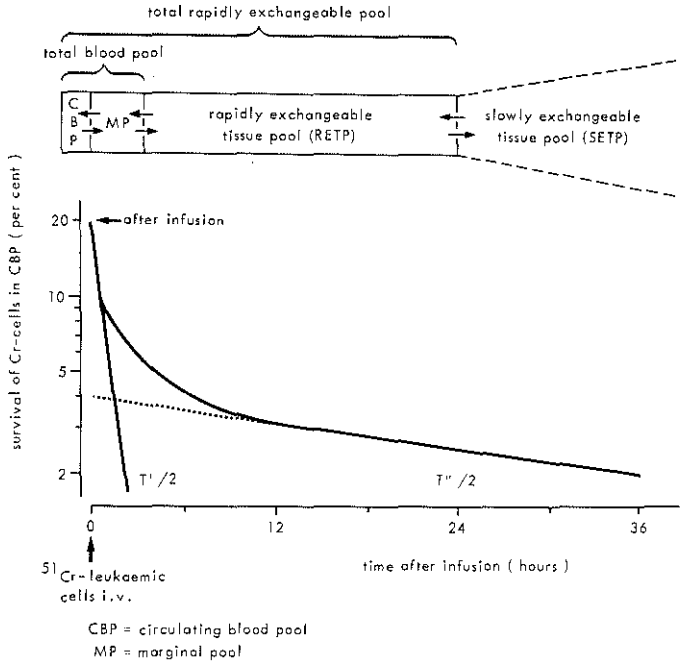

Fig. 2.38 *Schematic representation of cell compartments in acute myelocytic leukaemia.*

and two other rat models for AML [Hoelzer et al., 1973a; Sadler and Alexander, 1976]. Apparently, initial trapping with subsequent release of cells occurs in the lungs.

With the isotope dilution method, the total blood pool (TBP) of leukaemic cells can be quantified as follows:

$$\text{TBP} = \frac{\text{CBP}}{\%\ ^{51}\text{Cr-cells in CBP shortly after infusion}} \times 100 \qquad (1)$$

The circulating blood pool (CBP) is calculated with the formula:

$$\text{CBP} = \text{N} \times \text{V}_\text{b} \qquad (2),$$

where N is the number of leukaemic cells per ml of blood and V_b is the total blood volume (ml). The "% ^{51}Cr cells in CBP" stands for the per cent of injected labelled cells recovered in the CBP from the total number of injected labelled cells shortly (i.e. 30 sec – 1 min) after infusion.

The marginal pool (MP) is easily derived from:

$$\text{MP} = \text{TBP}-\text{CBP} \qquad (3).$$

After the extremely rapid equilibration of cells in the TBP, leukaemic cells disappear from this compartment according to a multiexponential function (see fig. 2.38). Two main components can be distinguished. A rapid decrease characterized by a low value for the half-disappearance time (T′/2) is noted over the first 10 h after reinfusion. Thereafter, a second slower component with a prolonged T/2 (T″/2) is observed.

The interpretation of the pattern of blood disappearance deserves some more consideration. In the experiments, leukaemic blood cells of various ages are labelled. If the disappearance of these cells from the blood compartment would only be a function of senescence, a linear decrease in radioactivity would be expected. However, as has been demonstrated, a multiexponential decrease is observed. It is therefore assumed that the cells are subject to random removal in addition to senescence. Another factor which might determine the pattern of blood disappearance could be the rapid removal of some cells damaged by the labelling procedure. However, as described earlier, the labelled cell suspension is as viable as its control, so that cell damage can be ruled out as an essential determinant of the blood disappearance curve. Early elution of ^{51}Chromium from labelled cells with subsequent urinary excretion of the isotope could also contribute to a rapid decrease in blood radioactivity. As mentioned earlier, many authors found no significant elution during the first 24 h after labelling. In our experience, a low daily urinary excretion argues against significant cell death and ^{51}Cr elution. Finally, studies in human AML using ^{3}H-Cytidine labelled leukaemic cells have yielded similar blood disappearance curves [Hoelzer et al., 1972]. This indicates that no significant artifacts were introduced inherent to the particular isotope involved in our studies with ^{51}Cr.

The multiexponential blood disappearance curve most probably represents the exchange of cells between intravascular and extravascular compartments, i.e., multiple compartments are available for equilibration. The first rapid component reflects the equilibrium established between the total blood pool (TBP) and the extravascular – so called – rapidly exchangeable tissue pool (RETP). This takes about 10 h. During this time, the various organs studied, such as the spleen, the liver and the femoral bone marrow, show an increase in radioactivity (figs. 2.29 and 2.30). The RETP should be regarded so far as a functional compartment without any definite anatomical localization. It may be assumed that this tissue pool is situated close to blood vessels.

The size of the total rapidly exchangeable pool (TREP), i.e., the total pool of dilution, in which leukaemic cells are distributed within the first 10 h after infusion and which comprises the CBP, the MP and the RETP, is calculated by extrapolating the second slow component of the disappearance curve towards the ordinate. From the point of intersection, i.e., the per cent of ^{51}Cr cells in the CBP at equilibrium, the TREP is calculated as follows:

$$\text{TREP} = \frac{\text{CBP}}{\%\ ^{51}\text{Cr-cells in CBP at equilibrium}} \times 100 \qquad (4).$$

From the TREP, the rapidly exchangeable tissue pool (RETP) can be derived:

$$\text{RETP} = \text{TREP} - (\text{CBP} + \text{MP}) \qquad (5).$$

With respect to the organ uptake of ^{51}Cr-labelled cells, a rough calculation of the size of the MP and the RETP of an individual organ (MP_0; $RETP_0$) can be made based on the per cent of injected ^{51}Cr-labelled cells present in the organ just after infusion (MP_0) and at equilibrium ($RETP_0$). The per cent at equilibrium is obtained from extrapolation of the slow component of the organ uptake curve; thus:

$$MP_0 = \frac{\text{CBP}}{\%\ ^{51}\text{Cr-cells in CBP after infusion}} \times \frac{\%\ \text{organ uptake of}}{^{51}\text{Cr-cells after infusion}} \qquad (6)$$

and:

$$RETP_0 = \frac{\text{CBP}}{\%\ ^{51}\text{Cr-cells in CBP at equilibrium}} \times \frac{\%\ \text{organ uptake of}}{^{51}\text{Cr-cells at equilibrium}} \qquad (7).$$

It should be noted that the radioactivity of an organ sample is built up by labelled leukaemic cells in the circulating blood volume of such an organ, its marginal pool and the extravascularly localized cells. The first two items have been generally neglected, because they do not significantly change the picture. By subtracting the MP_0 and the $RETP_0$ from the total tumour cell load of a given organ, which can be approximated by its weight (10^9 cells per gram; table 2.4) corrected for the remaining normal tissue, the more sessile tissue compartment, i.e., the slowly exchangeable tissue pool ($SETP_0$), can be roughly quantified.

The T''/2 of the second slow component of the blood disappearance curve indicates the duration of the blood transit time (BTT) of leukaemic cells. This applies only to the slowly disappearing cells. The BTT(h) is calculated as follows:

$$\text{BTT} = \frac{\text{T''/2}}{\log_e 2}\ \text{h} \qquad (8).$$

From the T''/2 and subsequent BTT values, an estimate can also be made of the turnover rate of leukaemic cells within the total rapidly exchangeable pool (TREP), i.e.,

$$\text{turnover rate} = \frac{\text{TREP}}{\text{BW} \times \text{BTT}}\ \text{cells/kg/h} \qquad (9),$$

where BW stands for body weight (kg).

All of the above-mentioned calculations are based on the assumption that, within the total rapidly exchangeable pool (TREP), leukaemic cells are continuously exchanging between the intra- and extravascular subcompartments as well as with the more sessile tissue compartments (SETP). Functional evidence for this comes from the following finding. During the development of the leukaemia, a second wave of invasion occurs in various organs (see Ch. 2.5). Apparently, this is caused by leukaemic cells which, after entering the intravascular compartment, lodge in other extravascular organ sites and multiply there. Within the relatively short observation period in our experiments, it can be safely assumed that there is a steady state condition with a constant inflow of leukaemic cells and an equal constant outflow from the measured compartment (TREP).

In order to gain insight into the kinetics of blood disappearance and organ uptake of ^{51}Cr-labelled leukaemic blood cells, these parameters were measured during 24 h after infusion on day 23 (figs. 2.28, 2.29 and 2.30). Shortly after infusion, the various organs, in particular the lungs, already contain a significant amount of radioactivity. This probably reflects the marginal pool (MP) as a subcompartment of the total blood pool in the organs. The initial per cent recovery from the CBP is 30% (fig. 2.28). The rapid initial decrease from the CBP (from 100 to 30 is 70%) is for the greater part recovered from the total initial organ uptake ($\pm$60%). It must be assumed that the rest of the radioactivity ($\pm$10%) will be present in organs such as the lymph nodes, the intestines, etc. which were not measured. Hereafter, the organs show an uptake pattern according to a multiexponential function with 2 main components, i.e., a rapid component during the first 2–4 h followed by a slower one which ends up in a plateau. As stated earlier, the multiexponential blood disappearance pattern is regarded as representing exchange of cells between various compartments. In this experiment, an equilibrium is established between the TBP and the RETP in the various organs within 10 h after infusion. However, the organ uptake plateau is already reached at 2–4 h after infusion. This discrepancy between the organ uptake curve and the blood disappearance curve can be explained in the following way. The first component of the blood disappearance curve is the resultant of: (1) outflow of cells from the blood to the MP and the RETP; and (2) outflow of cells from the MP of the lungs where 40% of the infused cells are initially trapped (fig. 2.30), to the blood. This apparently results in a slower net blood disappearance as opposed to the rapid organ uptake of labelled cells from the blood via the MP into the RETP. From this study, it can be concluded that, within a few hours after infusion, an equilibrium between the TBP and the total RETP has already been established. Thereafter, cells will either enter the more sessile tissue compartments (SETP) or will exchange with the blood compartment again. It is suggested that the number of leukaemic cells leaving an organ per unit time roughly equals the

number entering that same organ, because the radioactivity in the organs studied does not change significantly between 8 and 24 h after infusion.

According to the formulas presented in this chapter, the following data were calculated:

$$\frac{\text{Leukaemic cells} \times 10^7}{}$$

CBP	= 21.5	circulating blood pool
MP	= 48.8	marginal pool
TBP	= 70.3	total blood pool
RETP	= 427.4	rapidly exchangeable tissue pool
TREP	= 497.7	total rapidly exchangeable pool

Per cent Cr-labelled cells in CBP (see fig. 2.28)
- shortly after infusion: 30.58
- at equilibrium : 4.32

Blood transit time (BTT) = 36.0 h
turnover rate (TOR) = 48.4×10^7 cells/kg/h
= 1160.9×10^7 cells/kg/day.

In a rat weighing 250 g, this means that only 2.4% of the TREP is renewed each hour.

Approximations of the marginal pools and the rapidly exchangeable tissue pools of the organs studied are presented in table 2.8. From this table, it is clear

Table 2.8 *Quantification of organ compartments at day 23 after inoculation with 10^7 BNML spleen cells*

	% uptake of ^{51}Cr cells after infusion	MP $\times 10^7$	% uptake of ^{51}Cr cells at equilibrium	RETP $\times 10^7$
liver	12.59	8.9	42.97	214
spleen	0.36	0.3	7.19	36
total bone marrow*	2.50	1.8	12.50	67
lungs	43.29	30.4	1.91	10
		41.4		327
calculated from blood disappearance (table 2.9)		48.8		
difference		7.4		

* 1 femur ≈ 1.2% of the total bone marrow.
MP = marginal pool; RETP = rapidly exchangeable tissue pool.
For further explanation: see text.

that the lungs possess the largest marginal pool of all organs studied, whereas the largest rapidly exchangeable tissue pools are found in the liver and in the bone marrow. The difference of 7.4×10^7 cells between the measured and calculated total MP will be due to MP present in organs which have not been studied.

In the next experiment, the blood disappearance and organ uptake of ^{51}Cr-labelled leukaemic cells were determined at various stages of the leukaemia (figs. 2.31 and 2.32). In tables 2.9–2.14, the various pool sizes calculated on the basis of either blood disappearance curves or the measured organ uptake are presented. The data from the previous experiment at day 23 after inoculation are also included. At days 0 and 10, no pool sizes can be calculated because of the virtual absence of leukaemic cells in the blood. Between days 23 and the terminal stage (day 28; table 2.9), the TREP does not change significantly. However, a shift occurs in the distribution of leukaemic cells over the various subcompartments within the TREP. At the terminal stage, the MP equals the CBP, whereas the rapidly exchangeable tissue pool (RETP) has increased relative to the total blood pool (TBP). Apparently, there is a tendency of leukaemic cells to pile up in the organ compartments. However, the turnover rate (TOR) within the total rapidly exchangeable pool (TREP) has increased

Table 2.9 *Quantification of functional compartments at two stages of leukaemia development*

	days after inoculation with 10^7 BNML cells			
leukaemic cells $\times 10^7$	0	10	23	28
CBP	–	–	21.5	27.0
MP	–	–	48.8	29.2
—— +			—— +	—— +
TBP	–	–	70.3	56.2
RETP	–	–	427.4	472.2
—— +			—— +	—— +
TREP	–	–	497.7	528.4
$\frac{MP}{CBP}$	–	–	2.3	1.1
$\frac{RETP}{TBP}$	–	–	6.1	8.4
% ^{51}Cr cells				
– after infusion	24.2	29.8	30.6	48.1
– at equilibrium	7.9	8.5	4.3	5.1

day 0 = non-leukaemic controls; CBP = circulating blood pool; MP = marginal pool; TBP = total blood pool; RETP = rapidly exchangeable tissue pool; TREP = total rapidly exchangeable pool.
For further explanation: see text.

Table 2.10 *Blood transit times and turnover rates of BNML cells at various stages of the leukaemia*

	days after inoculation with 10^7 BNML cells			
	0	10	23	28
T′/2 (h)	1.1	1.1	1.3	0.6
T″/2 (h)	23.9	23.2	24.9	8.2
BTT (h)	34.4	33.5	36.0	11.9
TOR				
– cells $\times 10^7$/kg/h	*	*	48.4	159.7
– cells $\times 10^7$/kg/day	*	*	1160.9	3833.4

* not measurable.
day 0 = non-leukaemic controls; BTT = blood transit time; TOR = turnover rate.
For further explanation: see text.

Table 2.11 *Blood transit times (BTT) of acute leukaemia cellls as compared with normal granulocytes*

	BTT (h)	investigator
human		
normal granulocytes	5.5–10.4	Athens, 1969
AML	32–46	Killmann et al., 1963 Clarkson et al., 1969, 1970
rat		
normal granulocytes	6.1–11.4	Gerecke et al., 1973
BNML	34–36	this study

Table 2.12 *Quantification of organ compartments at two stages after inoculation with 10^7 BNML spleen cells*

	days after inoculation	% uptake of ^{51}Cr cells at equilibrium	RETP $\times 10^7$	SETP $\times 10^7$	total organ load $\times 10^7$	$\frac{\text{SETP}}{\text{RETP}}$
liver	23	42.97	214	716	930	3.4
	28	46.13	244	1430	1674	5.9
spleen	23	7.19	36	338	374	9.4
	28	4.93	26	361	387	13.9
total bone marrow*	23	12.50	67	416	483	6.2
	28	8.33	42	542	584	12.9
lungs	23	1.91	10	70	80	7.0
	28	2.77	15	51	66	3.4

* 1 femur ≈ 1.2% of the total bone marrow.
RETP = rapidly exchangeable tissue pool
SETP = slowly exchangeable tissue pool
} RETP+SETP = total organ load.
For further explanation: see text.

more than threefold at the terminal stage as compared with day 23 (table 2.10). This is due mainly to the shortened T''/2 and thus to a decreased average blood transit time of leukaemic cells. During the first 23 days of the disease, the BTT of BNML cells is significantly prolonged as compared with normal rat granulocytes [Gerecke et al., 1973]. A similar relationship between normal granulocytes [Athens, 1969] and AML cells [Killmann et al., 1963; Clarkson, 1969; Clarkson et al., 1970] has been described in man (table 2.11). In this respect, the BNML is highly relevant as a model for human AML. The abrupt decrease in the BTT of leukaemic cells in the terminal stage of the BNML leukaemia is an unexpected finding. It might have to do with the increased fragility of leukaemic cells (see Ch. 2.4.1).

From the number of LC in the circulating blood (CBP, table 2.9) and the blood transit time (BTT, table 2.10), the influx of leukaemic cells into the blood per hour can be calculated according to the formula:

$$\frac{\text{CBP}}{\text{BTT}}\ \text{LC/h} \qquad (10)$$

This holds only for a steady state condition. At days 23 and 28, the influx of LC per hour is 0.6×10^7 and 2.3×10^7, respectively.

The individual organ pool sizes at days 23 and 28 calculated as described above are presented in table 2.12. Except for the lungs, it is clear that, in the terminal stage of the leukaemia, the magnitude of the slowly exchangeable tissue pool (SETP) has increased markedly relative to the rapidly exchangeable tissue pool (RETP).

The added RETP, SETP and organ load of the 4 organs lead to the total leukaemic cell load per rat as presented in tables 2.13 (day 23) and 2.14 (day 28; "calculated from organ uptake"). In these 2 tables, the total RETP, SETP and organ load are also given, calculated from the blood disappearance curves (table 2.9). The difference between both methods, e.g. 5.71×10^9 leukaemic cells for the total body load at day 23 (table 2.13) will be due to organs such as the lymphatic apparatus, the kidneys, the gastrointestinal tract, etc., not measured. Apparently, the blood disappearance curve gives the most accurate information on total pool sizes, however, without specification of single organs. In this case, the SETP is derived from the RETP by assuming the same quantitative relationship between both compartments as was measured from the organ uptakes (SETP/RETP: 4.7 on day 23 and 7.3 on day 28).

The total body load of leukaemic cells at the terminal stage is about 4×10^{10} cells (± 40 g of leukaemic tissue), of which about 0.5×10^{10} (12.5%) are present in the exchangeable compartment. The total tumour load calculated in this way is much smaller than the one obtained after simple extrapolation of the dose-survival curve (fig. 2.15). This discrepancy has been explained earlier.

Table 2.13 *Comparison of pool size calculations based on blood disappearance or organ uptake at day 23 after inoculation with 10^7 BNML spleen cells*

	RETP $\times 10^9$	SETP $\times 10^9$	$\frac{\text{SETP}}{\text{RETP}}$	total organ load $\times 10^9$	TBP $\times 10^9$	total body load** $\times 10^9$
calculated from blood disappearance (table 2.9)	4.27	20.11	4.7	24.38	0.70	25.08
calculated from organ uptake* (table 2.12)	3.27	15.40	4.7	18.67	0.70	19.37
difference	1.00	4.71		5.71		5.71

* total uptake by liver, spleen, bone marrow and lungs.
** total body load = total organ load + TBP.
RETP = rapidly exchangeable tissue pool
SETP = slowly exchangeable tissue pool } RETP + SETP = total organ load.
TBP = total blood pool (see table 2.9).

Table 2.14 *Comparison of pool size calculations based on blood disappearance or organ uptake at day 28 after inoculation with 10^7 BNML spleen cells*

	RETP $\times 10^9$	SETP $\times 10^9$	$\frac{\text{SETP}}{\text{RETP}}$	total organ load $\times 10^9$	TBP $\times 10^9$	total body load** $\times 10^9$
calculated from blood disappearance (table 2.9)	4.72	34.41	7.3	39.13	0.56	39.69
calculated from organ uptake* (table 2.12)	3.27	23.84	7.3	27.11	0.56	27.67
difference	1.45	10.57		12.02		12.02

* total uptake by liver, spleen, bone marrow and lungs.
** total body load = total organ load + TBP.
RETP = rapidly exchangeable tissue pool
SETP = slowly exchangeable tissue pool } RETP + SETP = total organ load.
TBP = total blood pool (see table 2.9).

Figure 2.39 gives a quantitative representation of the various functional compartments in the terminal stage of the leukaemia. As can be seen, the majority of the LC is present in the slowly exchangeable tissue compartments. The liver has the greatest tumour load, followed by the bone marrow and the spleen. The arrows indicate the continuous exchange of LC between the organs and the blood compartments. The constants for the various flow rates will be derived from a computer program which is being developed at present [Freriks, personal communication).

The per cent of ^{51}Cr-labelled BNML cells taken up by the organs at various

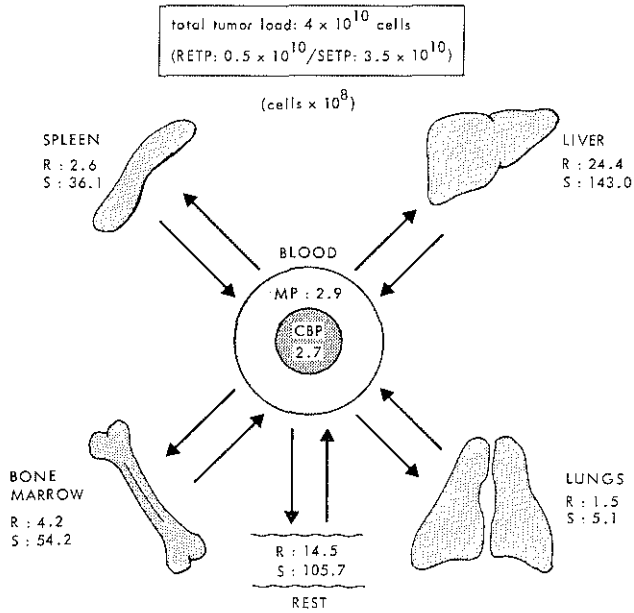

Fig. 2.39 *Sizes of functional compartments at the terminal stage of the BN myelocytic leukaemia.* Terminal stage: day 28 after inoculation with 10^7 BNML spleen cells.
R(ETP) = rapidly exchangeable tissue pool.
S(ETP) = slowly exchangeable tissue pool.
CBP = circulating blood pool.
MP = marginal pool.

stages of the leukaemia deserves more attention at this point. In nonleukaemic rats, one femur contains 0.6% of the labelled cells at 24 h after infusion (fig. 2.32). This figure corresponds very well with the one determined by a bioassay (fig. 2.23). In this experiment, about 1% of injected leukaemic cells (10^5 out of 10^7) is recovered in one femur at 24 h. As one femur represents approximately 1.2% of the total bone marrow [Pegg, 1962; Harker, 1968), it can be concluded that about 50% of the infused cells lodge in the bone marrow. The remaining 50% is fully recovered from the added percentages of the liver (42%), the spleen (8%) and the lungs (1.6%). The relative density of LC lodging in the spleen is a factor of 3 greater as compared with the bone marrow: in the spleen, 8% of the infused cells are distributed among 4×10^8 cells (400 mg of tissue), and, in the bone marrow, 50% is distributed among 80×10^8 cells (1 femur, representing 1.2% of the total bone marrow cellularity, contains 10^8 cells; see fig. 2.22a), or: in the spleen 1% per 0.5×10^8 cells and, in the bone marrow, 1% per 1.6×10^8 cells.

With respect to the spleen, there is a discrepancy between the uptake of infused labelled blood cells (8%) and the percentage of infused leukaemic

spleen cells recovered from the spleen (1%; fig. 2.27) by means of a bioassay at 24 h after infusion. This would indicate that leukaemic blood cells enter the spleen more easily as compared with leukaemic spleen cells.

Other organs in nonleukaemic rats such as lymph nodes, thymus and kidneys do not contain a significant amount of radioactivity after infusion of labelled cells. As the leukaemia progresses, the uptake by the bone marrow decreases markedly. At the terminal stage (days 23–28) when the entire bone marrow compartment has been replaced by leukaemic tissue (see Ch. 2.5.1), a very low uptake is observed. In fact, the leukaemic spleen also shows a decreasing uptake, whereas the liver and the lungs take up a few more labelled BNML cells towards the terminal stage. This might be explained by stating that, for organs which are heavily infiltrated with leukaemia, the capacity to exchange leukaemic cells with the blood has decreased. This argument is based on the assumption that, in a steady state, every cell leaving an organ is replaced by a cell entering it. Another point might be that, in the late stage of the leukaemia, more developing foci of leukaemic tissue, e.g., in the lymph nodes, kidneys, etc., are exchanging with the blood compartment in competition with the organs measured. From fig. 2.32 it can be calculated that, at day 28, about 30% of the infused cells are taken up by organs other than those which were studied. However, if the decreased uptake of the bone marrow and the spleen would be simply a matter of dilution of infused cells in a larger volume, it can be calculated that the femoral bone marrow and the spleen would take up 0.4% and 6%, respectively. These figures are higher than the ones actually measured. Besides, the decreasing uptake per gram organ (fig. 2.33) cannot solely be explained by a greater random dilution in the enlarged organ, because the relationship between the per cent uptake per gram organ and the organ weight is an exponential and not a linear one (fig. 2.34). This holds in particular for the spleen. It may be noted that the body weight has not been used in these calculations, because it does not change significantly during leukaemia development (fig. 2.24). Together, this supports the idea that the exchange rates of leukaemic cells between the bone marrow and the blood and the spleen and the blood are decreasing during leukaemia development. The return of leukaemic cells from the blood to the bone marrow has also been reported by Killmann et al. (1971) and Rosen et al. (1975) in human AML. It is quite possible that, after their return, the leukaemic cells can be released into the circulation again. This possibility is supported by Tarocco et al. (1972), who found that, in acute lymphoblastic leukaemia, leukaemic cells return to the marrow in S-phase, divide, and then reenter the circulation.

So far, kinetics of proliferation of BNML cells have been thoroughly investigated in the spleen. Up to day 6 after inoculation, the number of LC was determined by means of bioassays (fig. 2.27). After that time, these data may be

Table 2.15 *Cell proliferation characteristics of BNML cells in the spleen after inoculation with 10^7 BNML spleen cells*

parameters	symbols	experimental results obtained from 3 individual animals studied at each time of observation					
days after inoculation	t (days)	4	8	14	18	23	28
doubling time of the leukaemic cell population	T_d (h)	37	37	96	120	182	250
fraction of labelled BNML cells (see table 2.5)	LI_{spleen}	*	*	0.42	0.39	0.43	0.30
fraction of proliferating BNML cells	GF_{spleen}	(1.0)	(1.0)	0.56	0.52	0.57	0.40
fraction of cell loss relative to cell production	φ_{spleen} (%)	(62)	(62)	78	81	88	88

* values relevant for BNML cell proliferation not measurable.
For further explanation: see text.

Table 2.16 *Comparison of cell kinetic parameters of normal and leukaemic myelopoiesis in man and in the rat*

	T_c (h)	T_s (h)	LI	GF	investigators
human					
normal					
myeloblasts	15–30	13	0.45–0.70		Cronkite, 1969
promyelocytes	25–40	13	0.55–0.65	0.7–1.0(?)	Killmann, 1972
myelocytes	50–60	13	0.25–0.50		
AML	50–60	13–20	0.03–0.25	0.10–0.35	
rat					
normal					
myeloblasts	?	?	0.58	?	Constable and
promyelocytes	?	?		?	Blackett, 1972
myelocytes	12.0	5–8	0.48	0.77	
L 5222	12.6	8.7	0.30–0.58	0.43–0.84	Harriss and Hoelzer, 1971
BNML	14.0	10.0	0.30–0.43	0.40–0.57	this study

T_c = duration of the cell cycle; T_s = duration of the DNA synthesis phase; LI = tritiated thymidine labelling index; GF = growth fraction; ? = unknown.

derived from changes in splenic weight which then become detectable (fig. 2.25). Values for the doubling time (T_d) of the leukaemic cell population are given in table 2.15. It is clear that the rate of accumulation of LC is maximally rapid during the first 6–8 days after inoculation. The same holds for the growth rate in the bone marrow: up to day 6, the T_d is 16.8 h (fig. 2.23). Thereafter, the doubling time of the promyelocyte population increases significantly (fig.

2.22b). In summary, during the first week after inoculation, the growth rate of the leukaemic cell population in the bone marrow is more rapid as compared with that in the spleen. Thereafter, similar values for both organs are obtained.

The per cent labelled mitoses (PLM) curve prepared at day 18 after inoculation shows 2 peaks (t = 6 h and t = 20 h; fig. 2.35). Theoretically, in between these 2 peaks, the curve should reach the X-axis and the peak at t = 20 h should be similar to the one at t = 6 h. However, this is not observed; this may be due to variation in cell cycle parameters within the leukaemic cell population and/or cells leaving the proliferative compartment during the experiment (enter G_0, start to differentiate, die).

Various kinetic parameters of both human and rat normal myelopoiesis and acute leukaemia are compared in table 2.16. In man, AML cells differ from normal granulocyte precursors in terms of a prolonged average cell cycle time and a decreased labelling index and growth fraction. It should be noted, however, that the cycle times of normal myelocytes are in the same range as those of AML cells. Besides the BNML, data on one other model in the rat (the L5222 acute undifferentiated leukaemia) are compared with scanty data on normal rat myelopoiesis. Both the BNML and the L5222 leukaemia show a decreased labelling index and growth fraction and their cell cycle times are close to that of normal myelocytes. In this respect, rat leukaemia cells have the same relationship with their normal counterparts as human AML cells with theirs. Therefore, from a kinetic point of view, the BNML may be regarded as a realistic model for human AML. Although the kinetic parameters of both rat models are rather similar, the L5222 shows a very rapid *in vivo* growth, i.e., the survival time after inoculation with 10^7 cells is 7 days. This is possibly due to a much lesser degree of cell loss during leukaemia growth as compared with the BNML.

Using the data on the phase durations of cell cycle (table 2.6) and the LI

Table 2.17 *Method to calculate the cell loss factor during leukaemia growth*

$$T_{d(pot)} = T_c \frac{\ln 2}{\ln \alpha} \qquad (15)$$

where:

$$\alpha = 1 + GF \qquad (16)$$

$$k_{prod} = \frac{\ln 2}{T_{d(pot)}} \qquad (17)$$

$$k = \frac{\ln 2}{T_d} \qquad (18)$$

$$\varphi = \frac{k_{prod} - k}{k_{prod}} \times 100\% \qquad (19)$$

$T_{d(pot)}$ = potential doubling time; T_c = cell cycle time; α = number of cells produced per division; GF = growth fraction; k_{prod} = expected rate constant for growth; k = actual rate constant for growth; T_d = measured doubling time; φ = cell loss factor.

values (table 2.5), the fraction of proliferating BNML cells (GF) can be calculated according to a model described by Steel (1968) in which both proliferating and nonproliferating cells are assumed to be present; i.e., for an exponentially growing cell population,

$$GF = \frac{N_c}{N} \tag{11}$$

where N_c and N stand for the number of cells in cycle and the total number of cells, respectively. However, because these cannot be directly measured, this formula is written as

$$GF = \frac{\frac{N_s}{N}}{\frac{N_s}{N_c}} \tag{12}$$

where N_s represents the number of cells in S phase.

From

$$\frac{N_s}{N} = LI \tag{13}$$

and

$$\frac{N_s}{N_c} = 2^{\left(\frac{T_{G_2+M}}{T_c}\right)} \cdot (2^{\frac{T_s}{T_c}} - 1) \tag{14}$$

the growth fraction can be derived.

The values thus obtained for the GF are presented in table 2.15. Because the LI values at days 4 and 8 could not be accurately measured, the growth fraction could not be calculated. However, in the early stage of the leukaemia, it seemed to be justified to assume a maximal GF, i.e., GF = 1.0. This decreases to 0.5–0.6 between days 14 and 23. In the terminal stage, the GF tends to become still smaller. With these calculations, it is assumed that the cell cycle parameters determined at day 18 do not change significantly throughout the disease period. In fact, the cell cycle time will not be very much shorter, since the duration of the G_1-phase (0.8 h) is already extremely short. Particularly the G_1-phase might show considerable variation, implying that an increase in G_1 will correspond with an increase in the calculated value for the GF [Hermens, 1973].

Recently, improved techniques have yielded preliminary data which suggest that the growth fraction in the bone marrow is even lower than in the spleen ($LI_{BM} = 0.20-0.25$ at day 16 after inoculation; Aglietta, personal communication). The LI of leukaemic cells in the peripheral blood was 0.20.

From the T_d values, the rate constant for growth (k) was calculated, i.e. the fraction of cells produced per hour (see formula 18, table 2.17). Besides these two parameters, which are derived from measured growth, the potential doubling time, $T_{d(pot)}$, and the rate constant for the production of new cells, k_{prod}, which describe the expected growth rate can be calculated (see formulas 15, 16 and 17; table 2.17). In this mathematical model based on the assumption that only cell production plays a role, the growth fraction (GF) and hence the number of cells produced per division (α) lead to $T_{d(pot)}$ and k_{prod}. From the measured and expected production rates, the cell loss factor (φ) can be calculated (see formula 19, table 2.17). In table 2.15 this factor, defined as the rate of cell loss relative to the rate at which cells are being added to the total population by mitosis, is given for the various stages of leukaemia growth. Concomitant with a decreasing GF, the cell loss factor increases as the leukaemia progresses. This can be regarded as the major factor responsible for the net slow growth rate of the leukaemia.

Cell loss may be due to differentiation, migration and cell death. To what extent these mechanisms contribute to the observed phenomenon is difficult to establish. That cell death plays an important role during leukaemia growth in the spleen can clearly be seen in fig. 2.26. On the other hand, indications for loss of proliferative activity, possibly by differentiation, come from the TD_{50} experiment (fig. 2.17). Clearly, in contrast with other animal leukaemia models such as the Shay myelocytic leukaemia and the L1210 leukaemia, more than one leukaemic cell is needed to transfer the disease. The plotted points in fig. 2.17 conform to theoretical Poisson curves.

The chance of obtaining at random an inoculum containing no "taking unit" for successful transplantation of the leukaemia can be calculated to be 37% [Hewitt, 1958]. A suspension which gives 63% of takes contains a mean of 40 cells. This means that, of every 40 cells, only 1 is likely to induce leukaemia. This cell might be called a "clonogenic cell" capable of unlimited proliferation. However, it must be taken into account that, in this experiment, 84% of the cells were morphologically recognizable leukaemic cells; of these, 17% were scored as dead cells. This would mean that, instead of 1 out of 40 cells, 1 out of 28 cells would be a clonogenic leukaemic cell. The existence of clonogenic cells is furthermore supported by the observation of leukaemic colonies in the spleens of nonirradiated rats 20 days after injection of leukaemic cells.

In conclusion, the BN myelocytic leukaemia may be classified as a realistic model for human acute myelocytic leukaemia based on its pathophysiology studied so far. In addition, its response to chemotherapy is also similar to that of human AML. Clinically applied chemotherapy regimens for AML are far more effective in terms of inducing a complete remission as compared with treatment schedules used in acute lymphocytic leukaemia [Colly and Hagenbeek, 1977].

CHAPTER 3

LEUKOCYTE MOBILIZING AGENTS

3.1 Introduction

One of the major factors determining the efficacy of ECIB treatment is the total number of leukaemic cells in the circulating blood, which is determined by the inflow and outflow of cells per unit time. If the inflow of cells could be made to exceed the outflow during the whole period of treatment, leukaemic cell depletion by ECIB would theoretically increase.

Certain synthetic polyanions (table 3.1) have been shown to mobilize cells from haemopoietic organs. In addition, they share many other biological properties such as inhibition of cancer metastasis formation [Suemasu and

Table 3.1 *List of polyanions with similar biological effects*

heparin	
heparinoids	
dextran	sulphate
pentosan	sulphate
chondroitin	sulphate
glycogen	sulphate
sucrose	sulphate
polyethylene sulphonate	
polymethacrylic acid	
polyvinyl sulphonic acid	
copolymers	

Ishikawa, 1970; Franchi et al., 1973; Niitani et al., 1974], a heparin-like blood anticoagulant activity [Ricketts, 1952; Bradfield and Born, 1974] and activation of lipoprotein lipase [Yamada et al., 1961; Boberg, 1972].

In normal animal species (mice, rats, dogs, goats, calfs and monkeys), the various polyanions induce a transient absolute lymphocytosis shortly after administration [Jansen et al., 1962; Sasaki, 1967; Ormai et al., 1973; Bradfield and Born, 1974; Ross et al., 1975]. Some examples are given in figures 3.1, 3.2, and 3.3. The peak of cell mobilization (up to a threefold increase in the number of lymphocytes) is generally found between 1 and 4 h after injection. Normal values are again reached a few hours later. Histology and quantitative measurements indicate that the spleen and the lymph nodes are the major target organs from which lymphocytes are mobilized [Sazaki and Suchi, 1967; Ormai et al., 1973]. The route along which cells reach the blood is either via the efferent lymphatics, including the thoracic duct, or via the "direct route"; i.e., from the organs directly into the circulation [Ormai and de Clercq, 1969;

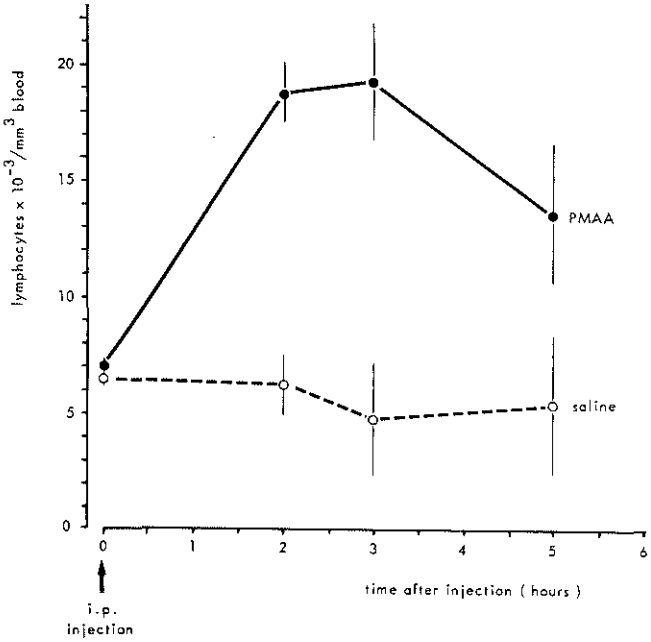

Fig. 3.1 *Effect of polymethacrylic acid (PMAA) on the number of lymphocytes in the peripheral blood of C57BL female mice.*
PMAA (MW 200,000): 40 mg/kg.
Each point represents means (±SE) of 5 mice.

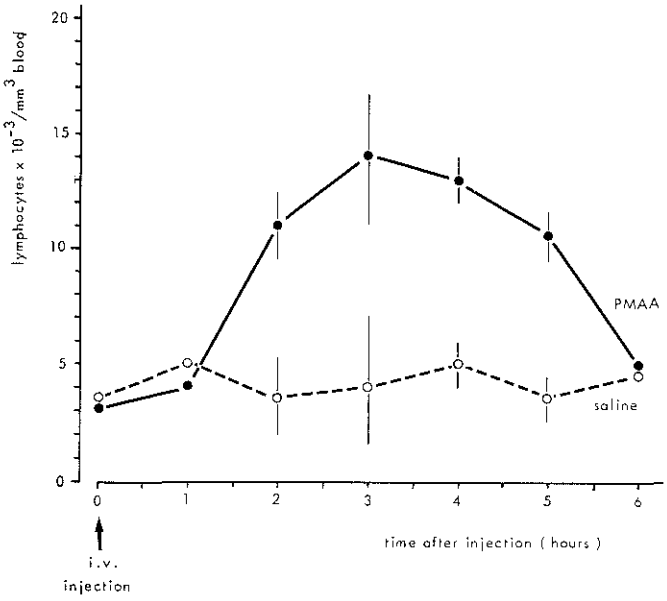

Fig. 3.2 *Effect of polymethacrylic acid (PMAA) on the number of lymphocytes in the peripheral blood of WAG/Rij male rats.*
PMAA (MW 200,000): 40 mg/kg.
Each point represents means (±SE) of 5 rats.

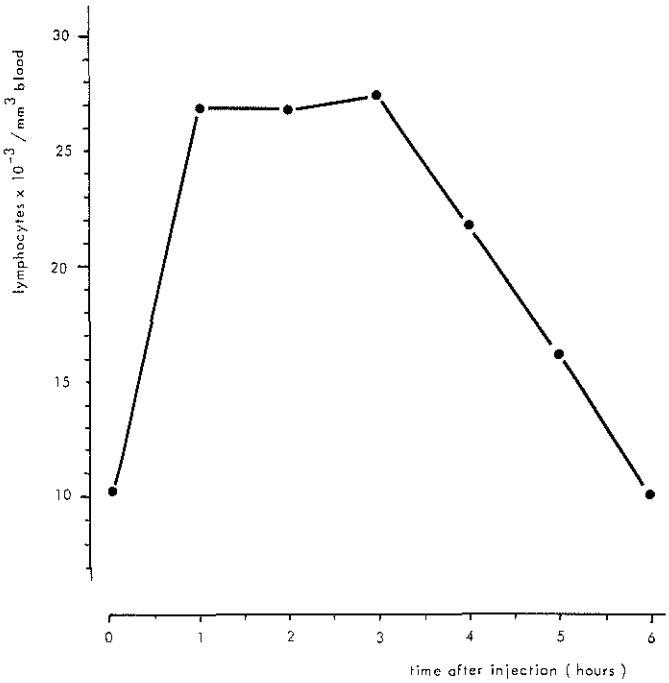

Fig. 3.3 *Effect of dextran sulphate (DS_{17}) on the number of lymphocytes in the peripheral blood of a rhesus monkey (Macaca mulatta).*
DS_{17} (MW 17,000): 5 mg/kg i.v.

Ormai et al., 1973]. Besides mobilization, leukocyte mobilizing agents (LMA) seem to inhibit lymphocyte recirculation to some extent; i.e., their rate of exit from the circulation is reduced [Bradfield and Born, 1969; Balow and Fauci, 1976]. This is an additional factor in the elevated peripheral cell counts. At the end of mobilization, cells return to the spleen and the lymph nodes [Ormai et al., 1973].

According to the morphology and biological properties of the mobilized lymphocytes which pour into the blood in large numbers via the thoracic duct, it was found that they belong to the small lymphocyte population [Ormai and Palkovits, 1972a]. There is also evidence that these small lymphocytes are immunoreactive and able to induce rejection of skin transplants in a hetero-transplantation model in mice. Immunosuppression was induced in this model by rabbit-antimouse antilymphocyte globulin [van Bekkum and Ormai, 1969].

It has been further observed that mobilization can also be effected in rats with hypoplastic lymphatic organs, induced either by breeding under germ-free conditions, by subjecting them to neonatal thymectomy or by treating them with hydrocortisone injections at birth [Ormai and Palkovits, 1972b]. By

means of the membrane fluorescence technique, it was demonstrated that both B and T lymphocytes are mobilized [van der Ham et al., 1977].

Apart from lymphocytes, LMA cause a moderate increase in the number of granulocytes in the blood. However, this marginal effect may be due to the anaesthesia and injection procedure. In mice, an increase in haemopoietic stem cells (HSC) in the peripheral blood has been reported [van der Ham et al., 1977]. This is in contrast with our findings in the rat, where, at 2 h after injection of dextran sulphate, no significant increase or decrease in HSC was found in the blood and the femoral bone marrow, respectively.

In view of a possible application of LMA in combination with ECIB, special attention has been paid to the mobilization of leukaemic cells. Polymethacrylic acid (PMAA) induces an absolute increase in the number of leukaemic cells in the blood, as was shown in the mouse lymphosarcoma 2028 and in the Shay myelocytic leukaemia in the rat [Ormai et al., 1970]. In this chapter, cell mobilization studies with the less toxic LMA Dextran Sulphate (DS) in the BN acute myelocytic leukaemia and in three patients with chronic lymphocytic leukaemia will be reported.

3.2 Specific experimental procedures

3.2.1 Dextran sulphates (DS)

For experimental purposes, DS_{17} (MW 17,000) was used (sulphur content: 18%; prepared by Dr. K. J. van den Berg) in a dose of 10 mg/kg i.v.

In an initial clinical trial, DS_4 (MW 4,000; sulphur content: 5%) supplied by the Kowa Company Ltd. (Nagoya, Japan) was injected i.v. in a dosage of 40 mg/kg. For clinical use, the solution in saline was sterilized through a millipore filter.

3.2.2 Experimental designs

a. Experimental studies

In a first experiment, 5 rats received DS_{17} i.v. on the 20th day after inoculation with 10^7 BNML cells. Following the injection, the number of lymphocytes and leukaemic cells in the blood were counted at hourly intervals up to 5 h. The increase in the number of cells is expressed as the increase factor (IF), calculated as follows:

$$IF = \frac{\text{number of cells at mobilization peak}}{\text{number of cells before injection}}$$

In a second experiment, the effects of repeated injections of DS_{17} on the number of blood cells were studied in 5 rats on four consecutive days (days 18, 19, 20 and

21 after inoculation with 10^7 BNML cells) with two injections at 3 h intervals per day. Each initial injection was given at 10.00 a.m.

b. Clinical study

As a preliminary to a possible combination of cell mobilization with clinical ECIB treatment, three patients with chronic lymphocytic leukaemia received DS_4 i.v. Their haematologic parameters determined before the infusion are given in table 3.2. Patients 1 and 3 had enlarged spleens and lymph nodes and showed a significant involvement of the bone marrow, i.e., about 55 and 95% of their bone marrow cells consisted of lymphocytes respectively. Patient 2 only had some slightly enlarged lymph nodes and no splenomegaly. However, the bone marrow was involved significantly (50% lymphocytes). In none of the patients was hepatomegaly found.

After the infusion of DS_4 at hourly intervals, up to 5 h after injection, venous blood samples were taken and the number of peripheral lymphocytes was determined.

Table 3.2 *Haematologic parameters of three patients with chronic lymphocytic leukaemia prior to injection with dextran sulphate (MW 4000)*

	Patients		
	1 ♂67 yr BW = 82 kg	2 ♂66 yr BW = 86 kg	3 ♂78 yr BW = 80 kg
Hb (mmol/l)	8.5	8.1	6.9
Ht (%)	39	39	35
platelets ($\times 10^9$/l)	122	129	366
leukocytes ($\times 10^9$/l)	18.0	14.0	51.0
differential counts (%)			
basophils	–	2.0	–
eosinophils	–	15.0	8.0
polymorphs	10.0	27.0	8.0
lymphocytes	88.0	53.0	82.0
monocytes	2.0	3.0	2.0

BW = body weight; Hb = haemoglobin; Ht = haematocrit.

3.3 Results

3.3.1 Experimental studies

In fig. 3.4, changes in the number of lymphocytes and leukaemic cells after one injection with DS_{17} are shown. The increase in the number of lymphocytes and leukaemic cells is at a maximum between 2–4 h and 1–3 h, respectively. At the peak of mobilization, the following increase factors were calculated:

$$IF_{\text{lymphocytes}} = 1.9; \qquad IF_{\text{leukaemic cells}} = 2.8.$$

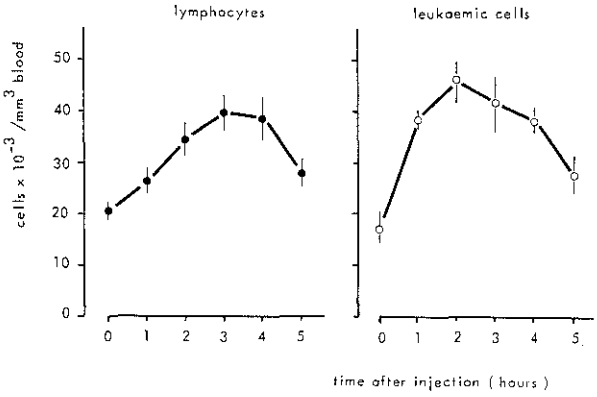

Fig. 3.4 *Effect of dextran sulphate (DS_{17}) on the number of lymphocytes and leukaemic cells in the peripheral blood of BN rats at day 20 after inoculation with 10^7 BNML spleen cells.*
DS_{17} (MW 17,000): 10 mg/kg i.v.
Each point represents means (±SE) of 5 rats.

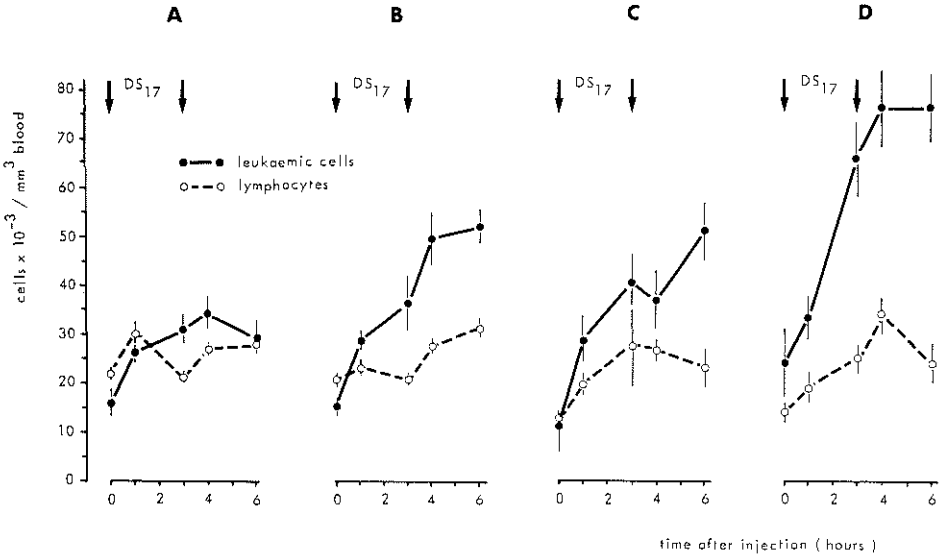

Fig. 3.5 *Effect of dextran sulphate (DS_{17}) given by repeated injections on four consecutive days during leukaemia growth on the number of lymphocytes and leukaemic cells in the peripheral blood of BN rats.*
A, B, C and D: days 18, 19, 20 and 21 after inoculation with 10^7 BNML spleen cells, respectively.
Each arrow represents one injection with DS_{17} (MW 17,000): 10 mg/kg i.v.
The same rats were used throughout the experiment.
Each point represents means (±SE) of 5 rats.

The values are close to normal at 5 h after injection.

Fig. 3.5 shows the effects of repeated injections with DS_{17} on 4 subsequent days. For leukaemic cells, it is clear that, except for day 18, the second injection of DS causes a further increase in number. The increase factor measured at 6 h after the first injection is 1.8 at day 18, 3.4 at day 19, 4.5 at day 20 and 3.2 at day 21. Before the first injection of DS at days 19, 20 and 21, cell counts were found to have decreased approximately to the original low preinjection level obtained at day 18. In this experiment, the increase in the number of normal lymphocytes is less striking.

3.3.2 Clinical study

In table 3.3, data on lymphocyte mobilization in patients with chronic lymphocytic leukaemia are presented. The total number of lymphocytes in the circulating blood volume was calculated by multiplying the number of lymphocytes per ml by the blood volume derived from normograms. All three patients show cell mobilization peaking at different times after injection, with increase factors ranging from 1.4 to 2.2. The absolute increase calculated

Table 3.3 *Effect of dextran sulphate (DS_4) on the number of peripheral blood lymphocytes in three patients with chronic lymphocytic leukaemia*

	patients		
	1 ♂67 year BV 5330 ml BW = 82 kg	2 ♂66 year BV = 5500 ml BW = 86 kg	3 ♂78 year BV = 5040 ml BW = 80 kg
time after DS_4	Tot. Ly $\times 10^9$	Tot. Ly $\times 10^9$	Tot. Ly $\times 10^9$
0 hr (before DS_4)	84.2	40.7	211.7
10 min	83.1	39.6	171.4
30	86.3	45.7	171.4
1 hr	101.3	28.6	252.0
2	113.5*	49.5	292.3
3	103.4	90.8*	216.7
4	108.7	73.2	272.2
5	98.6	74.8	302.4*
*IF	1.4	2.2	1.4
*maximal absolute increase	29.3×10^9 (113.5−84.2)	50.1×10^9 (90.8−40.7)	90.7×10^9 (302.4−211.7)

BV = blood volume; BW = body weight; Tot. Ly = total lymphocytes = Ly/ml × BV (ml); DS_4 = dextran sulphate (MW 4000), 40 mg/kg i.v.;

$$\text{IF} = \text{increase factor} = \frac{\text{number of lymphocytes at peak*}}{\text{number of lymphocytes at } t = 0}$$

Stars indicate maximal values measured.

by subtracting the initial number of cells from the number at the peak of mobilization ranges from 30 to 90×10^9 cells.

3.4 Discussion

From the foregoing, it is clear that, besides normal lymphocytes, LMA mobilize leukaemic cells in both various animal leukaemia models and in human CLL. The mobilization phenomenon is dose-dependent with the dose-effect curve showing a plateau [Ross et al., 1975; Ormai and Palkovits, 1975]. With DS_{17}, the optimal dose was 10 mg/kg. Above this dose, no further mobilization of leukaemic cells occurred, while the risk of haemorrhages due to the heparin-like anticoagulant activity increased [Hagenbeek et al., 1976]. The clinically applied dose of DS_4 (40 mg/kg) was derived from studies in patients treated for pulmonary metastases [Niitani et al., 1974]. Prior to its use in our CLL patients, the compound was subjected to various *in vitro* coagulation studies using human plasma. The thrombin time (TT), which is very sensitive to heparin-like activity in the plasma, was only slightly prolonged when DS_4 was incubated with plasma in a final concentration of 1 mg/ml. Moreover, DS_4 did not induce platelet aggregation *in vitro*. In fact, after injection of DS_4 in our patients, no changes were observed in their clinical condition. Except for a short-lasting prolonged cephaline time, no haematological abnormalities were noted.

Although the anatomical site of origin of mobilized leukaemic cells cannot be identified histologically, it may be assumed that the rapidly exchangeable tissue pool (RETP; see Chapter 2.6.5) is the major target compartment for cell mobilization. From fig. 3.4, it can be calculated that, at 2 h after injection with DS_{17}, a net number of 29.3×10^6 leukaemic cells per ml has been added to the circulating blood. With an average blood volume of 12.5 ml, the total number of mobilized cells is 3.7×10^8. This number is about 10% of the size reported for the RETP at day 23 ($3.27-4.27 \times 10^9$ cells; see Chapter 2.6.5, table 2.13). With repeated injections of DS_{17} (fig. 3.5), the second injection except for day

Table 3.4 *Net increase in the number of leukaemic cells in the circulating blood volume after repeated injections with dextran sulphate (DS_{17}) at four consecutive days after inoculation with 10^7 BNML spleen cells*

	number of leukaemic cells added to the circulating blood ($\times 10^8$)			
days after inoculation	18	19	20	21
3 h after the first injection with DS_{17}	1.8	2.7	3.6	5.3
3 h after the second injection with DS_{17}	1.6	4.6	5.0	6.5

DS_{17} = dextran sulphate (MW 17,000), 10 mg/kg i.v. at t = 0 h and t = 3 h (see fig. 3.5). The increases were calculated related to the values at t = 0 h.

18, significantly increases the number of leukaemic cells added to the circulating blood (table 3.4). A total net increase of 6.5×10^8 cells, i.e. 15–20% of the RETP, was measured when mobilization was induced at day 21 after leukaemia inoculation.

In the clinical study, the number of leukaemic cells mobilized ($3 \times 10^{10} - 10^{11}$; table 3.3) is between 5 and 20% of the RETP if the number of cells in the RETP in these patients is assumed to range from 2×10^{11} to 1.5×10^{12}, based on studies by Bremer et al. (1973a).

Although the mechanism underlying the mobilization phenomenon is still unclear, studies with many copolymers indicate that the mobilizing capacity of a polyanion is related to the overall anionic charge or the density of charge on the polyanion (Ross, personal communication). The present working hypothesis is that polyanions attach to the cell surface of the target cells, thereby changing their electronegative charge. This possibly alters the normal interaction between the cells and the surrounding structures (e.g. the reticuloendothelial system; RES), which may in turn result in an increased release to and possibly an impaired migration from the blood. This hypothesis is supported by the following facts. As is shown in table 3.5, *in vitro* incubation of cells from another rat leukaemia (L5222) and normal rat lymphocytes with either polymethacrylic acid or DS_{17} significantly increases their electrophoretic mobility towards the positive pole (Roeder and Haemmerli, personal communication). The effect is dose-dependent until a plateau is reached. The same phenomenon has been described by Suemasu et al. (1971) for cultured endothelium-like cells after *in vitro* incubation with DS. From *in vitro* studies with ^{35}S-labelled DS, these authors concluded that the compound interacts with the cell surface. For heparin also, *in vivo* attachment to endothelium was reported by Hiebert and Jaques (1976). *In vivo*, the electrostatic repulsion

Table 3.5 *Changes in electrophoretic mobility of L5222 rat leukaemia cells and normal rat lymphocytes after in vitro incubation with polyanions* [Roeder and Haemmerli, 1975]

		% increase of epm over controls	
	concentration (%)	L5222	lymphocytes
PMAA	0.1	4	7
	0.25	14	14
	0.5	28	12
	1.0	31	16
DS_{17}	0.1	16	15
	0.2	23	23

PMAA = polymethacrylic acid (MW 200,000); DS_{17} = dextran sulphate (MW 17,000).
Incubation: 50 min at 37 °C, concentration of cells: $10^7 - 2 \times 10^7$/ml.
epm = electrophoretic mobility.

induced by the increased surface negative charge might be responsible for the alteration in adhesion between leukaemic cells and the RES.

A second argument in favour of the hypothesis is derived from the observation that the lymphocytosis induced by polyanions can be abruptly abolished by the administration of polycations such as protamine chloride (fig. 3.6). Within 10 min after injection of the polycation, the number of lymphocytes has already decreased significantly. Apparently, the altered cell surface charge is neutralized. This might enhance migration of cells from the blood stream. The suggestion is that, in combination with mobilization from tissue stores, inhibition of recirculation at the stage of migration of cells from the peripheral blood

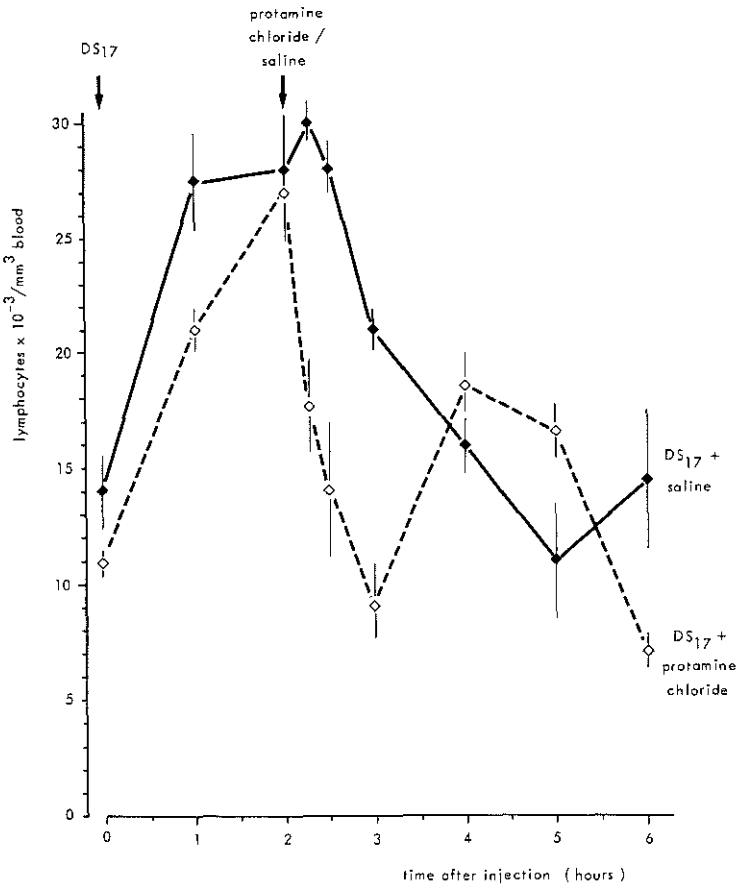

Fig. 3.6 *Effect of dextran sulphate (DS_{17}) and subsequent protamine chloride on the number of lymphocytes in the peripheral blood of WAG/Rij male rats.*
DS_{17} (MW 17,000): 10 mg/kg i.v.
Protamine chloride: 10 mg/kg i.v.
Each point represents means (±SE) of 5 rats.

is responsible for the elevated peripheral cell counts. When mixtures of DS and protamine are injected, the mobilization of cells can be completely prevented [Sasaki, 1967]. This phenomenon is less clear in leukaemia, possibly because of a less specific interaction of leukaemic cells with endothelial cells at the site of migration.

It has been clearly shown that the anticoagulant effect of various polyanions does not correlate with their ability to cause lymphocytosis [Bradfield and Born, 1974]. To what extent other biological properties of these compounds, e.g. activation of lipoprotein lipase with possible effects on endothelial structures, play an essential role in the mechanism of cell mobilization remains to be established.

Finally, it should be mentioned that chronic daily administration of DS_{17} does not change the growth rate of the BNML.

In summary, although the specific mode of action is yet unknown, LMA, particularly after repeated injections, add a significant number of leukaemic cells to the circulating blood. Their usefulness in terms of enhancing the efficacy of ECIB in cell depletion will be discussed in Chapter 4.

CHAPTER 4

ECIB IN THE BN ACUTE MYELOCYTIC LEUKAEMIA

4.1 Introduction

After introducing the ECIB model which was used in the rat, including the cannulation procedure, the ECIB procedure, the radiation equipment and the dosimetry, the various effects of ECIB treatment in the BN acute myelocytic leukaemia will be described. Three major questions are considered: (1) is there a different efficacy in terms of cell depletion between one long-lasting session of ECIB and several repeated sessions?; (2) to what degree do leukocyte mobilizing agents enhance the cell depletion induced by ECIB?; and (3) does ECIB change the proliferation characteristics of the leukaemic cell population?

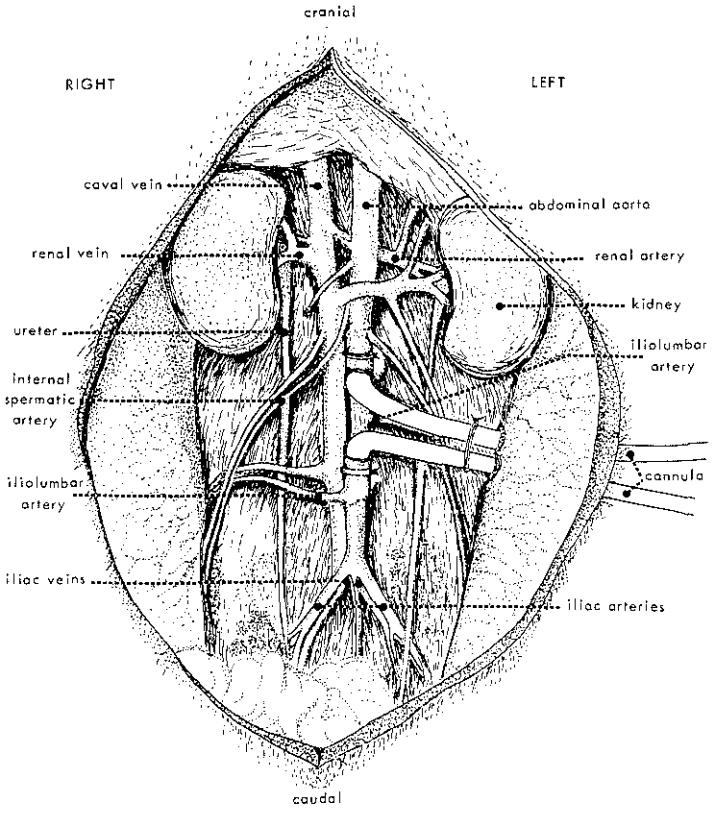

Fig. 4.1 *A schematic representation of the anatomical site of aorta cannulation in the rat.*

4.2 The ECIB model in the rat

4.2.1 Cannulation procedure

A schematic presentation of the cannulation of the abdominal aorta is presented in fig. 4.1. The cannula is inserted between both renal arteries and the aortic bifurcation. Only the left iliolumbar artery is cut off from its blood supply; however, this is without any harmful effects because of the rapid development of collaterals. The operation procedure is described in detail below.

The rat is anaesthetized with ether, the abdomen is shaved and the animal is fixed to a cork board. The abdominal wall is opened via a midline incision over a length of approximately 3 cm. After flexible wound retractors have been placed, the intestines, protected with moistened gauze, are retracted to the right side of the abdominal cavity to expose the aorta. The liver and the spleen are kept out of the operation field by compression with cotton wool rolls. With 2 rolls, the peritoneum overlying the abdominal aorta and the adjacent inferior caval vein is divided (fig. 4.2). Subsequently, both vessels are dissected with 2 forceps. Then loose ligatures (linen thread no. 40, Ethicon, Lameris, Utrecht, Holland) are placed around the aorta at the 2 sites where the cannula is to be inserted. The appropriately curved polyethene cannula (PP100, external diameter: 1.52 mm, internal diameter 0.86 mm; Portex Ltd., Hythe, Kent, England) is placed in the right position after it has been exteriorized by piercing the dorsal musculature with a trochar (fig. 4.3). It is filled with physiological saline. The cannula is cut off obliquely at both ends to facilitate insertion into the aorta. Two bulldog clamps are placed on the abdominal aorta, just distal from the renal arteries and proximal to the bifurcation. After 2 small incisions have been made in the aorta, both ends of the cannula are inserted and fixed by tightening the ligatures. Two additional ligatures are then placed at both ends before the clamps are removed (fig. 4.4). The abdomen is closed in two layers (linen thread no. 40, Ethicon, Lameris, Utrecht, Holland).

In this way, an extracorporeal aorta by-pass is established. The blood flow in the cannula can be judged on the basis of the light-red colour of the arterial blood. A broad piece of tape (Scotch Brand Tapes, Minnesota Mining and Mfg. Co., St. Paul, Minnesota, U.S.A.) is wrapped around the thorax to prevent the rat from committing suicide by biting through its own cannula (fig. 4.5). The total operation takes about 15 min, with a mortality of about 5%. The rats are caged individually. The cannula remains open for 1–2 weeks without the administration of anticoagulants.

4.2.2 ECIB procedure

To perform a session of ECIB, the rat is placed in a specially designed fixation

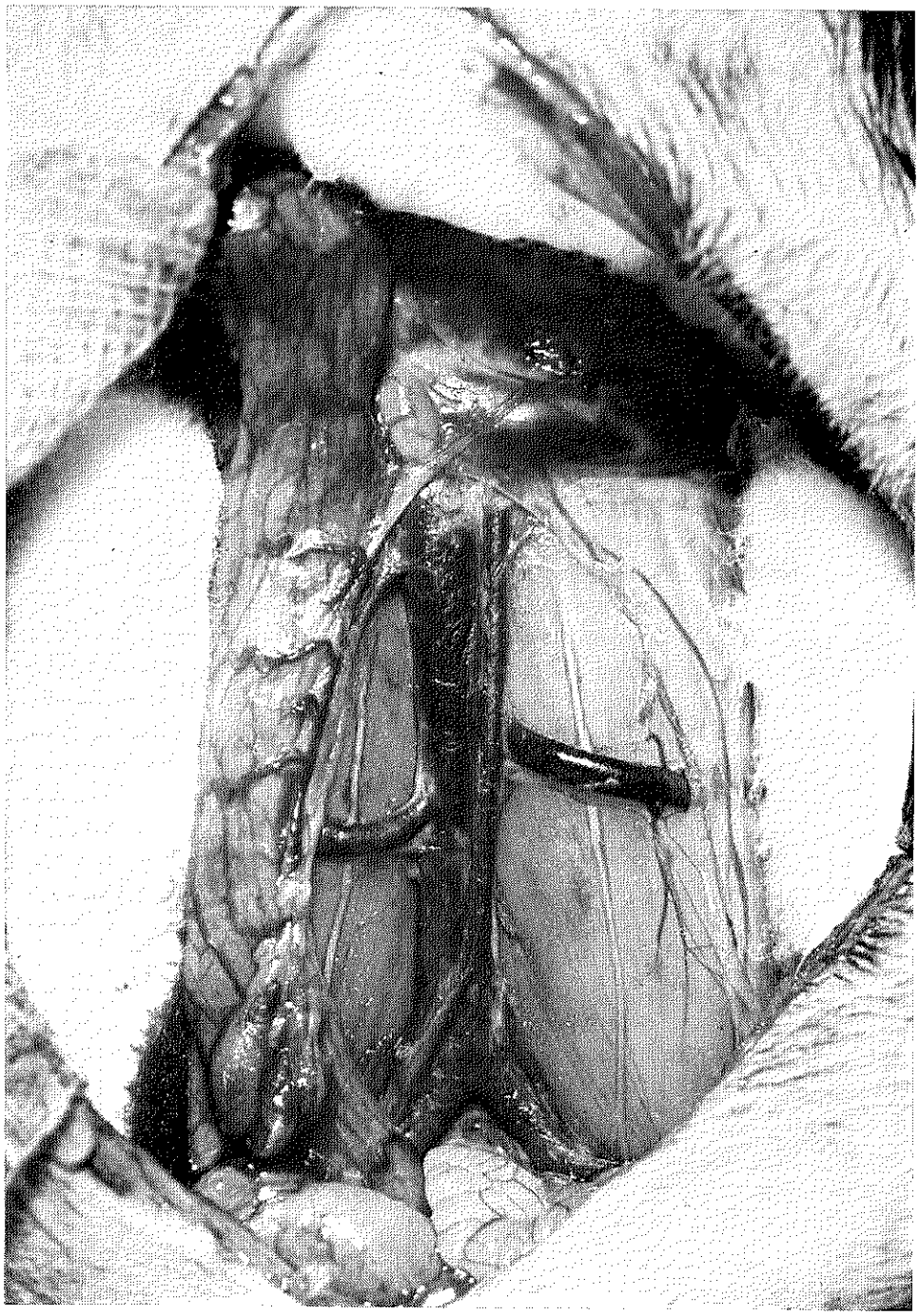

Fig. 4.2 *An overview of the operation field showing the abdominal aorta and the inferior caval vein.*

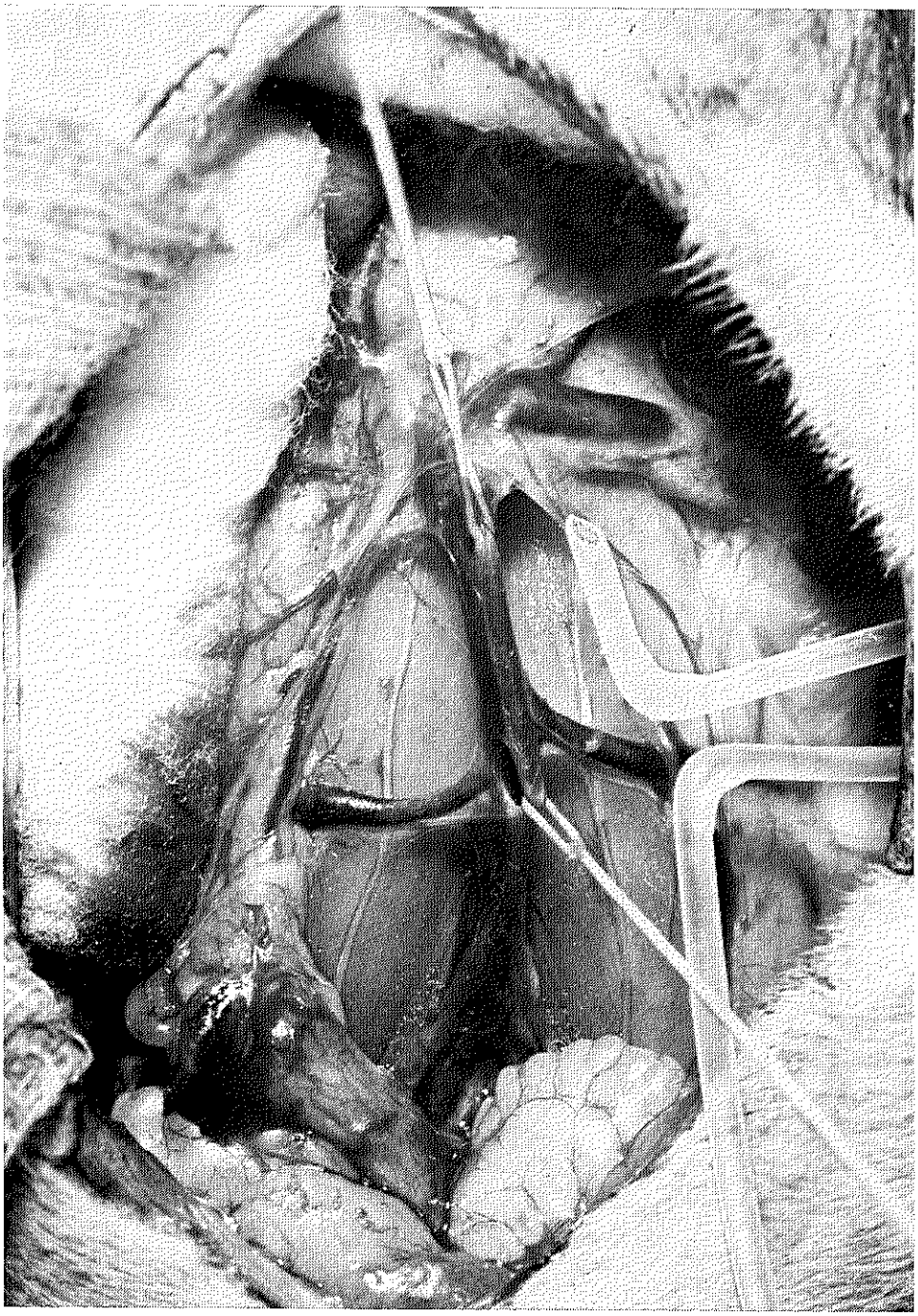

Fig. 4.3 *Two loose ligatures are placed around the aorta which is dissected from the caval vein. The cannula is placed in the right position.*

Fig. 4.4 *Both ends of the cannula have been inserted into the aorta. Arterial blood is flowing through the cannula.*

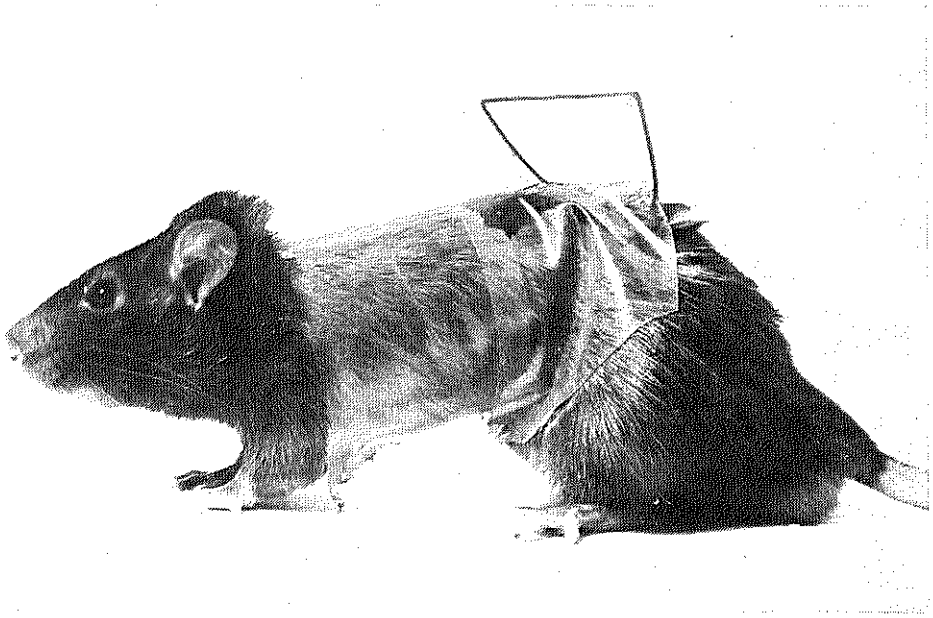

Fig. 4.5 *The exteriorized part of the cannula.*

cage which makes long-lasting anaesthesia unnecessary. Then the exteriorized part of the cannula is closed at 2 sides with forceps and cut in the middle. The cannula is flushed with physiological saline containing 200 IU (2 mg) of heparin (Thromboliquine, Organon, Oss, Holland). A silicone tube (external diameter: 2.5 mm, internal diameter: 1.5 mm; SR3H, Talas, Zwolle, Holland), is then connected to both ends of the cannula. The tube, which is filled with physiologic saline, has a length of 115 cm and a volume of 2 ml, which is about 1/7–1/8 of the total blood volume. It is placed as a coil in the radiation field. The volume which is irradiated is 1.5–1.6 ml. In this way, an extracorporeal blood circuit is established after removing the forceps (fig. 4.6). Twelve rats can be subjected to ECIB at one time. The flow rate in the loop is measured by determining the transit time of an air bubble introduced at the cranial end of the coil over a known volume part of the coil. It is regulated by a small screw clamp which is placed around the tube. The air surrounding the extracoporeal circuit is kept at a temperature of about 30 °C by a hot air blower in order to prevent the circulating blood from cooling. To avoid coagulation of the blood in the coil, 200 IU of heparin are injected every 2 h at the cranial end of the tube. With this method, a functioning extracorporeal blood circulation can be maintained for at least 24 h.

After the completion of each ECIB session, the flow rate of the blood in the coil is again measured and, after a forceps is placed at the cranial part of the

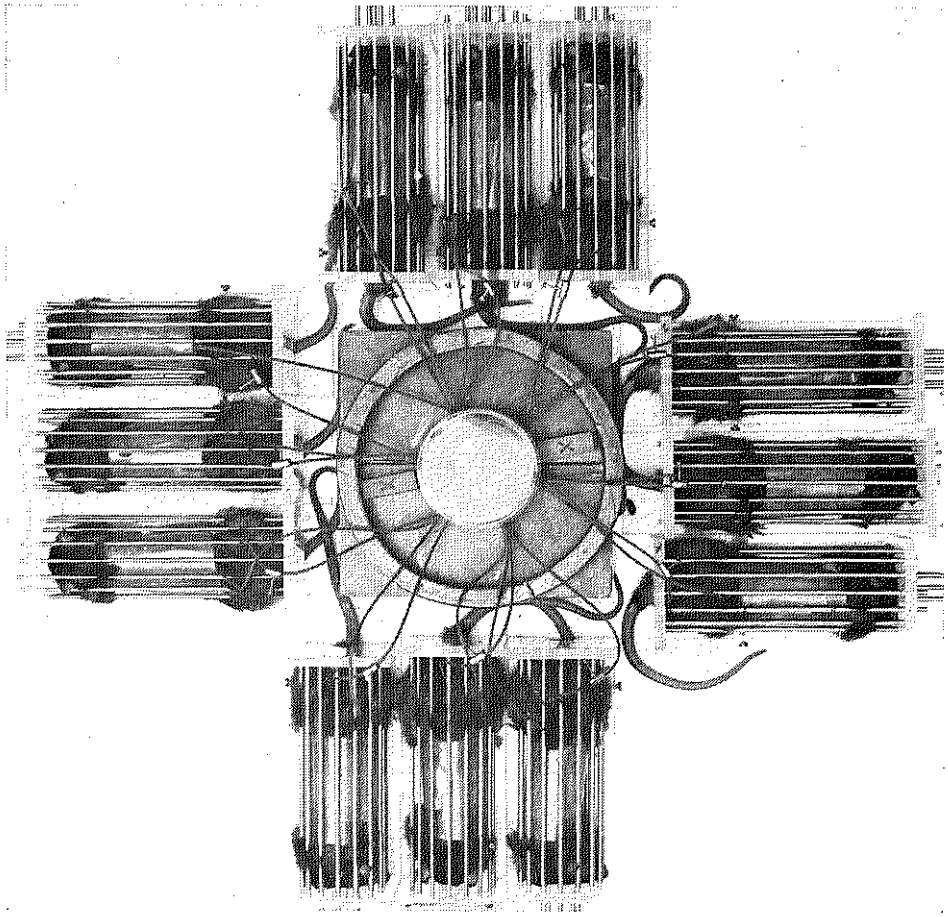

Fig. 4.6 *The extracorporeal blood circuit has been established. The tubes are placed as coils in the radiation field.*

cannula, the blood in the coil is pushed back into the caudal part of the cannula by means of air pressure from an air-filled syringe. The two parts of the cannula are joined with a small piece of silicone tube so that the original circuit is restored. The rat is then released from its fixation cage.

4.2.3 Radiation equipment

A conventional X-ray machine (Philips-Müller 300) is used to irradiate the blood. The physical parameters of the irradiation are: 300 kV, 10 mA, HVL of the beam 3.0 mm Cu, distance from the focus to the bottom of the cup 14–16 cm. To avoid Bremstrahlung, the cup with the coils is placed on a lead block of 20 by 20 cm with a thickness of 5 cm. The rats are placed adjacent to this block

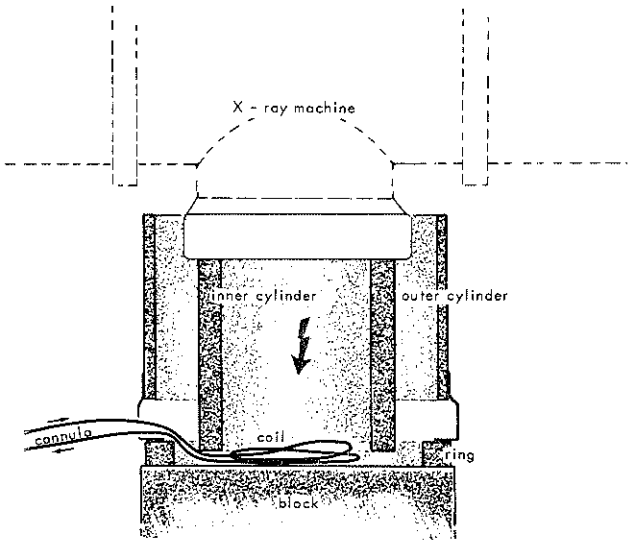

Fig. 4.7a. *A transverse section of the radiation set-up.*

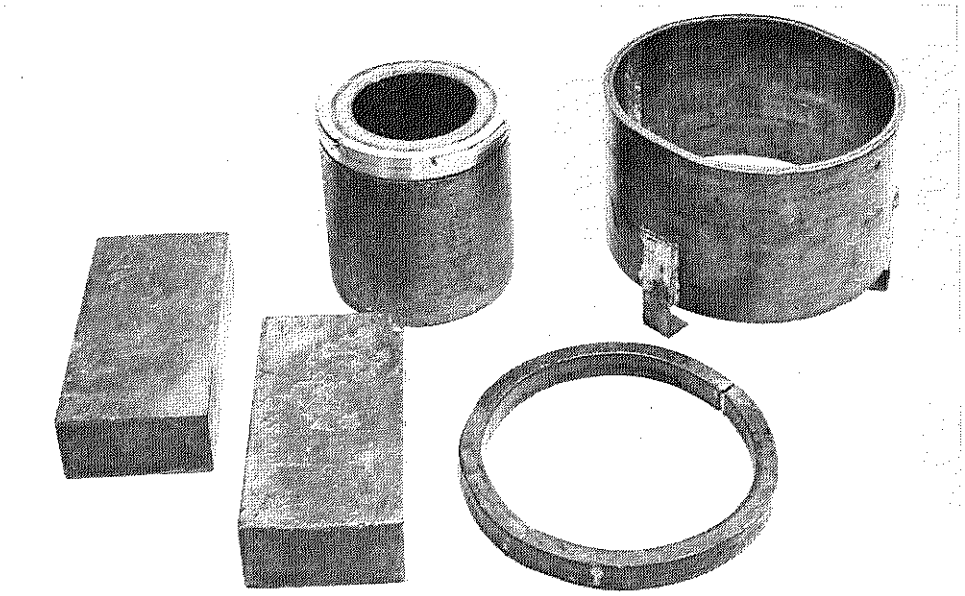

Fig. 4.7b *The separate lead units used for efficient shielding (inner and outer cylinders, ring and basement blocks).*

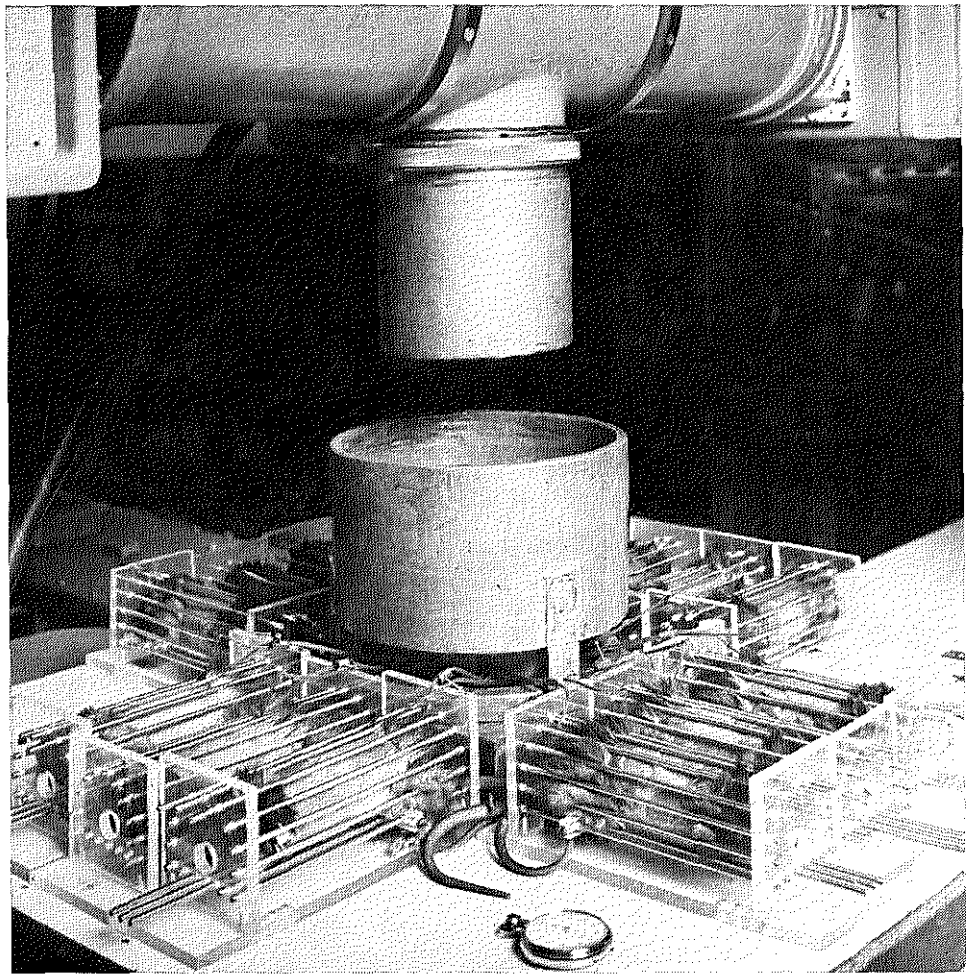

Fig. 4.7c *The radiation set-up with the two lead cylinders separated.*

(fig. 4.6). Because the BN leukaemia is very radiosensitive (see Ch. 4.3), efficient shielding is of utmost importance; therefore, two lead cylinders were constructed to focus the radiation to the cup. The inner one (thickness of the wall: 1.6 cm) is directly attached to the X-ray machine. The cup with the coils fits into its internal diameter (11 cm). Between the inner conus and the lead block, 0.5 cm is left open to give entrance to the coils. The outer cylinder (thickness of the wall: 0.8 cm) is placed around the inner one and, with 3 legs (length: 3 cm), rests on a lead ring (height: 1.9 cm, thickness: 1.6 cm), which in turn is placed on the lead block. In this way optimal shielding is provided (figs. 4.7 a, b, c, d and e).

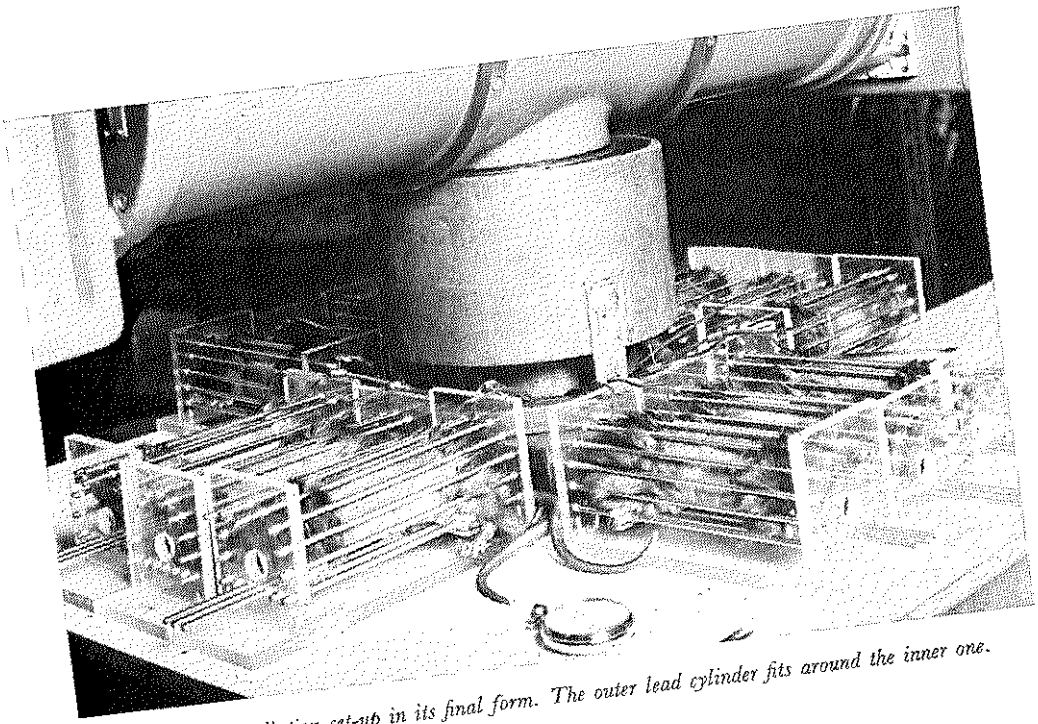

Fig. 4.7d *The radiation set-up in its final form. The outer lead cylinder fits around the inner one.*

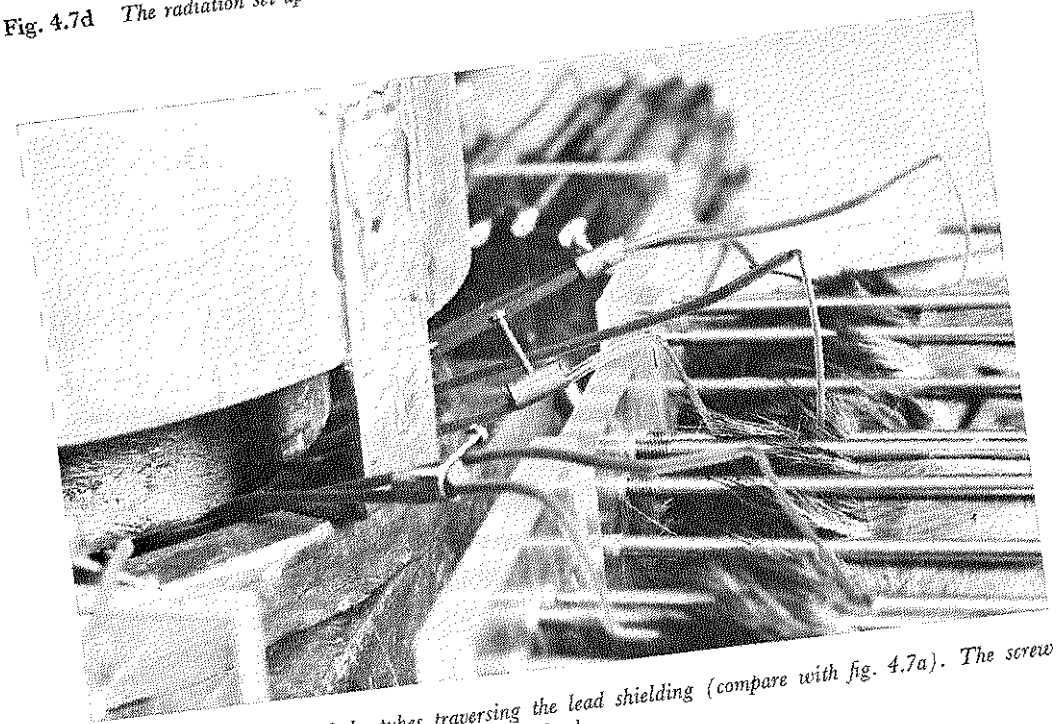

Fig. 4.7e *A close-up of the tubes traversing the lead shielding (compare with fig. 4.7a). The screw clamps regulate the flow rate of the blood.*

4.2.4 Dosimetry

With 300 kV, 10 mA X-rays the dose rate to the blood measured with a Baldwin Ionex ionization chamber is 200 ± 20 rad/min. Radiation leaks and back scatter are obviously unavoidable in this construction; therefore, the radiation dose received at the midline of the body of the rats was carefully determined. The average of a number of measurements ($n = 10$) was 0.6 ± 0.2 rad/h. This is acceptable, because no significant effect on the growth rate of the leukaemia was noted after subjecting rats to this protracted low-dose irradiation in control experiments.

With a fixed volume of the part of the coil which is irradiated (1.5–1.6 ml) and a fixed dose rate to the blood, the transit dose (TD) can be chosen within a wide range by changing the flow rate of the blood through the coil (see formula 1, Ch. 1.3.2). The TD during a session of ECIB is taken by the average of the measured flow rates before and after the irradiation. Generally, the flow rate measured after ECIB had decreased by 10–30% as compared with the initial flow rate. In practice, the TD was varied between 350 and 650 rad.

The mean total accumulated dose received by erythrocytes during one or more sessions of ECIB ($D_{\bar{x}}$, see formula 3, Ch. 1.3.2) never exceeded 35,000 rad. This is far below the haemolytic dose for rat erythrocytes (100,000–200,000 rad) as derived from data in the literature [Pellerin et al., 1960; Pellerin and Remy, 1968] and from our own experiments on *in vivo* survival of irradiated ^{51}Cr-labelled erythrocytes.

4.3 Radiosensitivity

4.3.1 Specific experimental procedures

In connection with the ECIB studies to be described, the D_0 for X-rays was determined for BNML blood cells. Leukaemic blood was irradiated *in vitro* at a concentration of 10^7 LC/ml with varying doses: 300, 600, 900, 1200 and 1500 rad, at a dose rate of 500 rad/min (for further physical parameters, see Ch. 4.2.3). The various suspensions were then inoculated into different groups of 6 rats in a dosage of 10^7 LC per rat. The per cent of rats dying from leukaemia and the survival times were determined. As controls, 4 groups consisting of 6 rats each received 10^6, 10^5, 10^4 and 10^3 nonirradiated leukaemic blood cells, respectively. From their dose-survival relationship (which was fitted by linear regression analysis) and the survival times of rats which received irradiated cells, the fraction of LC surviving a certain radiation dose could be calculated. In this way, the D_0 value was derived.

4.3.2 Results

The dose-survival curve for nonirradiated cells is given in fig. 4.8. The mean

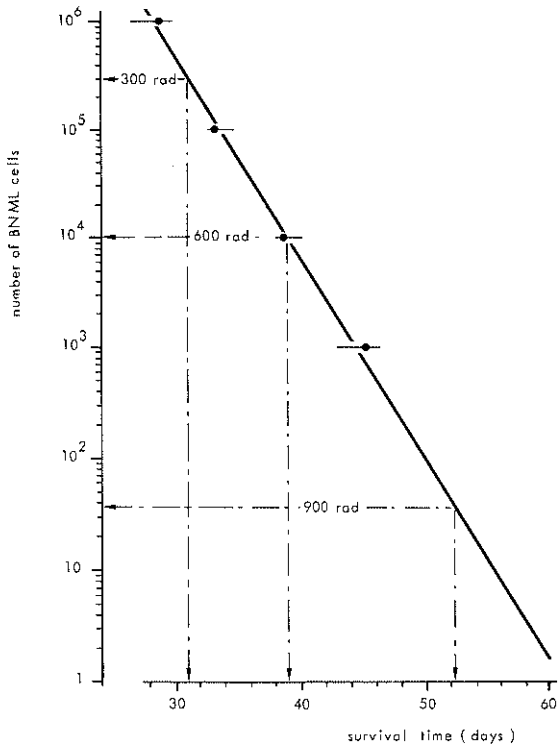

Fig. 4.8 *Dose-survival time curve for BNML blood cells.*
From the survival times scored after inoculation with 10^7 BNML cells in vitro irradiated with different doses of X-rays, the corresponding number of surviving cells was derived (see dotted lines). Horizontal bars represent 95% confidence limits. Each point of the dose-survival time curve for non-irradiated BNML cells represents means of 6 rats. For further explanation: see text.

survival times after injection with irradiated cells are also indicated. By extrapolation towards the Y-axis, the corresponding numbers of viable BNML cells are determined. Rats which received cells irradiated with 300 and 600 rad and all controls died from leukaemia. However, in the 900 rad group, 1 of the 6 animals (17%) survived. Furthermore, no rats receiving heavily irradiated BNML cells (1200, 1500 rad) developed leukaemia within the 250 days observation period.

By dividing the number of surviving cells by 10^7, the fraction of surviving cells for each radiation dose is found. Based on the surviving fraction of cells after 300, 600 and 900 rad irradiation, the curve in fig. 4.9 could be fitted. From this curve, the D_0 was calculated to be 68.1 rad, the 95% confidence limits ranging from 58.2 to 82.2 rad. The extrapolation number (N) was 3.7; this gives a D_q value of 89.0 rad, according to the formula:

$$D_q = D_0 \times \ln N.$$

4.3.3 Discussion

BNML blood cells are characterized by a rather low D_0 value in comparison with other experimental leukaemias. Most leukaemias studied, including the L5178Y, the CBA lymphocytic leukaemia, the AKR lymphoma and the P388 leukaemia, show D_0 values ranging from 60–160 rad [Whitmore and Till, 1964; Broerse and van Oosterom, 1974].

The small shoulder of the survival curve (fig. 4.9) characterized by low values for D_q and N indicates that repair of sublethal damage is not a pronounced phenomenon in the BNML. It indicates that the total cell kill after fractionated irradiation will be significantly greater than in the case of leukaemias showing survival curves with broader shoulders. This favours the application of repeated sessions of ECIB.

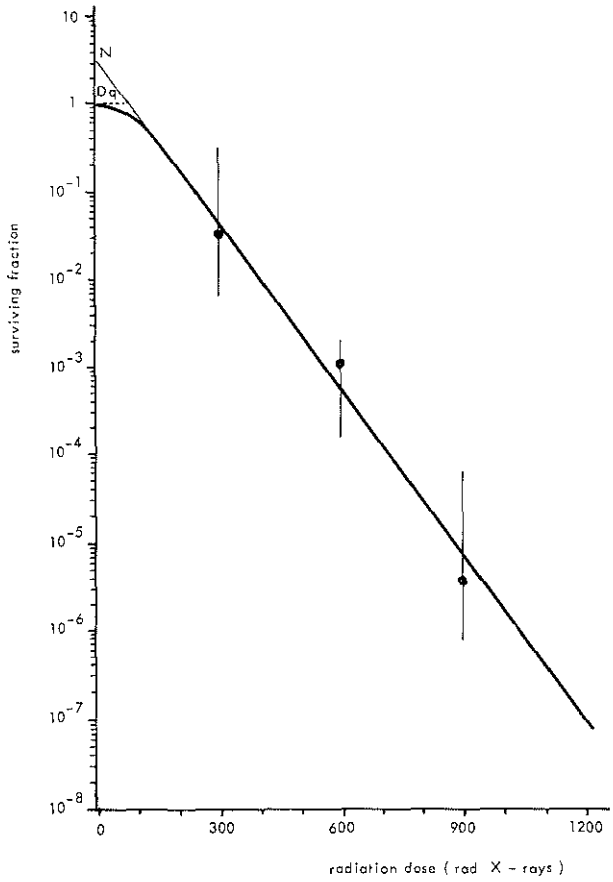

Fig. 4.9 *Survival curve for BNML blood cells after in vitro irradiation with X-rays.*
The fraction of surviving cells was calculated by dividing the number of surviving cells (see fig. 4.8) by 10^7 (the total number of injected irradiated cells). Vertical bars represent 95% confidence limits.

A dose of 900 rad results in an average surviving fraction of 3.6×10^{-6} (fig. 4.9). Thus, it can be calculated that, of 10^7 irradiated cells, 36 cells survive. This number of cells induces leukaemia in 83% (5 out of 6) of the rats. These results can be compared with those of the TD_{50} study. From the TD_{50} curve (fig. 2.17) it would be expected that 36 cells would induce leukaemia in 60% of the rats. If the 95% confidence limits in the TD_{50} experiment are taken into account, 83% is not significantly different from 60%. Thus, both experiments strongly confirm each other.

4.4 The appropriate controls for ECIB treated rats

4.4.1 The effect of aorta cannulation on haematologic parameters

Thirteen rats were inoculated with 10^7 BNML spleen cells. At day 14, eight of them were cannulated. Seven days later (day 21), blood samples were obtained from the tails of both cannulated and noncannulated rats and the absolute numbers of leukaemic cells, platelets, reticulocytes, erythrocytes and haematocrit values were determined.

In table 4.1, haematologic parameters derived from noncannulated leukaemic rats and cannulated rats are compared. Except for the number of platelets, all other parameters have changed significantly due to the aorta cannulation.

Table 4.1 *The effect of aorta cannulation on haematologic parameters determined 7 days after the operation*
(day 21 after inoculation with 10^7 BNML spleen cells)

	leukaemic cells ($\times 10^6$/ml)	platelets ($\times 10^6$/ml)	erythrocytes ($\times 10^6$/ml)	Ht (%)	reticulocytes (‰)
non-cannulated rats (n = 5)	15.2 ± 1.3	83.4 ± 11.9	1109 ± 47	44 ± 1	44.0 ± 7.9
cannulated rats (n = 8)	12.2 ± 0.8	104.0 ± 11.3	710 ± 41	38 ± 1	102.0 ± 16.4
student "t" test	$p < 0.05$	N.S.	$p < 0.0005$	$p < 0.0025$	$p < 0.0125$

Ht: haematocrit; N.S.: nonsignificant difference.
Values represent means (±SE).

In particular, the number of erythrocytes has decreased. The most likely explanation is that erythrocytes may have a shortened life span due to mechanical damage sustained during their repeated passage through the cannula. An increased number of reticulocytes, indicates increased erythropoiesis to compensate for the loss of erythrocytes. The number of leukaemic cells also decreases in the cannulated rats, most probably due to their increased fragility

(see Ch. 2.4.1) which makes them more vulnerable to damage during their passage through the cannula.

In summary, because the cannulation per se induces distinct haematologic changes, noncannulated rats cannot be used as controls for the ECIB studies.

4.4.2 Comparison between cannulated controls and rats submitted to extracorporeal circulation

Two other candidate control groups for ECIB were investigated: cannulated controls (CC) and rats which were subjected to repeated sessions of extracorporeal circulation (ECC) without irradiation. The experimental protocol was as follows. Eleven rats were inoculated with 10^7 BNML cells at day 0. Fourteen days later, all rats were cannulated. At days 21, 22, 23 and 24, six rats were subjected to ECC. Each session lasted for 5 h; the average flow rate of the blood in the extracorporeal circuit ($F_{\bar{x}}$) was 1.0 ml/min, i.e., 22 times the blood volume (BV) passed through the shunt per session (BV = 13.5 ml). The 5 remaining rats received only heparin according to the schedule described in Ch. 4.2.2. At day 25, blood samples were obtained from the aorta and the various haematologic parameters were determined. The results are given in table 4.2. No significant differences were found for any of the parameters studied. Therefore, both cannulated rats and rats undergoing ECC can serve as optimal controls for ECIB treated rats.

Table 4.2 *The effect of repeated extracorporeal circulation on haematologic parameters determined one day after the last session*
(day 25 after inoculation with 10^7 BNML spleen cells)

	leukaemic cells ($\times 10^6$/ml)	platelets ($\times 10^6$/ml)	erythrocytes ($\times 10^6$/ml)	Ht (%)	reticulocytes (‰)
CC (n = 5)	15.0±1.0	58.0±4.9	339±33	13±2	178.0±33.7
ECC (n = 6)	16.4±1.0	60.0±5.7	361±34	16±2	139.0±22.6
student "t" test	N.S.	N.S.	N.S.	N.S.	N.S.

Ht: haematocrit; N.S.: nonsignificant difference; values represent means ±S.E.
CC: cannulated controls; ECC: 4×5 h of extracorporeal circulation at days 21–24 after inoculation with 10^7 BNML spleen cells.
For further explanation: see text.

4.5 Comparison of different ECIB schedules

Three different ECIB protocols in terms of sessions per unit of time were investigated: 1×20 h, 2×10 h and 4×5 h. With the repeated regimens, the sessions were performed on consecutive days. The irradiations usually started between 10 and 11 a. m. Cannulated rats or ECC rats (which received repeated

injections with heparin; Ch. 4.2.2) were used as controls in all experiments. As a rule, cannulation was performed at day 13 or 14 after inoculation with 10^7 BNML cells.

The parameters of evaluation were: haematologic follow up before and after irradiation performed on blood samples obtained from the cannula; compartment analysis by means of the ^{51}Chromium method (Ch. 2.6.3; 2.6.5), which was started 1–2 h after completing (the last session of) ECIB; and fresh liver and spleen weights at the time of killing, i.e., 24–25 h after infusion of ^{51}Cr-labelled leukaemic cells. A list of symbols for the various physical parameters to be described is given in table 4.3.

Table 4.3 *Symbols for the various physical parameters used in ECIB*

parameter	symbol
body weight	BW (g)
blood volume	V_b (ml)
volume of the irradiated coil	V (ml)
flow rate of the blood in the coil	F (ml/min)
number of blood volumes passing through the coil	xV_b
dose rate	R (rad/min)
transit dose	TD (rad)
mean dose to the total blood	$D_{\bar{x}}$ (rad)

4.5.1 1 × 20 h of ECIB

Five rats were subjected to 1 × 20 h of ECIB which was started at day 21 after inoculation. In this experiment, 5 ECC rats served as controls. The physical parameters are shown in table 4.4.

One hour after completing the ECIB/ECC procedure, all rats received 4.5×10^7 ^{51}Cr-labelled BNML blood cells through the aorta cannula and the blood disappearance was investigated during the subsequent 25 h. At t = 25 h, the rats were killed and their organ uptake was determined.

Changes in the number of both lymphocytes and leukaemic cells (LC) are plotted before and after ECIB in fig. 4.10. The number of lymphocytes has already decreased significantly shortly after the session. However, it takes 24 h more to observe a significant decrease in the number of LC as compared with the steadily increasing number in the ECC group. In fact, in the ECIB treated group, the number of LC remains at a constant level rather than decreasing. Table 4.5 shows the liver and spleen weights which were determined 26 h after completion of the ECIB session. No significant differences were noted.

Based on calculations described in Ch. 2.6.5, changes in the size of the various functional compartments of LC were determined. From the blood disappearance curve, the total blood pool (TBP), the marginal pool (MP), the

Table 4.4 *Physical parameters of 1×20 h, 2×10 h and 4×5 h of ECIB/ECC*

	1×20 h of ECIB		2×10 h of ECIB				4×5 h of ECIB
			session I		session II		
	ECIB (n=5)	ECC (n=5)	ECIB (n=4)	ECC (n=4)	ECIB (n=4)	ECC (n=4)	ECIB (n=8)
BW (g)	210	220	220	210	220	210	260
V_b (ml)	10.5	11.0	11.0	10.5	11.0	10.5	13.0
V (ml)	1.5	1.5	1.5	1.5	1.5	1.5	1.6
F (ml/min)	0.55	0.57	0.43	0.43	0.52	0.62	0.80
xV_b	62.9	62.2	23.5	24.6	28.4	35.4	18.5
R (rad/min)	160	–	190	–	190	–	190
TD (rad)	436	–	663	–	548	–	380
$D_{\bar{x}}$ (rad)	24,000	–	13,680	–	13,680	–	6247

For explanation of symbols: see table 4.3; ECC: extracorporeal circulation.
Values represent means of the indicated number of rats. In the 4×5 h experiment no significant differences were found between the separate sessions; cannulated rats served as controls.

Table 4.5 *Organ weights measured 26 h after 1×20 h of ECIB/ECC*

	liver weight (g)	spleen weight (g)
ECC	19.8±0.5	2.4±0.3
ECIB	19.2±0.8	2.2±0.3
student "t" test	N.S.	N.S.

Values represent means (±SE) of 5 rats.
ECC: extracorporeal circulation; N.S.: nonsignificant difference.

Table 4.6 *Changes in the size of the various functional compartments after 1×20 h of ECIB* (leukaemic cells $\times 10^7$)

	% ^{51}Cr-cells in CBP after infusion	CBP	MP	TBP	% ^{51}Cr-cells in CBP at equilibrium	RETP	TREP
ECC	27.1	15.4	41.4	56.8	13.1	60.8	117.6
ECIB	32.0	11.8	24.9	36.7	14.1	46.7	83.4
% reduction by ECIB		24	40	35		23	29

Values represent means of 5 rats.
ECC: extracorporeal circulation; CBP: circulating blood pool; MP: marginal pool; TBP: total blood pool; RETP: rapidly exchangeable tissue pool; TREP: total rapidly exchangeable pool.

Table 4.7 *Changes in the size of the rapidly exchangeable tissue pools (RETP) in various organs after 1 × 20 h of ECIB*
(leukaemic cells $\times 10^7$)

	liver	spleen	bone marrow*	lungs
ECC	57.1	3.9	12.7	1.2
ECIB	37.5	3.0	6.6	0.8
% reduction by ECIB	34	23	48	30

* Total bone marrow was calculated (1 femur = 1.2%).
Values represent means of 5 rats.
ECC: extracorporeal circulation; RETP: rapidly exchangeable tissue pool.
For further explanation: see text.

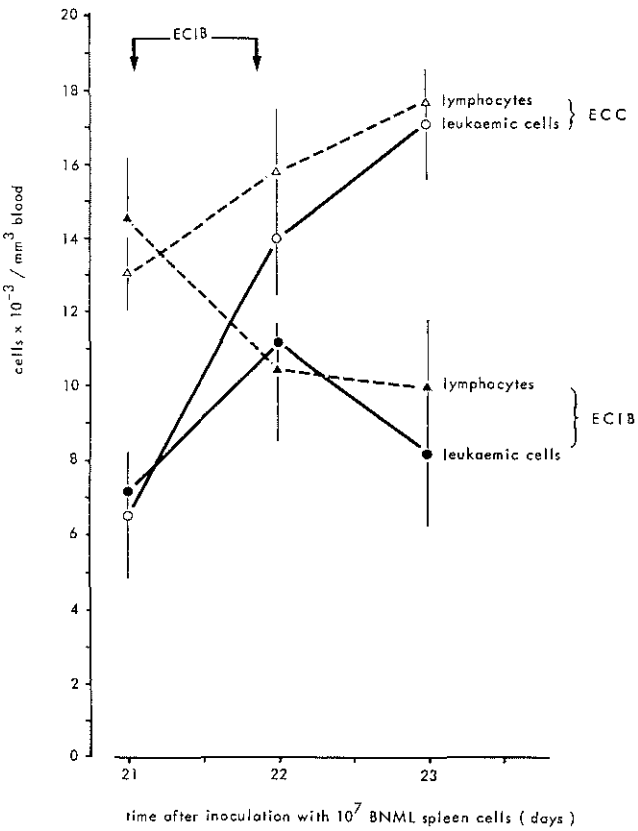

Fig. 4.10 *Effect of 1 × 20 h of ECIB/ECC on the number of leukaemic cells and lymphocytes in the peripheral blood.*
Each point represents means (± SE) of 5 rats.
ECC: extracorporeal circulation.

rapidly exchangeable tissue pool (RETP) and thus the total rapidly exchangeable pool (TREP) could be quantified (table 4.6). The TBP is more effectively depleted (35% reduction) as compared with the RETP (23%). This is mainly due to a reduction in the MP to 60% of its original size. The entire compartment of rapidly exchangeable LC (TREP) is reduced by 29%. In table 4.7, the values for the RETP are specified for various organs, based on ^{51}Cr-organ uptake studies. After 20 h of ECIB, the RETP of LC in the bone marrow is the most reduced (48%), followed by the liver, the lungs and the spleen. The slowly exchangeable tissue pools, calculated by means of total organ weights, have only been reduced by 11 and 4% in the spleen and the liver, respectively (table 4.8).

Table 4.8 *Changes in the size of the slowly exchangeable tissue pools (SETP) in the spleen and the liver after 1 × 20 h of ECIB*
(leukaemic cells $\times 10^7$)

		RETP	SETP	% reduction of SETP by ECIB
spleen	ECC	3.9	176.1	
	ECIB	3.0	157.0	11
liver	ECC	57.1	1122.9	
	ECIB	37.5	1082.5	4

Values represent means of 5 rats.
ECC: extracorporeal circulation; RETP: rapidly exchangeable tissue pool; SETP: slowly exchangeable tissue pool.

4.5.2 2 × 10 h of ECIB

At days 20 and 21, four rats with BNML underwent 2 sessions of ECIB, each lasting for 10 h. Four leukaemic ECC rats were used as controls. The physical parameters are presented in table 4.4. After the second session, all rats were injected with 3×10^7 ^{51}Cr labelled BNML cells for compartment analysis. Organ uptake was determined at 24 h after infusion.

Fig. 4.11 shows changes in the number of peripheral lymphocytes and leukaemic cells. In the ECIB treated group, both cell types show a decline which is much more striking than in the 1 × 20 h experiment (fig. 4.10).

With respect to organ weights, only the spleen weight is significantly reduced (table 4.9). Changes in the size of the functional compartments are given in tables 4.10–4.12. Both the blood compartment (TBP) and the rapidly exchangeable tissue compartment (RETP) are depleted to the same extent, i.e., to approximately 30% of their original size, by this treatment (table 4.10); this is about 2–3 times as much as in the 1 × 20 h regimen (table 4.6). The RETP as measured in the various organs is decreased to a similar degree as the TBP and

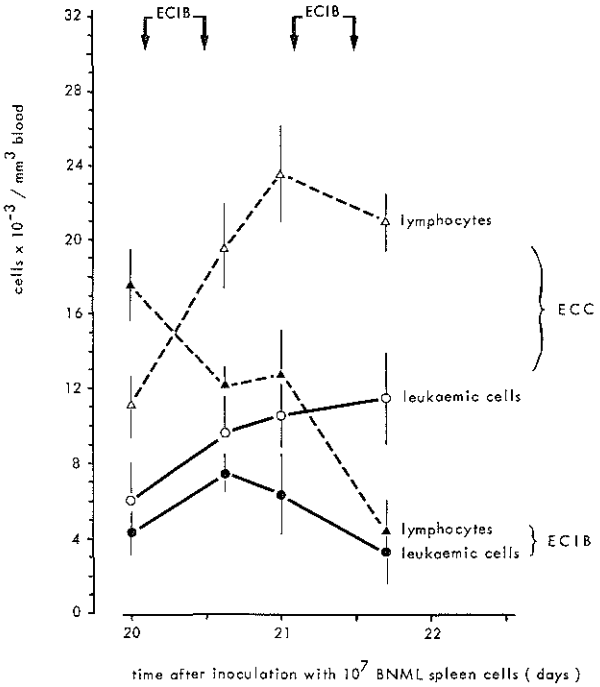

Fig. 4.11 *Effect of 2×10 h of ECIB/ECC on the number of leukaemic cells and lymphocytes in the peripheral blood.*
Each point represents means (±SE) of 4 rats.
ECC: extracorporeal circulation.

the RETP calculated from the blood disappearance curve (table 4.11). The depletion of the RETP per organ is a factor of 1.5–2.5 more than after 1×20 h of ECIB (table 4.7). The SETP in the spleen is reduced by 28% (table 4.12), i.e., 2.5 times as much as compared with the first regimen (11%, table 4.8). As in that experiment, the SETP in the liver is again not significantly influenced by 2×10 h of ECIB (compare tables 4.8 and 4.12).

Table 4.9 *Organ weights measured 25 h after 2×10 h of ECIB/ECC*

	liver weight (g)	spleen weight (g)
ECC	17.3±1.2	3.7±0.5
ECIB	17.8±0.3	2.8±0.4
student "t" test	N.S.	$p<0.05$

Values represent means (±SE) of 4 rats.
ECC: extracorporeal circulation; N.S.: nonsignificant difference.

Table 4.10 *Changes in the size of the various functional compartments after 2× 10 h of ECIB* (leukaemic cells $\times 10^7$)

	% ^{51}Cr-cells in CBP after infusion	CBP	MP	TBP	% ^{51}Cr-cells in CBP at equilibrium	RETP	TREP
ECC	20.7	12.1	46.2	58.3	10.2	60.1	118.4
ECIB	19.7	3.6	14.8	18.4	9.5	19.8	38.2
% reduction by ECIB		70	68	68		67	68

Values represent means of 4 rats.
ECC: extracorporeal circulation; CBP: circulating blood pool; MP: marginal pool; TBP: total blood pool; RETP: rapidly exchangeable tissue pool; TREP: total rapidly exchangeable pool.

Table 4.11 *Changes in the size of the rapidly exchangeable tissue pools (RETP) in various organs after 2× 10 h of ECIB* (leukaemic cells $\times 10^7$)

	liver	spleen	bone marrow*	lungs
ECC	57.2	6.4	16.8	3.1
ECIB	19.5	2.5	4.8	0.9
% reduction by ECIB	66	61	72	72

* Total bone marrow was calculated (1 femur = 1.2%).
Values represent means of 4 rats.
ECC: extracorporeal circulation; RETP: rapidly exchangeable tissue pool.
For further explanation: see text.

Table 4.12 *Changes in the size of the slowly exchangeable tissue pools (SETP) in the spleen and the liver after 2× 10 h of ECIB* (leukaemic cells $\times 10^7$)

		RETP	SETP	% reduction of SETP by ECIB
spleen	ECC	6.4	303.6	
	ECIB	2.5	217.5	28
liver	ECC	57.2	872.8	
	ECIB	19.5	960.5	–

Values represent means of 4 rats.
ECC: extracorporeal circulation; RETP: rapidly exchangeable tissue pool; SETP: slowly exchangeable tissue pool.

4.5.3 4×5 h of ECIB

Eight rats were treated with 4 sessions of ECIB each lasting for 5 h at days 20, 21, 22 and 23 after inoculation with leukaemia. For physical parameters, see table 4.4. Eight cannulated rats served as controls. In 6 ECIB rats and 6 CC rats, compartment analysis was performed as soon as the last session was ended; 2.5×10^7 ^{51}Cr-cells were infused intra-arterially.

During this treatment regimen, the number of leukaemic cells in the blood initially decreases (fig. 4.12), but starts to rise again 24 hours after the second session of ECIB. This is in contrast with the 1×20 h regimen, where, at 24 h after ECIB, a slight decrease as compared with the number of LC counted directly after the irradiation is noted (fig. 4.10). A small decrease is also observed during the subsequent 24 h, after the first session of 1×10 h of ECIB; this continues after the second session (fig. 4.11). The increase in the 4×5 h

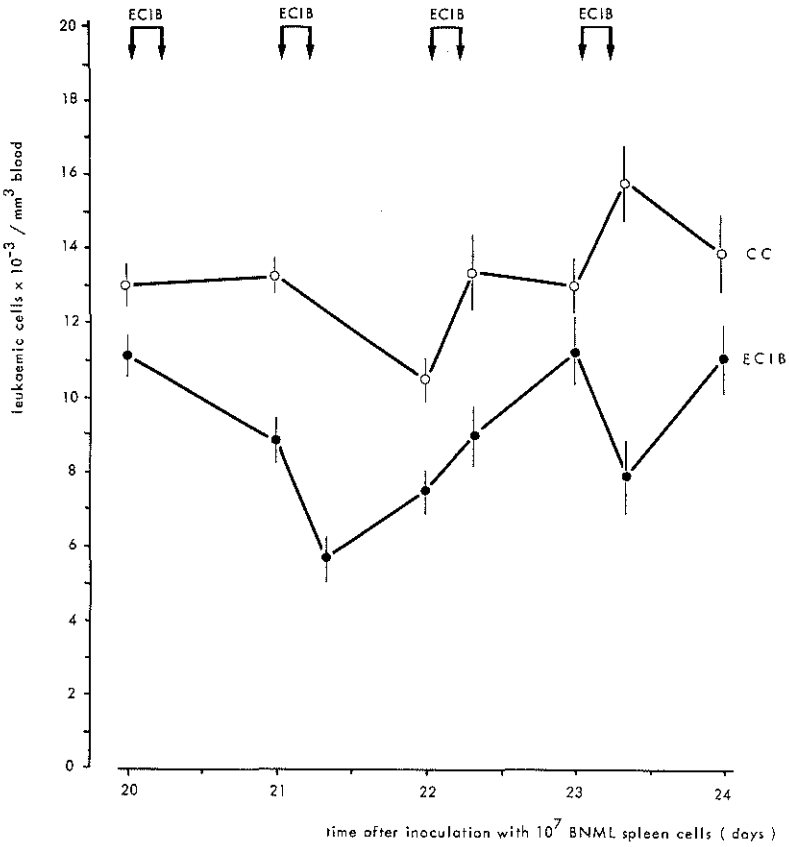

Fig. 4.12 *Effect of 4×5 h of ECIB on the number of leukaemic cells in the peripheral blood.* Each point represents means (±SE) of 8 rats.
CC: cannulated controls.

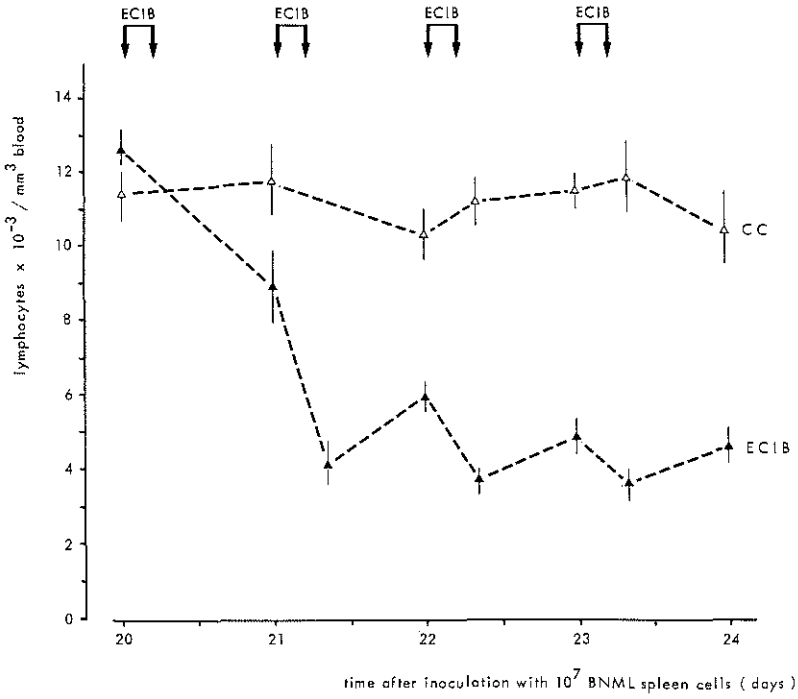

Fig. 4.13 *Effect of 4×5 h of ECIB on the number of lymphocytes in the peripheral blood.*
Each point represents means (±SE) of 8 rats.
CC: cannulated controls.

experiment runs approximately parallel – although at a lower level – to the control curve. Just as in the other two experiments, lymphocytes show a rapid decline in the ECIB treated group (fig. 4.13). After the second session, the lymphocyte count remains at a constant low level up to the end of the observation period.

In this experiment, changes in the number of platelets, haematocrit (Ht) values, erythrocytes and reticulocytes were also determined. Platelets show a similar decrease in both the ECIB and the control group (fig. 4.14). Haematocrit values also decrease (fig. 4.14) which correlates very well with decreasing erythrocyte counts (table 4.13). Despite the developing anaemia, which is mainly due to the unavoidable blood loss associated with the repeated ECIB procedure, reticulocytes do not change significantly during the course of treatment (table 4.13). Apparently, at this stage of the disease, normal erythropoiesis fails to keep pace with the increased demand for red cells.

With respect to organ weights, both the liver and the spleen show a significant decrease after 4 sessions of ECIB (table 4.14). In table 4.15, it is shown that cell depletion in the RETP (by 74%) is two times as great as in the TBP

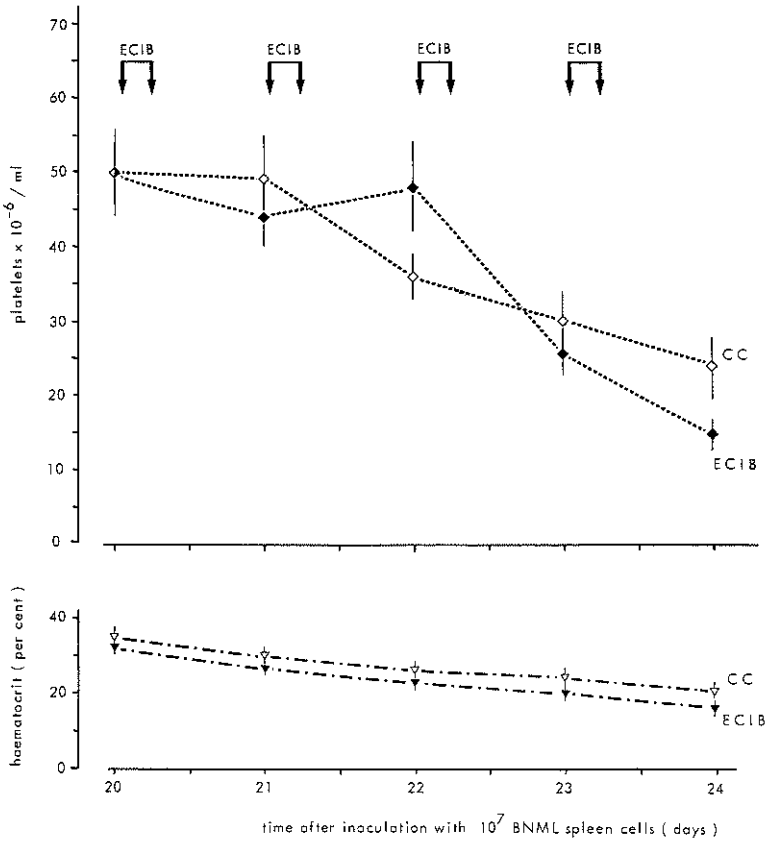

Fig. 4.14 *Effect of 4×5 h of ECIB on the number of blood platelets and haematocrit values.*
Each point represents means (±SE) of 8 rats.
CC: cannulated controls.

Table 4.13 *Effect of 4×5 h of ECIB on the number of erythrocytes and reticulocytes*

	erythrocytes (×10^6/ml)		reticulocytes (‰)	
	before ECIB	after 4×5 h ECIB	before ECIB	after 4×5 h ECIB
	day 20	day 24	day 20	day 24
CC	702±34	363±21	45±12	53±9
ECIB	694±45	324±41	54±7	50±5
student "t" test	N.S.	N.S.	N.S.	N.S.

Values represent means (±SE) of 8 rats.
CC: cannulated controls; N.S.: nonsignificant difference.

Table 4.14 *Organ weights measured 25 h after 4 × 5 h of ECIB*

	liver weight (g)	spleen weight (g)
CC	24.4±0.9	4.4±0.3
ECIB	21.0±0.8	3.6±0.2
student "t" test	$p < 0.01$	$p < 0.025$

Values represent means (±SE) of 6 rats.
CC: cannulated controls.

Table 4.15 *Changes in the size of the various functional compartments after 4 × 5 h of ECIB* (leukaemic cells × 10^7)

	% ^{51}Cr-cells in CBP after infusion	CBP	MP	TBP	% ^{51}Cr-cells in CBP at equilibrium	RETP	TREP
CC	21.1	20.5	76.9	97.5	2.1	880.6	978.1
ECIB	16.7	10.3	51.4	61.7	3.5	231.8	293.4
% reduction by ECIB		50	33	37		74	70

Values represent means of 6 rats.
CC: cannulated controls; CBP: circulating blood pool; MP: marginal pool; TBP: total blood pool; RETP: rapidly exchangeable tissue pool; TREP: total rapidly exchangeable pool.

Table 4.16 *Changes in the size of the rapidly exchangeable tissue pools (RETP) in various organs after 4 × 5 h of ECIB* (leukaemic cells × 10^7)

	liver	spleen	bone marrow*	lungs
CC	488.1	20.3	97.8	23.7
ECIB	148.3	9.4	35.5	8.9
% reduction by ECIB	70	54	64	62

* Total bone marrow was calculated (1 femur = 1.2%).
Values represent means of 6 rats.
CC: cannulated controls; RETP: rapidly exchangeable tissue pool.
For further explanation: see text.

(reduction by 37%). The efficient size reduction of the RETP is confirmed by data derived from the individual organs (table 4.16): the RETP in all organs measured decreases by more than 50%. This is similar to results obtained with the 2 × 10 h regimen (table 4.11). However, the TBP is more drastically reduced after 2 × 10 h of ECIB, i.e., 68% reduction (table 4.10) as compared with 37% after 4 × 5 h of irradiation. When the 1 × 20 h and the 4 × 5 h protocols are

compared, both the TBP and the RETP are depleted to a greater degree in the latter protocol.

After 4×5 h of ECIB, the SETP in the spleen has decreased by 19% (table 4.17), which is more than after 1×20 h of ECIB (11%; table 4.8) but less than after the 2×10 h protocol (28%; table 4.12). As after the other two schedules, the size of the SETP in the liver is not reduced (table 4.17).

Table 4.17 *Changes in the size of the slowly exchangeable tissue pools (SETP) in the spleen and the liver after 4×5 h of ECIB*
(leukaemic cells $\times 10^7$)

		RETP	SETP	% reduction of SETP by ECIB
spleen	CC	20.3	359.7	
	ECIB	9.4	290.6	19
liver	CC	488.1	1151.9	
	ECIB	148.3	1151.7	–

Values represent means of 6 rats.
CC: cannulated controls; RETP: rapidly exchangeable tissue pool; SETP: slowly exchangeable tissue pool.

4.6 ECIB in combination with cell mobilization

Fifteen rats were used in this study. Five of them were subjected to two 8 h sessions of ECIB on days 20 and 21 after inoculation with leukaemia. The second group of 5 animals was treated similarly. In addition, these rats were given three injections with dextran sulphate (DS_{17}; see Ch. 3.2.1) at 10 mg/kg each. The injections were given through the cannula at $t = -1$ h, $t = 3$ h and $t = 6$ h, where the start of an ECIB session is designated as $t = 0$ h. Physical

Table 4.18 *Physical parameters of 2×8 h of ECIB in combination with cell mobilization*

	session I		session II	
	ECIB	ECIB+DS_{17}	ECIB	ECIB+DS_{17}
BW (g)	290	295	290	295
V_b (ml)	14.5	14.7	14.5	14.7
V (ml)	1.5	1.5	1.5	1.5
F (ml/min)	0.44	0.56	0.45	0.54
x V_b	14.6	18.2	14.9	17.5
R (rad/min)	190	190	190	190
TD (rad)	648	509	633	528
$D_{\bar{x}}$ (rad)	8550	8418	8550	8418

Values represent means of 5 rats.
DS_{17}: dextran sulphate (MW 17,000).
Cannulated rats served as nonirradiated controls.
For explanation of symbols: see table 4.3.

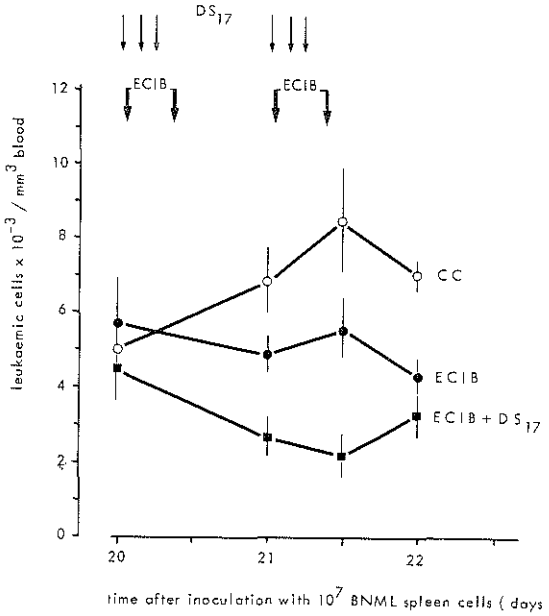

Fig. 4.15 *Effect of 2×8 h of ECIB in combination with cell mobilization on the number of leukaemic cells in the peripheral blood.*
Each point represents means (±SE) of 5 rats.
CC: cannulated controls.
DS_{17}: dextran sulphate (MW 17,000): 10 mg/kg i.a.; each arrow represents one injection.

parameters are given in table 4.18. Five cannulated rats served as controls. All animals were heparinized during both sessions.

As described earlier, a complete haematologic follow-up was performed, organ weights were determined and compartment analysis was carried out by infusing 2.4×10^7 ^{51}Cr-labelled leukaemic cells into all rats at 1 h after the second session was completed.

Changes in the number of peripheral leukaemic cells are plotted in fig. 4.15. As LC increase steadily in the CC group, the decrease is most marked in the DS-treated ECIB group. In the group treated with ECIB alone, LC remain at a fairly constant level and finally decrease. Lymphocytes decrease significantly in both ECIB groups with lowest values reached in the DS-treated ECIB group (fig. 4.16).

Liver and spleen weights are only significantly reduced in the DS-treated ECIB group both as compared with the CC group and with the group treated with ECIB alone (table 4.19).

From table 4.20, it is clear that ECIB in combination with DS induces a more marked reduction in both the blood compartments (TBP: 68%) and the RETP (87%) as compared with ECIB alone (TBP: 35%; RETP: 48%). In

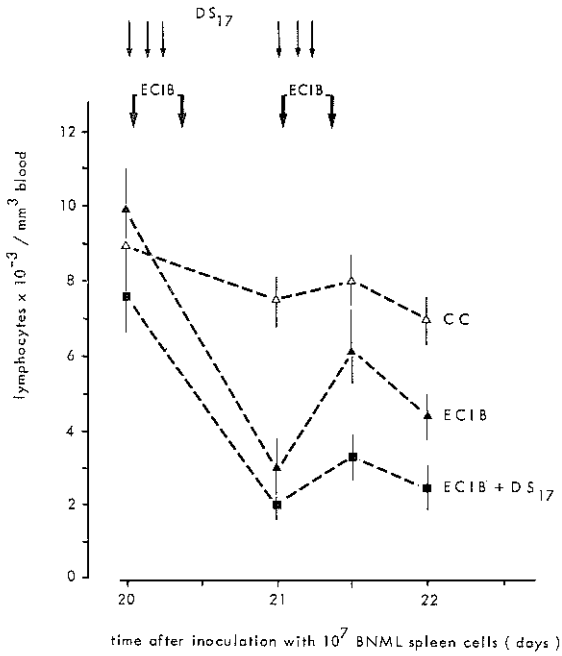

Fig. 4.16 *Effect of 2×8 h of ECIB in combination with cell mobilization on the number of lymphocytes in the peripheral blood.*
Each point represents means (±SE) of 5 rats.
CC: cannulated controls.
DS_{17}: dextran sulphate (MW 17,000): 10 mg/kg i.a.; each arrow represents one injection.

particular, the RETP is depleted. The organ uptake of infused ^{51}Cr-labelled leukaemic cells is given in table 4.21. In all organs, except for the lungs, the uptake measured in the DS-treated ECIB group has significantly increased as compared with ECIB alone. This may be due to the fact that the capacity of the

Table 4.19 *Organ weights measured 25 h after 2×8 h of ECIB in combination with cell mobilization*

	liver weight (g)	spleen weight (g)
CC	18.6±1.5	4.1±0.2
ECIB	18.0±0.9	3.7±0.1
ECIB+DS_{17}	13.8±1.1	2.9±0.3
student "t" test		
CC–ECIB	N.S.	N.S.
CC–ECIB+DS_{17}	$p<0.025$	$p<0.01$
ECIB–ECIB+DS_{17}	$p<0.025$	$p<0.001$

Values represent means (±SE) of 5 rats.
CC: cannulated controls; DS_{17}: dextran sulphate (MW 17,000); N.S.: nonsignificant difference.

Table 4.20 *Changes in the size of the various functional compartments after 2×8 h of ECIB in combination with dextran sulphate*
(leukaemic cells $\times 10^7$)

	% ^{51}Cr-cells in CBP after infusion	CBP	MP	TBP	% ^{51}Cr-cells in CBP at equilibrium	RETP	TREP
CC	36.6	12.3	21.4	33.7	12.8	62.6	96.3
ECIB	37.2	8.1	13.7	21.8	15.0	32.3	54.1
ECIB+DS_{17}	30.1	3.3	7.6	10.8	17.3	8.0	18.8
% reduction by:							
ECIB		34	36	35		48	44
ECIB+DS_{17}		74	65	68		87	80

Values represent means of 5 rats.
CC: cannulated controls; DS_{17}: dextran sulphate (MW 17,000); CBP: circulating blood pool; MP: marginal pool; TBP: total blood pool; RETP: rapidly exchangeable tissue pool; TREP: total rapidly exchangeable pool.

Table 4.21 *Uptake of ^{51}Cr-labelled leukaemic cells (%) per organ measured at 20 h after infusion into rats which were submitted to 2×8 h of ECIB in combination with dextran sulphate*

	liver	spleen	bone marrow*	lungs
CC	35.6	4.0	5.0	2.7
ECIB	32.7	4.3	8.3	2.4
ECIB+DS_{17}	40.1	7.7	14.3	2.5

* Total bone marrow was calculated (1 femur = 1.2%).
Values represent means of 5 rats.
CC: cannulated controls; DS_{17}: dextran sulphate (MW 17,000).
For further explanation: see text.

Table 4.22 *Changes in the size of the rapidly exchangeable tissue pools (RETP) in various organs after 2×8 h of ECIB in combination with dextran sulphate*
(leukaemic cells $\times 10^7$)

	liver	spleen	bone marrow*	lungs
CC	34.3	3.8	4.8	2.6
ECIB	17.7	2.3	4.5	1.3
ECIB+DS_{17}	7.6	1.5	2.7	0.5
% reduction by:				
ECIB	48	40	6	50
ECIB+DS_{17}	78	62	45	82

* Total bone marrow was calculated (1 femur = 1.2%).
Values represent means of 5 rats.
CC: cannulated controls; DS_{17}: dextran sulphate (MW 17,000); RETP: rapidly exchangeable tissue pool.
For further explanation: see text.

depleted RETP to take up cells from the peripheral blood has increased. These data also clearly demonstrate that the migration of leukaemic cells from the blood to the tissues is not inhibited by DS. This is in contrast with observations on normal lymphocytes, which show a reduced rate of exit from the circulation after administration of polyanions [Bradfield and Born, 1969; Balow and Fauci, 1976, see Ch. 3.1).

From data on organ uptake, the size of the RETP in the various organs was calculated (table 4.22). ECIB in combination with DS causes an extra reduction in this compartment in all organs measured, in particular in the bone marrow. It should be mentioned however, that, in this experiment, 2×8 h of ECIB alone was less effective in terms of cell depletion than 2×10 h of ECIB (table 4.11). Whether this is due to 4 hours more irradiation in the latter regimen or to variations between experimental animals and the stage of the disease remains to be established.

The most striking difference between ECIB and ECIB in combination with DS is the degree of reduction in the SETP (table 4.23). In the DS-treated ECIB group, the reduction in the SETP in the spleen and the liver is 3 and 11-fold greater, respectively, as compared with the group which only underwent ECIB. In contrast to all other ECIB experiments described earlier, the SETP in the liver is significantly decreased by 44%. The reduction of the SETP in the spleen (with 34%) is also the most effective one found so far.

Table 4.23 *Changes in the size of the slowly exchangeable tissue pools (SETP) in the spleen and the liver after 2×8 h of ECIB in combination with dextran sulphate* (leukaemic cells $\times 10^7$)

		RETP	SETP	% reduction of SETP by ECIB
spleen	CC	3.8	346.2	–
	ECIB	2.3	307.7	11
	ECIB + DS_{17}	1.5	228.5	34
liver	CC	34.3	1025.7	–
	ECIB	17.7	982.3	4
	ECIB + DS_{17}	7.6	572.4	44

Values represent means of 5 rats.
CC: cannulated controls; DS_{17}: dextran sulphate (MW 17,000); RETP: rapidly exchangeable tissue pool; SETP: slowly exchangeable tissue pool.

4.7 The effect of ECIB on the proliferation of leukaemic cells

At day 22 after leukaemia transfer, 4 rats were treated with ECIB. After 6 h and 13 h of irradiation, respectively, ECIB was completed in two groups of two rats (see table 4.24 for physical parameters). Thereafter, they received 1 μCi tritiated thymidine (^{3}H-TdR) per gram body weight through the cannula

Table 4.24 *Physical parameters of 6 h – 13 h of ECIB*

	6 h of ECIB	13 h of ECIB
BW (g)	260	260
V_b (ml)	13.0	13.0
V (ml)	1.6	1.6
F (ml/min)	0.89	0.90
x V_b	24.6	54.0
R (rad/min)	195	195
TD (rad)	351	347
$D_{\bar{x}}$ (rad)	7,693	16,668

Values represent means of 2 rats.
Cannulated rats served as controls.
For explanation of symbols: see table 4.3.

Table 4.25 *^{3}H-TdR labelling indices of leukaemic cells in the spleen after 6 h – 13 h of ECIB*

		LI
		$\bar{x} \pm SE$
	CC	0.32 ± 0.02
	after 6 h of ECIB	0.29 ± 0.01
	after 13 h of ECIB	0.25 ± 0.01
student "t" test	CC – 6 h of ECIB	N.S.
	CC – 13 h of ECIB	$p < 0.05$
	6 h of ECIB – 13 h of ECIB	$p < 0.05$

CC: cannulated controls; LI: tritiated thymidine (^{3}H-TdR) labelling index; N.S.: non-significant difference.

(specific activity 2 Ci/mmol; The Radiochemical Centre, Amersham, England). The rats were killed thirty minutes later and impression slides of the spleen were made for determining the labelling index (LI) of leukaemic cells by means of direct autoradiography according to the procedures described in Chapter 2.6.4. In this experiment, 3 cannulated rats served as controls. Two impression slides from each rat were counted (500 cells per slide).

The results are given in table 4.25. During the first 6 h of ECIB, the LI tends to decrease, although it is not significantly different from the nonirradiated control group (CC). However, after 13 h of irradiation, a significantly lower LI is found both as compared with the CC group and with the group which received 6 h of continuous irradiation.

4.8 Discussion

The ECIB model described offers the unique possibility to apply repeated sessions of ECIB. Moreover, the exteriorized aorta by-pass can also be used for continuous flow leukapheresis, intra-arterial infusions, cross circulation studies,

regular sampling of arterial blood, etc. The mortality due to the cannulation procedure is low (approximately 5%), the main cause of failure being damage to the inferior vena cava. During extracorporeal circulation sometimes, clotting occurs in the shunt despite heparinization. These clots can be easily removed, thereby restoring the circulation.

With respect to the radiation dose, transit doses ranging from 350 to 650 rad have been used, because of the different blood flow rates and radiation dose rates in the various experiments. If a cell suspension should be irradiated with these doses, the fraction of surviving cells would range from 2×10^{-2} to 2.6×10^{-4} (see fig. 4.9, Chapter 4.3). However, during a session of ECIB, the majority of peripheral leukaemic cells (LC) passes the irradiator more than one time, thereby receiving twice or more times the transit dose. Given the prolonged blood transit time of LC (table 2.11) and their rather slow exchange rate with the tissue compartments, the difference in terms of cell depletion between ECIB regimens applied with different transit doses will be insignificant.

By comparing the three ECIB treatment schedules as described in Chapter 4.5 it can be concluded that repeated sessions (2×10 h; 4×5 h) are far more effective in terms of cell depletion than one long-lasting continuous irradiation (1×20 h). No complete or even partial remissions were obtained, as judged by blood and bone marrow cytology. However, the objectives of these experiments were to study the quantitative relationships among the various functional compartments and changes induced by the selective depletion of one of these (CBP).

Comparing the reduction in the blood compartments (CBP+MP) with that of the rapidly exchangeable tissue pool (RETP) leads to striking discrepancies between the three treatment regimens. In table 4.26, it is shown that, after 1×20 h of ECIB, the decrease in the TBP is greater relative to the decrease in the RETP. The reverse, however, holds for the 4×5 h regimen, whereas the 2×10 h schedule reduces both compartments to a similar degree. This might be explained by assuming different patterns of mobilization of LC from the RETP. Repeated short-lasting sessions apparently result in a more marked traffic of cells to the blood as is seen in fig. 4.12 (4×5 h of ECIB). After the second session of ECIB, the number of LC in the blood starts to increase despite

Table 4.26 *The size reduction of the total blood pool relative to that of the rapidly exchangeable tissue pool after three different ECIB treatment regimens*

ECIB	% reduction of TBP	% reduction of RETP	% reduction of TBP / % reduction of RETP	
1×20 h	35	23	1.5	table 4.6
2×10 h	68	67	1.0	table 4.10
4×5 h	37	74	0.5	table 4.15

TBP: total blood pool; RETP: rapidly exchangeable tissue pool.

two more treatment sessions. Apparently, cell depletion in the peripheral blood induces a certain degree of mobilization of cells from the tissues. In the 4×5 h treatment schedule, the number of cells mobilized exceeds the number of cells killed by irradiation. Individual organ studies indicate that the mobilized cells come from the RETP, which is significantly depleted (table 4.16). The induction of a significant degree of mobilization apparently takes some days (fig. 4.12). In general, it might be stated that the entrance rate of LC into the blood increases with diminishing numbers of peripheral LC. In the 2×10 h treatment regimen, besides a significant reduction of the RETP (tables 4.10 and 4.11) which is similar to the 4×5 h regimen, the TBP and MP are depleted to a greater extent (a factor of 2) than after the 4×5 h protocol (compare tables 4.10 and 4.15). This might be due to the fact that, in these longer lasting sessions, cell depletion keeps pace with cell mobilization (fig. 4.11). Thus, the 2×10 h regimen has been an optimal choice for balanced size reduction in all exchangeable compartments. Although some mobilization occurs during the 20 h irradiation session (fig. 4.10), this schedule – in which about as much depletion of the TBP is observed as in the 4×5 h schedule (compare tables 4.6 and 4.15) – has remarkably less influence on the sizes of the rapidly exchangeable tissue compartments (tables 4.6 and 4.7).

In all three experiments, a completely different picture is obtained for normal lymphocytes. Cell kill by irradiation clearly exceeds cell mobilization, as indicated by the significant decrease in the number of lymphocytes in the blood (figs. 4.10, 4.11 and 4.13). That mobilization occurs is demonstrated by slightly rising peripheral cell counts between two sessions of ECIB (figs. 4.11 and 4.13).

The slowly exchangeable tissue compartments of LC (SETP) only decrease to a small degree (tables 4.8, 4.12 and 4.17). In fact, only the SETP of the spleen shows a significant depletion especially after repeated sessions of ECIB. It is clear from these tables that the RETP of a given organ is rather small as compared with the SETP (see also table 2.12 and fig. 2.39). This explains why ECIB reduces the total tumour load only to a small degree.

These data support the hypothesis of the presence of various functional compartments in acute leukaemia (see fig. 2.38), each having its own exchange rate parameters with the adjacent compartment. The data obtained thus far will be further analyzed by a computer program for compartment analysis which is presently being developed (Freriks, personal communication). With the recently acquired knowledge on the presence of at least two populations of LC in the BNML, i.e. clonogenic-(LCFUs) versus nonclonogenic cells, the question remains as to whether or not there is a preferential mobilization and subsequent killing of one of these cell types.

When ECIB is combined with dextran sulphate, the induction of extra mobilization followed by killing results in a significantly greater reduction in all functional compartments studied as compared with ECIB alone. From

table 4.20, it can be calculated that the extra reduction factor, which is similar for all compartments, is in the order of 1.9. Essentially the same factor is calculated for the RETP of the various individual organs (table 4.22). However, in case of the bone marrow, ECIB in combination with DS causes a 7.5-fold extra decrease as compared with ECIB alone. It should be mentioned that the measured reduction in the size of the RETP is the resultant of (1) cell mobilization from this compartment to the blood compartments, and (2) influx of cells mobilized from the SETP (table 4.23). In summary, there is an increased rate of exchange of LC from the tissue compartments into the direction of the peripheral blood. The increased influx in the latter compartment apparently does not exceed the capacity of the irradiation regimen used to kill these cells. This is indicated by the decreasing number of LC in the blood measured before and after ECIB (fig. 4.15). Essentially the same pattern is observed for normal lymphocytes (fig. 4.16). The major adjuvant effect of DS seems to be a significant mobilization effect on the SETP, which is only slightly influenced by ECIB alone (table 4.23).

From the follow-up of a few small groups of rats which were not sacrificed for ^{51}Cr-determinations after ECIB, it was concluded that the different ECIB regimens did not significantly change the survival time of the leukaemic rats. Both the ECIB treated rats and their controls die between days 26 and 29 after

Table 4.27 *Reduction of the total number of leukaemic cells after 2×8 h of ECIB in combination with dextran sulphate*

	leukaemic cells ($\times 10^8$)	
	CC	ECIB+DS_{17}
TBP	3.4	1.1
liver RETP	3.4	0.8
liver SETP	102.6	57.2
spleen RETP	0.4	0.2
spleen SETP	34.6	22.9
bone marrow	50.0 *	27.5 **
lungs	7.8	1.7
rest	50.0	30.0
total tumour load	2.5×10^{10}	1.4×10^{10}

TBP (total blood pool): see table 4.20.
Pool sizes in liver and spleen: see table 4.23.
The total tumour load in the bone marrow was calculated from 1 femur = 1.2%.
* CC: 1 femur contains 6.0×10^7 leukaemic cells (see fig. 2.22b);
** ECIB+DS_{17}: 1 femur contains 3.3×10^7 leukaemic cells (±45% reduction, table 4.22).
The total tumour load in the lungs was derived from measured leukaemic lung weights corrected for the normal lung weight.
The "rest" was derived from table 2.13; ECIB+DS_{17}: 40% reduction.

CC: cannulated controls; RETP: rapidly exchangeable tissue pool; SETP: slowly exchangeable tissue pool; DS_{17}: dextran sulphate (MW 17,000).

inoculation. After treatment, two factors determine the length of the remaining life span: (1) the total tumour load which is left; and (2) the growth rate of the remaining leukaemic cells. With respect to the reduction in the tumour load, table 4.27 represents the most effective regimen used, i.e., ECIB in combination with dextran sulphate. The total tumour load was roughly quantified by adding the various compartments. As can be seen, the total reduction is about 50%. This means that 1 doubling of the leukaemic cell population is required to reach the initial tumour mass again. From the data on cell proliferation in the spleen in nontreated leukaemic rats, it is derived that, after ECIB, the remaining total tumour load at day 21 is similar to that between days 14 and 18 in nontreated leukaemic rats (see table 2.15; Ch. 2.6). In other words, at day 16, 1 doubling of the leukaemic cell population takes about 4.5 days (96–120 h). Thus, from this calculation, a prolongation of the life span of ECIB treated rats by 4.5 days as compared with the controls would be expected. However, as stated earlier, this is not observed. In theory, this discrepancy might be due to an increased proliferation rate after ECIB with shortened cell cycle parameters and/or an increased growth fraction (GF). If it is assumed that the cell cycle parameters remain unchanged, then the GF will be reduced, as concluded from the observation that the tritiated thymidine LI measured in the spleen tends to decrease under the influence of ECIB treatment (table 4.25); i.e., the proliferation rate will certainly not have increased. The decreasing LI in the spleen might also be due to a return of significant numbers of nonlabelled irradiated LC to the spleen or a preferential mobilization of cycling cells from the tissues during ECIB. The decrease in the LI is in contrast with observations of other authors applying ECIB treatment to human acute leukaemia (see Ch. 1.4.7c; tables 1.4 and 1.5). However, they also reported on patients in whom no changes in the proliferation parameters were noted. It should be kept in mind that, with the available data, it cannot be ruled out that cell proliferation (i.e. the LI and the GF) increases at a later stage after ECIB has been completed. This hypothesis is not supported by preliminary data on the effectiveness of chemotherapy with cycle active cytostatic drugs (cytosine arabinoside) at various time intervals after ECIB. This treatment further reduces the tumour load in a pure additive way without any signs of synergism.

The most likely explanation for the discrepancy between the expected and the measured survival time of ECIB treated rats is a combination of two factors. Firstly, the repeated ECIB procedure might adversely affect the general condition of the leukaemic rat. Secondly, a prolongation of the life span by only 4–5 days may not have been distinguished from control rats because of a large spread in the survival times of the treated and nontreated animals.

In summary, ECIB significantly reduces the total tumour burden, in particular in combination with cell mobilization. The maximal reduction is 50% with different degrees of cell depletion in the various functional cell compartments.

This indicates the presence of a regulatory mechanism, at least in this rat model for acute myelocytic leukaemia, which controls the distribution of leukaemic cells over the various compartments. On the other hand, ECIB treatment, with its technical limitations, used either alone or in combination with chemotherapy, certainly cannot compete with other treatment modalities in acute myelocytic leukaemia, such as chemotherapy (2–7 log cell kill) or daily low-dose whole body irradiation (2–3 log cell kill), which have been shown to significantly prolong the survival time of leukaemic rats [Hagenbeek et al., to be published]. Obviously, ECIB treatment should be regarded as palliative rather than curative.

CHAPTER 5

GENERAL DISCUSSION

As most of the specific data have been discussed in detail in the previous chapters, only general ideas emerging from these studies will be presented in this chapter.

5.1 The suitability of the rat leukaemia model as a model for human acute myelocytic leukaemia; implications for future (clinical) research

Animal models for human diseases generally serve the purpose of making the disease accessible to experimentation, which is often impossible in patients. The objectives of model research in leukaemia are: to extend the knowledge on the pathogenesis, to develop new methods of investigation and therapy and finally to extrapolate the information to humans for the purpose of devising more efficient treatment regimens for human leukaemia.

The first prerequisite is to establish leukaemia models which are more relevant to human leukaemia. Many leukaemia models, both of "spontaneous" origin and induced by chemicals, radiation or tumour viruses have been investigated during the past decades. Although some of them have yielded very useful information (e.g., the L1210 transplantable mouse leukaemia for the screening of potential antileukaemia drugs), only few show a reasonable resemblance to human leukaemia in terms of growth characteristics.

It is likely that one leukaemia model in an inbred strain of animals stands for one leukaemic patient, because of the large variety of growth patterns among individual patients. However, this does not necessarily limit the usefulness of basic studies, as they are meant to discover fundamental characteristics of leukaemia growth applicable to man.

As described in Chapter 2, two of the most important features which make the BN myelocytic leukaemia relevant to human AML are its slow growth and the severe suppression of normal haemopoiesis during leukaemia development. In addition, many other characteristics, such as the cytological and cytochemical characterization, the type of coagulation defects, cell surface properties and the response patterns to various treatment modalities, are reminiscent of human acute promyelocytic leukaemia. This was concluded at a recent workshop during which many aspects of the BNML were analysed in detail [Hagenbeek and van Bekkum, 1977].

A. Kinetics of proliferation

Although the kinetics of proliferation in the BNML cannot be directly compared with human acute leukaemia, an identical relationship was found between proliferation parameters of normal and leukaemic haemopoiesis in the rat and normal and leukaemic haemopoiesis in human AML (see table 2.16). However, if the time from diagnosis to death is related to the total life span of the species, a different picture is obtained. In the BNML, the period between the time of diagnosis and death (10–15 days) is 1.1–1.7% of the median normal rat life span (30 months; J. Burek, personal communication). In untreated human AML, the median survival time is 2–3 months (Killmann, personal communication), i.e., about 0.3% of human life span (70 years). Untreated human chronic myelocytic leukaemia (CML) gives a median survival of 2 years, which is about 3% of human life span. Thus, in this respect, the BNML should be classified as an intermediate form between acute and chronic leukaemia, assuming that this direct interspecies comparison is allowable and valid. However, another method to compare the BNML with human AML might be to relate the time from diagnosis to death to the cell cycle time. In the BNML, 10–15 days take 17–26 cell cycle times (T_c = 14 h; table 2.16); in human AML, 24–43 cell cycle times (T_c = 50–60 h; table 2.16) are equal to 2–3 months. Thus, from this intercomparison where the growth fraction is assumed to be similar in both leukaemias, the progression of the disease in the BN rat is about equally acute as in human AML. Also, because of the immaturity of the BNML cells ("hiatus leukaemicus") and their failure to produce colonies *in vitro*, it is concluded that the BNML model is much closer to human AML than to CML.

In contrast to spontaneous rodent leukaemias, which might serve as models for studying the pre-diagnosis stage of human leukaemia, each passage of a transplantable leukaemia such as the BNML should be compared with each of a succession of remissions and relapses in a single patient [van Bekkum and Hagenbeek, 1977].

B. Kinetics of distribution

The studies on the distribution of leukaemic cells after injection (Chapter 2) have yielded important information, particularly with respect to the question as to at which point can one interfere with the progression of the disease. After infusion of leukaemic cells, a pattern of initial trapping was observed. This presumably takes place in the capillary beds in various parts of the body, but particularly in the lungs. The cells are subsequently released and this is followed by a secondary pattern of homing in organs such as the bone marrow, the spleen and the liver where they start to proliferate. Questions that remain are: What is the mode of interaction between leukaemic cells and the reticuloendo-

thelial system in the process of trapping and secondary homing?; What is the significance of specific characteristics of the tumour cell surface in relation to this process?; Are there means to prevent trapping or perhaps induce trapping followed by immediate tumour cell destruction?

Studies on distribution kinetics confirmed the hypothesis concerning the presence of various functional cell compartments in leukaemia, i.e., rapidly versus slowly exchangeable compartments. On determining the sizes of these pools, it appeared that the further the leukaemia progresses, the greater the fraction of cells present in the slowly exchangeable tissue pools, or, the lower the exchange rates of leukaemic cells between the tissues and the blood. This is of particular interest with respect to the efficacy of specific treatment modalities such as ECIB and leukapheresis, which require a certain degree of exchange to yield a significant therapeutic benefit (see Chapter 5.2).

C. The fate of the normal haemopoietic stem cell

The suppression of normal haemopoiesis at the level of the haemopoietic stem cell (HSC) is another interesting feature of the BNML which reminds one of human AML. In human AML, a significant decrease in the number of CFUc in the bone marrow has been reported [Moore, 1974; van Bekkum, 1976a]. In the BNML, apart from an absolute numerical decrease (see table 2.7), a redistribution of HSC seems to take place, i.e., the number of HSC decreases in the bone marrow but increases in the blood and in the spleen as the leukaemia progresses (see fig. 2.37). Whether extramedullary haemopoiesis is due to lodging of circulating HSC or to stimulation of resting HSC by a feed back mechanism or to a combination of these two should be further analysed. Other specific questions to be answered are: does the proliferative status of the HSC change during leukaemia development, and: is there again a redistribution of HSC after remission induction chemotherapy? Obviously, it would be very useful to investigate similar phenomena in human acute leukaemia. However, this will be a difficult task with the presently available methods. Regular CFUc determinations in the bone marrow and the blood might be possible, but, one is faced with technical and ethical problems as regards the extramedullary sites. In particular, it would be important to answer the question as to whether or not there is an increase in the number of CFUc in the blood and/or other organs at a certain stage during remission or relapse. These studies might certainly have implications for clinical leukaemia treatment, e.g., harvesting HSC from the peripheral blood for autologous bone marrow transplantation at the time of the virtual absence of HSC in the bone marrow.

D. Subpopulations of leukaemic cells

A final essential point deals with the leukaemic cell population *per se*. There

appeared to be at least two populations of leukaemic cells: clonogenic (LCFUs) and nonclonogenic cells. Further experiments are needed to determine their functional and quantitative relationship. In this respect, an important question is what are the conditions for *in vitro* culturing of leukaemic cells so that they give rise to colonies. Although some authors claim to have achieved *in vitro* colony formation by AML cells after stimulation with phytohaemagglutinin [Dicke et al., 1976b], this was not reproducible with BNML cells. *In vitro* culturing of leukaemic cells would also make it possible to test drug sensitivity, to detect residual leukaemic cells after treatment and to study factors which might influence proliferation and even maturation.

5.2 Recommendations for application of ECIB in the future

In the BNML model, the different regimens of ECIB studied did not result in a significant prolongation of the life span of the rats. When the exchange rate of leukaemic cells between the tissues and the blood was increased by means of leukocyte mobilizing agents (Chapter 3), ECIB (2×8 h) reduced the total tumour load by about 50% (Chapter 4). In this case, the calculated survival time would increase by 4.5 days, i.e., 0.5% of the total life span of a normal rat (30 months) would be added. If the same calculation is applied to man, a similar ECIB regimen would result in a prolongation of the life span by about 4 months. If more sessions of ECIB were applied, this figure might even become greater. In this respect, ECIB might be of therapeutic value, particularly in cases of drug resistance or toxicity. This has been shown in four patients with chronic lymphocytic leukaemia who received repeated sessions of ECIB. Their total tumour load as measured by the ^{51}Cr-method was reduced by $3-6 \times 10^{12}$ cells [Hagenbeek et al., to be published]. Although no complete remissions were achieved, the progression of the leukaemia could be significantly retarded. With 4–6 series of ECIB sessions per year, a satisfying general condition and a good quality of life could be maintained in these patients who were resistant to chemotherapy. Thus, in selected cases of leukaemia, ECIB is certainly beneficial from a therapeutic point of view.

From the experimental studies on the quantitative changes in the various functional cell compartments induced by ECIB, it appeared that the traffic of leukaemic cells is subject to a regulatory mechanism. The more the peripheral compartment is depleted, the more cells are mobilized from the rapidly and slowly exchangeable tissue compartments. Whether this mobilization phenomenon is induced by degradation products from irradiated leukaemic cells remains to be established. In this respect, it should be worthwhile to perform similar studies with leukapheresis by which cells are removed instead of being destroyed.

The ^{51}Cr-method has provided rough quantitative information on the degree

of tumour cell depletion in response to ECIB in both the rat and man. In the rat, a rather precise insight into the changes in the rapidly exchangeable and more sessile tissue compartments could be obtained by the principle of isotope dilution in combination with organ weight measurements. For obvious reasons, one is faced with serious problems with respect to evaluating the reduction in the total tumour load in response to therapy in man. A combination of isotope dilution methods, methods of *in vitro* culturing of clonogenic leukaemic cells and measurements of specific biochemical markers (e.g., muramidase) might lead to achieving this goal.

SUMMARY

In 1962, extracorporeal irradiation of the blood (ECIB) was introduced as a possible therapeutic measure in the treatment of leukaemia, particularly in patients who are unresponsive to chemotherapy [Lajtha et al., 1962]. The rationale for this treatment modality is that leukaemic cells are far more radiosensitive than other types of blood cells.

Besides its theoretical therapeutical usefulness, it was thought that the ECIB technique might make a significant contribution to the study of the kinetics of proliferation and distribution of leukaemic cells, as is pointed out in Chapter 1. In this chapter, an historical review on the development of ECIB is also presented and the various techniques and parameters in general use are described. In addition, recent developments in experimental and clinical ECIB are reviewed. It appears from the literature that, particularly in chronic lymphocytic leukaemia, the tumour cell load can be significantly reduced by ECIB, whereas, in chronic myelocytic leukaemia and in the acute leukaemias, varied results are obtained. In some patients, a partial remission is achieved at best; in others, the growth rate of the leukaemia increases in response to ECIB. Because of the lack of comprehensive clinical and experimental studies, it seemed justified to study the effects of ECIB in leukaemia from both a kinetic and a therapeutic point of view.

The general experimental procedures and the experimental model are described in detail in Chapter 2. A chemically induced leukaemia in the Brown Norway inbred rat strain was used. This leukaemia gives a reproducible growth pattern upon cell transfer. It is classified as an acute promyelocytic leukaemia, with a high degree of similarity to human acute myelocytic leukaemia (AML). Firstly, in contrast to most other animal leukaemia models, it has a rather slow growth rate. After inoculation with 10^7 leukaemic cells, the rats survive 28 ± 2 days. The responsible factors in the death of the animal are bleeding, infections in the lungs and impaired functions of vital organs which are infiltrated with leukaemic cells. The cellular kinetics of proliferation essentially show deviations which are identical with those seen in human AML. With a cell cycle time of 14.0 h, the decreasing growth fraction (to 0.40) and the increasing cell loss (to 88%) during the development of the disease fully explain the net slow growth.

A second feature of analogy with the human disease is the severe suppression of normal haemopoiesis during the development of leukaemia. By means of methods to assay normal haemopoietic stem cells (HSC), it was found that these stem cells decrease in the bone marrow and increase in the blood and extramedullary sites such as, e.g., the spleen. Replacement by leukaemic cells,

possibly in combination with a decreasing proliferation rate of HSC, are the major factors responsible for the significantly decreased normal haemopoiesis.

Studies on the histological pattern of spread and the kinetics of distribution of injected ^{51}Chromium-labelled leukaemic cells are also reported in Chapter 2. After inoculation, the majority of cells lodges in the bone marrow, the spleen and the liver, where they start to proliferate. During the development of the disease, most organs become heavily infiltrated with leukaemia. It was concluded from the isotope studies that two functional compartments can be distinguished in acute leukaemia, i.e., a rapidly exchangeable pool of cells (including the circulating blood pool, the marginal noncirculating blood pool and the rapidly exchangeable tissue pool; RETP) and a slowly exchangeable tissue pool (SETP). The sizes of these various compartments were roughly quantified at various stages of the disease by calculations based on the principle of isotope dilution and organ weight measurements. It was found that, as the leukaemia progresses, the size of the SETP increases significantly relative to the size of the RETP. Simultaneously, the exchange rates of leukaemic cells between the organs and the blood decrease. The blood transit time of leukaemic cells was also significantly prolonged, as is the case in human AML.

Finally, evidence was provided for the presence of at least two subpopulations of leukaemic cells: clonogenic and nonclonogenic cells.

With the purpose of increasing the leukaemic cell depletion by ECIB, leukocyte mobilizing agents were introduced (Chapter 3). Within a few hours after injection, these synthetic polyanions induce a marked increase in the number of peripheral lymphocytes and leukaemic cells, as has been demonstrated in various animal species, in animal leukaemia models and in three patients with chronic lymphocytic leukaemia. The phenomenon is no longer evident within a few hours after the peak value has been reached. Although the mechanism underlying this mobilization of cells from the organs is still unclear, studies are reported which indicate that the polyanions attach to the surface of these cells and alter their interaction with the reticuloendothelial system by changing their electronegative charge. This would then result in an increased release to and possibly an impaired migration from the blood.

In Chapter 4, the ECIB model in the rat is described in terms of the cannulation technique, the ECIB procedure, the radiation equipment and dosimetry. The D_0 for X-rays of leukaemic cells in the blood was 68.1 rad.

After the appropriate controls were selected, ECIB studies in the BN acute myelocytic leukaemia were performed to answer the following questions:

(1) Is there a different efficacy in terms of cell depletion between one long-lasting session of ECIB and several repeated sessions? It was found that repeated sessions of ECIB (2×10 h; 4×5 h) are far more effective in

terms of cell depletion than one long-lasting session (1×20 h). ^{51}Chromium studies indicated that the sizes of the blood compartments and of the rapidly exchangeable tissue pool are markedly reduced (maximally by about 70%). The more sessile organ compartments are only slightly influenced.

(2) To what degree do leukocyte mobilizing agents enhance the cell depletion induced by ECIB? Besides a depletion of the rapidly exchangeable compartments, ECIB (2×8 h) in combination with cell mobilization by dextran sulphate induces a significant depletion in the slowly exchangeable tissue pool as measured in the various organs. The reduction in the total tumour load was about 50%.

(3) Does ECIB change the proliferation characteristics of the leukaemic cell population? Tritiated thymidine studies performed during a prolonged session of ECIB suggested that this treatment does not change the proliferation rate of the leukaemia.

In summary, ECIB significantly reduced the tumour burden, particularly in combination with cell mobilization, causing different degrees of cell depletion in the various functional cell compartments. This suggests the presence of a regulatory mechanism (at least in this rat model for acute myelocytic leukaemia) which controls the distribution of leukaemic cells over the various compartments. From a therapeutic point of view, ECIB treatment should be regarded as palliative rather than curative. The method certainly cannot compete with chemotherapy, which more effectively reduces the total tumour load.

The suitability of the BN rat leukaemia model as a model for human acute myelocytic leukaemia is discussed in Chapter 5. Based on studies described in this thesis, implications for future clinical research are indicated. Each passage of this transplantable rat leukaemia should be compared with each of a succession of remissions and relapses in a single patient.

From the data on experimental ECIB treatment, extrapolations are made towards the clinical treatment of leukaemia. It is concluded that ECIB in man can certainly be of therapeutic value, especially in cases of drug resistance or toxicity.

SAMENVATTING/SUMMARY IN DUTCH

In 1962 werd extracorporale bestraling van het bloed (ECBB) als een mogelijke behandelwijze van leukaemie geintroduceerd, in het bijzonder voor patiënten die niet reageren op chemotherapie [Lajtha et al., 1962]. De rationale bij deze behandelingsmethode ligt in het feit dat leukaemiecellen veel meer stralingsgevoelig zijn dan andere soorten cellen in het bloed.

Afgezien van de therapeutische bruikbaarheid van ECBB, zou deze methode een significante bijdrage kunnen leveren aan kinetiek studies betreffende de proliferatie en distributie van leukaemiecellen, zoals in Hoofdstuk 1 uiteengezet wordt. In dit hoofdstuk wordt tevens een historisch overzicht gegeven van de ontwikkeling van de ECBB methode, terwijl ook de verschillende technieken en parameters, die erbij worden gebruikt, worden beschreven. Daarnaast wordt een samenvatting gegeven van recente ontwikkelingen op het gebied van experimentele en klinische toepassingen van ECBB. Uit de literatuur blijkt dat ECBB, met name bij chronische lymphatische leukaemie, het totaal aantal tumorcellen aanzienlijk vermindert. In chronische myeloide leukaemie en in de acute leukaemieën worden zeer wisselende resultaten verkregen. In sommige patiënten wordt een partiële remissie bereikt, in anderen wordt een toename van de groeisnelheid van de leukaemie in het beenmerg gemeld ten gevolge van ECBB. Vanwege het gebrek aan systematisch klinisch en experimenteel onderzoek leek het gerechtvaardigd om, zowel vanuit het oogpunt van kinetisch leukaemie-onderzoek als vanuit een therapeutisch standpunt bezien, de effecten van ECBB in leukaemie te bestuderen.

In Hoofdstuk 2 worden de algemeen gebruikte experimentele procedures alsmede het experimentele model beschreven. Er werd gebruik gemaakt van een chemisch geïnduceerde leukaemie in de ingeteelde Brown Norway rattenstam. Deze leukaemie vertoont een reproduceerbaar groeipatroon na elke transplantatie van leukaemiecellen. De leukaemie is geclassificeerd als een acute promyelocyten leukaemie, die op vele punten een sterke gelijkenis vertoont met acute myeloïde leukaemie (AML) in de mens. In de eerste plaats groeit deze leukaemie, in tegenstelling tot de meeste andere transplanteerbare leukaemieën in proefdieren tamelijk langzaam. Na het inspuiten van 10^7 leukaemiecellen overleven de ratten 28 ± 2 dagen. De belangrijkste doodsoorzaken zijn bloedingen, longontsteking en verslechterde funkties van vitale organen, die met leukaemiecellen zijn geïnfiltreerd. De proliferatiekinetiek vertoont dezelfde afwijkingen van normaal als in humane AML. Hoewel de cel cyclus tijd vrij kort is (14.0 uur), kan de lage groeisnelheid volledig worden verklaard uit het afnemen van de groeifraktie (tot 0.40) en toenemend celverlies (tot 88%) gedurende de ontwikkeling van de ziekte.

Een tweede overeenkomst met humane AML is de sterke onderdrukking van de normale haemopoiese gedurende de ontwikkeling van de leukaemie. Met behulp van methoden om normale haemopoietische stamcellen (HSC) te detecteren, werd een afname van deze stamcellen in het beenmerg geconstateerd en een toename in het bloed en in extramedullaire organen zoals de milt. Complete vervanging door leukaemiecellen, mogelijk in combinatie met een verminderde groeisnelheid van HSC, zijn de belangrijkste factoren op grond waarvan de sterk verminderde haemopoiese verklaard wordt.

In Hoofdstuk 2 worden ook onderzoekingen beschreven betreffende het histologisch groeipatroon en de distributiekinetiek van ingespoten ^{51}Chroom-gelabelde leukaemiecellen. Na inspuiting nestelen de meeste cellen zich in het beenmerg, de milt en de lever, waar ze beginnen te prolifereren. In de loop van de ontwikkeling van de ziekte worden de meeste organen massaal geïnfiltreerd met leukaemiecellen. Uit de isotoop-studies werd geconcludeerd, dat er in acute leukaemie twee functionele celcompartimenten onderscheiden kunnen worden, te weten: een snel uitwisselbaar compartiment (bestaande uit het circulerende bloed-, het marginale niet-circulerende bloed- en een snel uitwisselbaar weefselcompartiment) en een langzaam uitwisselbaar weefselcompartiment. De grootten van deze verschillende compartimenten konden bij benadering worden bepaald in verschillende stadia van de ziekte door middel van berekeningen gebaseerd op het principe van isotoop verdunning en orgaan gewichten. Het langzaam uitwisselbare weefselcompartiment bleek in verhouding tot de snel uitwisselbare compartimenten, het sterkst in grootte toe te nemen naarmate het ziekteproces voortschreed. Tegelijkertijd neemt de uitwisselingssnelheid van leukaemiecellen tussen de organen en het bloed af. Evenals in humane AML werd ook een verlengde verblijfsduur van leukaemiecellen in het bloed gevonden.

Tenslotte werden er aanwijzingen gevonden voor het bestaan van tenminste twee subpopulaties leukaemiecellen: clonogene- en niet-clonogene cellen.

Met het doel om de leukaemiecel depletie door ECBB te vergroten werden studies betreffende leukocyten mobilizerende stoffen geïntroduceerd (Hoofdstuk 3). Binnen een paar uur na injectie veroorzaken deze synthetische polyanionen een sterke toename in het aantal perifere lymphocyten en leukaemiecellen, zoals werd aangetoond in verschillende soorten normale proefdieren, in diermodellen voor leukaemie en in drie patiënten met chronische lymphatische leukaemie. Dit verschijnsel verdwijnt weer binnen een paar uur nadat de piekwaarde is bereikt. Hoewel het mechanisme dat aan deze mobilizatie van cellen uit organen ten grondslag ligt nog niet geheel is opgehelderd, worden er onderzoekingen beschreven, die erop wijzen dat de polyanionen zich hechten aan het oppervlak van deze cellen en hun interactie met het

reticulo-endotheliale systeem beïnvloeden door verandering van de elektronegatieve lading van het celoppervlak. Dit zou dan kunnen resulteren in een toename van de afgifte van cellen aan het bloed, mogelijk in combinatie met een belemmerde migratie uit de bloedstroom naar de weefsels.

In Hoofdstuk 4 wordt het ECBB model in de rat beschreven met betrekking tot de cannulatietechniek, de ECBB procedure, de bestralingsopstelling en de dosimetrie. De D_0 voor Röntgenstralen van leukaemiecellen in het bloed was 68.1 rad.

Nadat de geschikte contrôles waren gekozen, werden er ECBB studies verricht in de BN acute myeloïde leukaemie met het doel om de volgende vragen te beantwoorden:

(1) Bestaat er een verschil in de graad van celdepletie geïnduceerd door één langdurige ECBB sessie en door enkele herhaalde sessies? Onderzoekingen wezen uit dat herhaalde ECBB sessies (2×10 uur; 4×5 uur) in dit opzicht veel effectiever zijn dan één langdurige sessie (1×20 uur). Uit ^{51}Chroom studies bleken de bloedcompartimenten en het snel uitwisselbare weefselcompartiment sterk gereduceerd te worden (met maximaal 70%). Het langzaam uitwisselbare weefselcompartiment wordt slechts in geringe mate beïnvloed.

(2) In welke mate verhogen leukocyten mobilizerende stoffen de door ECBB veroorzaakte celdepletie? Behalve een afname in de grootte van de snel uitwisselbare compartimenten, induceert ECBB, in combinatie met celmobilizatie door dextraansulphaat, in de verschillende organen een duidelijke depletie in de langzaam uitwisselbare compartimenten. Het totale aantal tumorcellen werd met ongeveer 50% gereduceerd.

(3) Beïnvloedt ECBB de proliferatiekarakteristieken van de leukaemiecel populatie? Tritium thymidine studies, uitgevoerd gedurende een langdurige ECBB sessie, gaven aan dat deze behandeling de groeisnelheid van deze leukaemie niet duidelijk verandert.

Samenvattend kan worden gesteld dat ECBB de tumorcelpopulatie aanzienlijk reduceert, in het bijzonder als de methode met celmobilizatie wordt gecombineerd. De verschillende functionele celcompartimenten worden in verschillende mate gedepleteerd. Dit wijst op het bestaan van een regulatiemechanisme (ten minste in dit rattenmodel voor acute myeloïde leukaemie) met betrekking tot de verdeling van leukaemiecellen over de diverse compartimenten. Vanuit een therapeutisch standpunt bekeken is ECBB veeleer een palliatieve dan een curatieve behandelingsmethode. Voor wat betreft de reductie van het aantal tumorcellen heeft chemotherapie een veel drastischer effect .

In Hoofdstuk 5 wordt de relevantie van het BN rattenleukaemie model als model voor de humane acute myeloide leukaemie besproken. Op grond van studies in dit proefschrift beschreven, worden implicaties voor klinisch leukaemie-onderzoek aangegeven. Vanaf het moment dat leukaemiecellen worden ingespoten kan deze transplanteerbare rattenleukaemie worden vergeleken met een remissie gevolgd door een relapse in een individuele patiënt.

Gegevens verkregen uit de experimentele ECBB behandeling worden geextrapoleerd naar de klinische behandeling van leukaemie. Concluderend, kan klinisch toegepaste ECBB behandeling zeker van therapeutisch belang zijn, met name in gevallen waar resistentie, toxiciteit of contraindicaties ten aanzien van chemotherapie bestaat.

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LIST OF SYMBOLS

ALL	acute lymphocytic leukaemia
AML	acute myelocytic leukaemia
BNML	acute myelocytic leukaemia in the Brown Norway rat
BTT	blood transit time
CBP	circulating blood pool
CC	cannulated control(s)
CFUc	colony forming units in culture
CFUs	colony forming units spleen
CLL	chronic lymphocytic leukaemia
CML	chronic myelocytic leukaemia
^{51}Cr	51-Chromium
DMBA	9,10-dimethyl 1,2-benzanthracene
DNA	deoxyribonucleic acid
DS	dextran sulphate
ECC	extracorporeal circulation
ECIB	extracorporeal irradiation of the blood
HSC	haemopoietic stem cell(s)
Ht	haematocrit
^{3}H-TdR	tritiated thymidine
i.a.	intraarterial
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
LBC	leukaemic blast cell(s)
LC	leukaemic cell(s)
LI	^{3}H-TdR labelling index
LMA	leukocyte mobilizing agents
MI	mitotic index
MP	marginal pool
PLM	per cent labelled mitoses
PMAA	polymethacrylic acid
RETP	rapidly exchangeable tissue pool
S.D.	standard deviation
S.E.	standard error
SETP	slowly exchangeable tissue pool
TBP	total blood pool
T_d	doubling time
TD_{50}	number of leukaemic cells which kills 50% of the animals
$T/_2$	halving time
TEP	total exchangeable pool
TOR	turnover rate
TREP	total rapidly exchangeable pool

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