FETAL LIVER CELL TRANSPLANTATION

Role and nature of the fetal haemopoietic stem cell

The work described in this thesis has been performed at the Radiobiological Institute TNO, Rijswijk and the Department of Radiobiology, Erasmus University, Rotterdam.

This thesis is available as a publication of the Radiobiological Institute of the Organization for Health Research TNO, Rijswijk (Z.H.), The Netherlands.

FETAL LIVER CELL TRANSPLANTATION

Role and nature of the fetal haemopoietic stem cell

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE ERASMUS UNIVERSITEIT TE ROTTERDAM, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. P. W. KLEIN EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN. DE OPENBARE VERDEDIGING ZAL PLAATS VINDEN OP WOENSDAG 29 JANUARI 1975 DES NAMIDDAGS TE 4.15 UUR

DOOR

BOB LÖWENBERG

geboren te Groningen

UITGEVERIJ WALTMAN - DELFT - 1975

PROMOTOR: PROF. DR. D. W. VAN BEKKUM

CO-REFERENTEN: PROF. DR. O. VOS
PROF. DR. J. ABELS

to my parents

to Joke

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Rationale for the study

In humans, transplantation of haemopoietic cells has shown to be an effective treatment for certain disorders originating in the haemopoietic stem cell (HSC) compartment: severe combined immunodeficiency disease (CID) [Buckley, 1971; Van Bekkum, 1972] and aplastic anaemia [Thomas et al., 1972; Storb et al., 1974; Floersheim and Storb, 1974]. The bone marrow aplasia induced by a radical chemotherapeutic treatment of acute leukaemia is also amenable to bone marrow transplantation [Graw et al., 1971; Fefer et al., 1973; Floersheim and Storb, 1974].

The rationale for bone marrow transplantation is that an injection of haemopoietic cells derived from a healthy donor can restore the damaged or depleted HSC compartment in the recipient, provided that the inductive microenvironment is not defective [Van Bekkum and De Vries, 1967]. In transplantation practice, bone marrow has been used most extensively as the source of haemopoietic cells. Bone marrow contains, along with cells of the erythroid, myeloid, thromboid, and lymphoid cell lines in all stages of differentiation, a small minority of HSC [Metcalf and Moore, 1971a]. During the last decade, it has been shown that this cell type is responsible for the extensive repopulation of the haemopoietic sites of the recipient following marrow transplantation [Till and McCulloch, 1961; Trentin and Fahlberg, 1963]; as such, the HSC population is the most important cellular constituent of the bone marrow graft.

The serious complications which arise from allogeneic bone marrow transplantation have prevented extensive application of this therapy. These problems are mostly related to the Graft versus Host (GvH) reaction, a dangerous immunological reaction against recipient tissues mediated by lymphocytes in the graft. When no special measures are taken in allogeneic transplantation, the GvH disease is invariably fatal [Bortin, 1970].

Many techniques have been devised in attempts to prevent or mitigate the GvH disease, but as yet none of them offers full protection. The selection of histocompatible donors has been relatively successful in reducing the severity of GvH disease [Gatti et al., 1968; De Koning et al., 1969; Levey et al., 1971;Rubinstein et al., 1971; Van Bekkum, 1972; Yamamura et al., 1972; Stiehm et al., 1972; Dupont et al., 1973]; however, in spite of selection, bone marrow transplantation still results in an approximately 50% incidence of GvH complications and a 25% mortality from GvH disease [Storb et al., 1974]. Moreover, it is worth considering that the selection procedure is of very restricted applicability, because of the limited availability of

matched donors. For these reasons, histocompatibility testing has not really solved the problem of GvH reactions.

In recent years, it has been shown that the acute GvH reaction which is directly caused by lymphocytes in the graft, can be prevented in principle by removal of mature lymphocytes from the transplant [Dicke et al., 1968]. Although this principle is already being used in the treatment of infants, its application to bone marrow transplantation in adults requires further development. The method, however, is of no value in preventing the so-called delayed GvH disease and, therefore, the latter reaction continues to be the most urgent issue to be approached at the experimental level [Van Bekkum and Dicke, 1972; Dicke and Van Bekkum, 1973]. It is supposed that the latter reaction is elicited by lymphocytes gradually arising from grafted HSC after the cell infusion [Van Bekkum and Dicke, 1972].

One of the promising approaches to mitigate delayed GvH disease is the use of another haemopoietic stem cell source, the embryonic liver. This organ is a major site of haemopoiesis during a long period of prenatal development [Metcalf and Moore, 1971a]. Cell grafts from fetal liver have previously been extensively investigated in animals [Uphoff, 1958; Crouch, 1959; Urso et al., 1959; Lengerova, 1959; Barnes et al., 1961; Van Putten et al., 1968; Bortin and Saltzstein, 1969]. The studies showed that the transplantation of these cells not only resulted in preventing acute GvH disease but also in the occurrence of a relatively moderate delayed GvH reaction with a markedly longer survival. Notwithstanding, the application of fetal liver cell grafting has so far not become an established procedure in man for several reasons. The main problem has been that difficulties were encountered in obtaining haemopoietic or immunologic restoration with these grafts [Van Putten et al., 1968; Soothill et al., 1971; Githens et al., 1973]. It was not possible to determine the biological basis for the low repopulation efficiency of fetal liver cells. Another obstacle was the logistical problem of collecting suitable embryo donors at the time when a transplantation is required.

A number of novel technical procedures has been developed in recent years, and these permit a new approach to these problems. The methods involve assays for HSC proliferation and differentiation and for the quantitation of HSC numbers [Till and McCulloch, 1961]. They seem to be of value in acquiring a better insight into the factors responsible for the take problems.

If the low incidence of takes following fetal liver cell grafting is due to an insufficient number of HSC in fetal liver, augmentation of the number of HSC prior to grafting might be a way to ensure that a take is obtained. The newly developed techniques for *in vitro* cultivation of haemopoietic cells [Pluznik and Sachs, 1965; Bradley and Metcalf, 1966] might offer practical possibilities for inducing HSC proliferation in culture [Dicke et al., 1971]. Furthermore, the HSC assays permit direct investigation of the question as to whether the relatively mild type of delayed GvH reaction following fetal liver cell grafting is due to the presence of low numbers of HSC in this tissue

or to special cellular properties of the embryonic HSC. In addition, it is envisaged that a recently developed method for preservation of HSC [Schaefer et al., 1972a] will enable the long term storage of fetal liver cells and diminish the logistical problem of having the transplants available at the appropriate time.

The progress in technical potentialities to approach and possibly solve these current questions has created new perspectives for fetal liver cell transplants. This is of extreme interest, from the standpoint of the beneficial outcome of fetal liver cell grafting as regards the severity of delayed GvH disease, of the unsatisfactory results with bone marrow cells, and of the urgent need for a solution to the delayed GvH problem. Therefore, fetal liver cell transplantation is the subject of this study.

1.2 Present status of bone marrow transplantation and GvH disease

1.2.1 Transplantation of haemopoietic cells

To obtain a take of grafted "foreign" (allogeneic or xenogeneic*) haemopoietic cells in a recipient, it is necessary to suppress the mechanism(s) of the host which controls immunological rejection. Lethal irradiation or treatment with immunosuppressive agents provide such a "conditioning". Due to the resulting destruction of the pre-existent marrow cells two other conditions are also accomplished which are usually favourable to the take of the grafted haemopoietic cells: space for the cells to lodge in the marrow sites and a stimulus for the cells to proliferate [Van Bekkum and De Vries, 1967]. Since the discovery that treatment with bone marrow can prevent mortality from the acute lethal irradiation injury (the "primary disease"), the irradiated animal has provided a profitable experimental model system to which we owe most of our current understanding concerning the basic principles of bone marrow transplantation [Micklem and Loutit, 1966; Van Bekkum and De Vries, 1967].

An infusion of a sufficient number of bone marrow cells is capable of repopulating the depleted haemopoietic sites and can thus prevent early death from the irradiation-induced bone marrow aplasia. In this way, the inoculation of syngeneic or autologous bone marrow cells produces a lasting recovery. Allogeneic marrow cells can produce a similar reconstitution; however, the recipients subsequently develop a complication which occurs, at least in the mouse, following the recovery from the radiation sickness and is therefore known as secondary disease. After this secondary disease was

* To indicate the immunogenetical relationship between donor and host, four categories of transplantations are distinguished in transplantation biology: autologous grafting – the graft is taken from the same individual to which it is transplanted; isogeneic or syngeneic grafting – host and donor are genetically identical (animals of the same inbred strain or identical twins in humans); allogeneic grafting – donor and recipient are genetically different but of the same species; xenogeneic grafting – donor and recipient belong to different species. In allogeneic and xenogeneic transplantations, immunological reactions (graft rejection, GvH reaction) between recipient and graft may be involved, whereas following autologous and isogeneic (syngeneic) transplantation such reactions do not occur.

recognized as being caused by an immune reaction mounted by graft-derived lymphoid cells against the host tissues [Barnes and Loutit, 1956; Trentin, 1956, 1957; Loutit, 1957; Congdon and Urso, 1957; Uphoff, 1957; Uphoff and Law, 1958; Van Bekkum et al., 1959], the term "secondary" disease was replaced by the operational term "Graft-versus-Host" disease. This disease was also encountered in transplantations in which conditioning of host other than irradiation was involved and appeared to be a characteristic complication of non-syngeneic haemopoietic cell grafts. The GvH reaction has continued to be the most dangerous complication of bone marrow transplantation, although much experimental work has been done concerning its pathogenesis, prevention, and therapy.

1.2.2 Acute and delayed GvH reactions

Two distinct forms of GvH disease can be recognized, each with clearly different clinical manifestations (table 1.1). The number of immunocompetent cells present in

Table 1.1 *Graft versus host disease*

	Acute	*Delayed*
Etiology:	Many immunocompetent cells present in the graft which react against the recipient tissue antigens Severe donor-recipient histo-incompatibility	Few immunocompetent cells present in the haemopoietic cell graft (the immunocompetent cells which originate from the grafted HSC react against the recipient (?)) Slight donor-recipient histo-incompatibility
Symptoms and pathology:	Diarrhoea (loss of crypts, denudation) Icterus (liver cell necrosis, loss of epithelial cells in the bile ducts) Dermatitis (lymphocyte infiltration, dyskeratosis, hyperkeratosis, vacuolisation of the basal layer)	Infections Immunodeficiency Diarrhoea Dermatitis Icterus
Onset:	5–10 days after transplantation	20–30 days after transplantation
Course:	Lethal within 15 days after transplantation	Less acute

Modified according to Van Bekkum and De Vries, 1967.

the graft and the degree of histo-incompatibility between donor and host determine the type of GvH disease that will occur [Van Bekkum and De Vries, 1967].

Monkey and human bone marrow contain large numbers of immunocompetent cells and transplantation leads to the rapid development of a violent GvH reaction, the acute or early type of GvH disease, which is usually fatal within 15 days following transplantation [Van Bekkum, 1964; Dicke et al., 1968, 1969; Dicke, 1970]. By contrast, marrow from rodents, containing relatively low numbers of lymphoid cells,

provokes a delayed or late type of GvH disease which is a milder and protracted variant of the reaction and which has its onset at about three weeks following transplantation.The acute GvH reaction is the result of a cell-mediated immune attack against the host tissues by mature immunocompetent cells which are already present in the graft [Simonsen and Jensen, 1959; Vos et al., 1959; Van Bekkum, 1964], whereas the delayed type is assumed to originate from an immunological reaction initiated by lymphoid cells which develop from grafted precursor cells after transfer to the host. Elimination of the lymphoid cells from primate bone marrow or mouse spleen prevents the normally occurring typical acute and vigorous GvH reaction and the delayed and mitigated form appears [Dicke et al., 1968, 1969; Dicke, 1970]; conversely, the addition of lymphocytes to rodent marrow grafts leads to an acute GvH reaction in the transplanted host [Vos et al., 1959; Cole et al., 1959; Goodman and Congdon, 1961].

The cells reacting in the GvH process represent thymus-dependent (T) lymphoid cells [Miller, 1961, 1962]. The recipient target organs affected by these cells are many; the multiple sites of damage (intestinal tract, liver, skin, spleen, lymph nodes, and bone marrow) as well as the varying degree of clinical expression of the disease make its symptomatology a variable one [Simonsen, 1962; Van Bekkum and De Vries, 1967]. The GvH syndrome in its most complete form may comprise a number of symptoms, only some of which are usually apparent in each individual case: diarrhoea, reduction in body weight ("wasting"), hepato-splenomegaly, icterus, dermatitis, anaemia, infections, and fever. It is important to note that, during the disease, a state of generally suppressed immune responsiveness [Barchilon and Gershon, 1970; Zaleski and Milgrom, 1973] exists which is thought to be largely responsible for the increased incidence of microbial infections [Simonsen, 1962; Van Bekkum and De Vries, 1967]. It is not known whether this diverse picture of GvH disease is related to differing activities of subpopulations of the cells which participate in cell-mediated immune reactions [Tigelaar and Asofsky, 1972; Cantor and Asofsky, 1972; Cantor, 1972].

The actual cause of death during the *acute* GvH reaction is attributed to severe damage to the organs and tissues involved. In the *delayed* GvH disease, functional and pathological changes that can be directly attributed to the GvH reaction are relatively mild, but they are aggravated by infections and inflammations. The overwhelming infectious complications represent the predominant cause of mortality in the delayed form of the disease.

1.2.3 Mitigation of GvH disease

Because of the life-threatening features of GvH disease, bone marrow transplantation research in recent years has been predominantly concerned with the prevention and the mitigation of GvH reactions. From the variety of methods explored so far, the selection of donors which are genotypically identical at the major histocompatibility

complex has been shown in experimental animals and in man to be a most valuable procedure to mitigate both the acute and the delayed GvH disease [Good et al., 1964; Storb et al., 1968; Gatti et al., 1968; De Koning et al., 1969; Van Bekkum, 1972; Storb et al., 1974]. Although this selection significantly reduces the severity of the disease, it does not provide absolute protection [Speck et al., 1971; Rubinstein et al., 1971; Graw et al., 1971; Stiehm et al., 1972]. In the treatment of panmyelopathy, in spite of donor-recipient HLA/MLC matching, the disease still develops in roughly 50% of the cases, half of which results in mortality [Storb et al., 1974]. Moreover, a major drawback of the selection method for clinical use is that the frequency of identical sibling donors in humans is low. If there are no immunogenetically identical family members available, one is restricted to histo-incompatible grafting which involves, when engraftment ensues from it, a 100% incidence of fatal GvH disease [Bortin, 1970]. It is due to this complication that we are not yet at the stage of general application of haemopoietic cell therapy. Therefore, continued research in the combat against GvH disease is still of a high priority.

The acute GvH problem has been essentially solved by the development of a cell fractionation technique (discontinuous albumin gradient centrifugation). Using this technique, which takes advantage of differences in cellular density between stem cells and lymphocytes, separation of lymphocytes from purified stem cell fractions has been achieved [Dicke et al., 1968]. The method has been shown to be not only effective in terms of prevention of acute GvH disease but is also reliable, since it can be controlled by *in vitro* assays for numerical changes in lymphocytes and stem cells in the different fractions [Dicke et al., 1969, 1970; Dicke, 1970]. The demonstration that the density gradient centrifugation technique may serve to prevent acute GvH disease, even in fully histo-incompatible situations [Dicke and Van Bekkum, 1972, 1973], implies that the acute GvH reaction is no longer of prime concern as an experimental issue and that further research should be centred on its application to human patients and on the prevention or control of delayed GvH reactions.

1.2.4 Approaches for the control of delayed GvH disease

From our present state of knowledge, experimental efforts to overcome the delayed GvH risks may focus on a number of different factors (table 1.2). In this respect,

Table 1.2 *Procedures for the control of delayed GvH reactions*

	directed at donor	directed at host
available methods	donor selection	
prospective methods (?)	improvement in donor-matching fetal liver cell transplants	strict pathogen-free conditions manipulation of thymus function immunological enhancement

five lines of research are to be distinguished: the improvement of donor selection; the application of gnotobiotic conditions; the use of embryonic liver cell grafts; the employment of immunological enhancement [Voisin and Kinsky, 1962; Batchelor and Howard, 1965; Voisin et al., 1968; Safford and Tokuda, 1970]; and the manipulation of thymus function. The latter two have not yielded very promising results so far and will not be discussed separately [Buckley et al., 1971; Van Putten, 1964]. The other three seem to be of more practical interest.

The first approach concerns the further development of histocompatibility testing. It aims at the identification of those individuals among the HLA/MLC identical donors, whose bone marrow is incapable of producing GvH disease; and furthermore, it is directed towards the selection of compatible donors among non-family members. The successful reconstitution of a CID infant with MLC-negative/HLA-incompatible bone marrow [Copenhagen Study Group of Immunodeficiencies, 1973] may represent a promising lead for expansion of the category of compatible donors.

A second line of work is based on the observations that, similarly to animals, a high proportion of patients suffering from delayed GvH disease, die from bacterial infections [Van Bekkum, 1972; Dicke and Van Bekkum, 1973]. It has been demonstrated in the mouse that almost complete prevention of the delayed GvH mortality can be achieved by the establishment of strict gnotobiotic conditions [Van Bekkum et al., 1974]. In similar experiments in monkeys, however, it has been found that allogeneic bone marrow transplantation in decontaminated recipients also had a beneficial effect on survival but still resulted in substantial mortality due to viral infections [Hendriks, 1974]. Apparently, the prevention of bacterial infections did not suffice to overcome the persistent state of immuno-incompetence. Although extension of this approach to patients has to determine its clinical merit in the inhibition of human GvH hazards, it is not expected that it will provide a complete safeguard in totally incompatible combinations.

The last avenue for research which will be referred to is the use of fetal liver cell grafts. In 1958, it was shown in mice that the transplantation of embryonic haemopoietic liver cell grafts instead of bone marrow resulted in the prevention of the acute GvH syndrome as well as suppression of delayed GvH mortality [Uphoff, 1958]. These results were afterwards confirmed by others in animal experiments [Crouch, 1959; Urso et al., 1959; Lengerova, 1959; Barnes et al., 1961]. The fact that widespread application in humans has not been effected is due to factors which are not related to GvH reactions. The main drawback with respect to clinical application has been the difficulties encountered in obtaining takes with fetal liver cells [Thomas et al., 1963; Van Putten et al., 1968; Githens et al., 1973]. Advances in experimental haematology and newly developed techniques have created new possibilities for solving or at least analysing this problem [Till and McCulloch, 1961; Pluznik and Sachs, 1966; Bradley and Metcalf, 1966; Dicke et al., 1968; Lahiri et al., 1970]; this is one of the subjects of this investigation.

1.3 *Fetal liver cell transplantation*

1.3.1 Origin and nature of fetal liver haemopoiesis

Embryonic haemopoiesis is initiated in the yolk sac [Aberle, 1927; Attfield, 1951; Borghese, 1959] and, during the regression of yolk sac function, the liver becomes the major fetal haemopoietic organ in most mammals [Rifkind et al., 1968, 1969; Niewisch et al., 1970; Moore et al., 1970]. In the mouse, fetal liver haemopoiesis begins in the liver anlage by the 10–12th day of gestation and the organ develops to be the main site of blood cell production until the time of birth. During the first week after birth, liver haemopoiesis disappears completely. The bone marrow, where haemopoiesis is established at 3–4 days before term, takes over the role of the liver and continues to function as the main site of haemopoiesis for the rest of the life of the individual [Moore et al., 1970; Niewisch et al., 1970].

In man, fetal liver haemopoiesis begins at about the 7th week and subsides at 22–24 weeks of gestation. Since most knowledge of developmental haemopoiesis has been obtained in small vertebrates and, since the mouse is the subject of investigation in this study, we will confine the more detailed discussion of the available information to the ontogenesis of this species.

HSC and progenitor cells which originate *de novo* in the yolk sac blood islands and subsequently migrate via the circulation to colonize developing haemopoietic tissues, have been proposed to establish self-replicating stem cell populations in the fetal liver, the spleen, and, later, in the bone marrow [Moore and Owen, 1967; Metcalf and Moore, 1971a]. This so-called unicentric theory accepts the primitive yolk sac stem cell as the common precursor of all other stem cells and proposes migration streams of stem cells which emigrate from the yolk sac and settle in the liver. Conversely, the multicentric theory is based on an independent evolution of stem cells from endogenous precursors in yolk sac, liver, and other haemopoietic sites [Niewisch et al., 1970].

Although, no definite verification of the unicentric theory can be made, it is supported by a number of observations. Extirpation experiments showed that the development of embryonic haemopoiesis in the liver requires the presence of a yolk sac in an early stage of gestation. Furthermore, a cellular traffic of HSC to the liver before the commencement of haemopoiesis has been observed by several investigators [Niewisch et al., 1970; Moore et al., 1970; Goss, 1928; Metcalf and Moore, 1971c].

Though models of haemopoiesis were postulated around 1930 [Maximow and Bloom, 1934] which included the existence of a HSC capable of taking alternative pathways of self-reproduction and differentiation towards fully developed elements [Vogel et al., 1969], only in 1961 could a cell be experimentally identified which fits the criteria for this theoretical entity. This was the haemopoietic cell which following its injection into lethally irradiated mice formed visible colonies on the surface of the spleen in 7–10 days after transfer of the cells [Till and McCulloch, 1961]. In brief,

the identity of the so-called spleen-colony-forming cell (colony forming unit spleen; CFU-s) with a multipotent HSC was supported by the following evidence:

a. By employing a unique chromosomal marker to identify member cells of a single clone, it was shown that the majority of the dividing cells within a colony are derived from a single precursor cell [Becker et al., 1963; Fowler et al., 1967; Wu et al., 1967].

b. Cells within such colonies were demonstrated to consist of erythropoietic, granulopoietic, and megakaryopoietic precursor cells [Wu et al., 1967]; furthermore, cells obtained from the same clone characterized by a specific karyotype were also found to give rise to lymphocytes [Wu et al., 1968; Nowell et al., 1970]. This represented elegant proof of a common multipotent lympho-haemopoietic cell as the initiator of spleen colonies.

c. The demonstration that spleen colony forming cells had the capacity to produce new colony forming cells within the colonies [Siminovitch et al., 1963] and that an individual colony could offer complete protection to a lethally irradiated host [Trentin and Fahlberg, 1963] was evidence of the extensive self-renewal capacity of the spleen colony forming cells.

Thus, it was established that the cells which give rise to macroscopic colonies in the spleens of irradiated mice fulfil the key requirements of pluripotency and "unlimited" self-replication capacity of HSC. It was also shown that fetal liver contained spleen colony forming cells.

Numerous studies have been involved with the properties of the fetal liver stem cells. Fetal liver stem cells produce colonies which closely resemble those produced by stem cells from adult animals [Silini et al., 1968; Vogel et al., 1970; Moore et al., 1970]. The embryonic stem cells have a more homogeneous and lighter density [Moore et al., 1970] and a larger volume [Haskill and Moore, 1970], than those of the adult bone marrow. Experiments on the morphological identification of the stem cell similar to those carried out on stem cells from adults [Van Bekkum et al., 1971; Dicke et al., 1973b, 1973c] have not been extended to the cells of fetal origin.

In vitro exposure of the cells to the lethal effect of tritiated thymidine showed that, while the majority of bone marrow stem cells are in the resting state, most fetal liver stem cells are rapidly proliferating [Becker et al., 1965; Duplan, 1970]. Furthermore, it was found that fetal CFU-s had a higher radiosensitivity and this was shown to be independent of the cell cycle state of the cell population [Duplan, 1970]. In addition to these physical differences between marrow and fetal liver stem cells, also functional differences have been observed, e.g., greater proliferative capacities of fetal liver stem cells following a transfer to lethally irradiated recipients as compared to bone marrow [Schofield, 1970; Metcalf and Moore, 1971e].

As to the more differentiated compartment of haemopoiesis in the embryonic liver,

erythropoiesis represents by far the most important component of haemopoietic activity. It differs from that in the adult animal by the electrophoretically different type of haemoglobin [Craig and Russel, 1964; Fantoni et al., 1967] and the very low level of haemoglobin content in early erythroblasts [Steiner and Vogel, 1973]. The experimental data on the hormonal control of liver haemopoiesis as studied by the responsiveness of fetal liver cells to erythropoietin are contradictory: some of them indicating a complete lack of sensitivity of liver erythropoiesis to the action of the hormone [Jacobson et al., 1959; Bleiberg et al., 1965]; others showing a positive requirement of the cells for erythropoietin [Chui et al., 1971; Stephenson et al., 1971; Gregory et al., 1973]. This contradiction may be explained by the finding that erythropoietin responsiveness is present only in young fetuses and that this responsiveness is lost during embryonic development [Cole and Paul, 1966].

Another indication of a different haemopoietic control in fetal and adult tissues is provided by the comparative paucity of granulopoiesis in fetal liver [Silini et al., 1967]. The findings of low numbers of granulocytic cells in the liver and the generation of normal proportions of granulocytic spleen colony numbers by fetal cells in adult hosts [Vogel et al., 1970; Moore et al., 1970] support the opinion that the liver microenvironment largely determines the suppressed granulopoiesis in the embryo.

On the other hand, evidence has been obtained that the embryonic cells have certain specific growth properties which are not dependent on environmental factors but rather on intrinsic cell properties [Schofield, 1970]. The latter may be particularly important in haemopoietic cell transplantation. Therefore, they will also be under discussion in this investigation.

1.3.2 The take of fetal liver cell transplants

For many years, investigations of the proliferative capacity of fetal liver cells have employed the parameters of protection of irradiated animals and histological repopulation of the haemopoietic tissue; later, when CFU assays were available, the proliferation of the stem cells was also taken as the endpoint. The data on the take capacity of fetal liver cell transplants in the literature show a remarkable divergence.

The results of a quantitative study conducted by Crouch [1959] demonstrated that, in comparison to marrow cells, more than twice the number of fetal liver cells were needed to obtain a similar percentage survival of lethally irradiated mice. The results of Crouch are difficult to reconcile with other experimental data which indicate a more pronounced proliferation capacity for embryonic liver cell transplants. The classical competition experiments of Micklem et al. using isogeneic chromosome-marked cell populations have shown that, beginning as early as 2 weeks following injection, the populations derived from the liver outgrew karyotypically-distinguishable bone marrow derived populations [Micklem and Loutit, 1966; Micklem and Ford, 1966].

In serial spleen colony passaging experiments, the CFU of embryonic origin did

not undergo the same decline as bone marrow CFU, which was consistent with the idea that fetal liver CFU can sustain a continuous proliferation over longer periods of time [Metcalf and Moore, 1971e; Micklem, 1972].

The shorter doubling time of the fetal-liver-derived CFU population in the exponential growth phase in the spleen following transplantation was indicative of a greater capacity of fetal liver CFU for self-renewal [Schofield, 1970]. The observation that stem cells in fetal liver produced spleen colonies of a larger size and with an increased rate of DNA synthesis per colony [Micklem, 1972] as compared to bone marrow was also suggestive of an enhanced growth rate of these cells. These findings, however, were inconsistent with results in which the cellularity and the size of the CFU-s and the CFU-c populations of individual spleen colonies of fetal liver origin were not significantly different from those of bone marrow derived colonies [Metcalf and Moore, 1971e].

Comparative bone marrow and fetal liver cell transplantation experiments performed on the basis of the equivalence of the numbers of CFU-s in the cell inocula did not allow clear-cut conclusions as to the relative proliferative efficiencies of the cells from both sources. The more pronounced splenic and femoral erythropoiesis during the first 6 days following transplantation of fetal liver cells in irradiated recipients may be ascribed to the transplantation of a relative excess of erythropoietic precursors in fetal liver rather than to differences in stem cell potencies [Kubanek et al., 1969]. Restoration experiments in lethally irradiated mice revealed that only 60% of the total number of fetal liver CFU-s were needed to provide a similar degree of irradiation protection. These investigations were also not conclusive, because they did not account for possible differences in spleen colony forming efficiency and related differences in absolute numbers of stem cells in the grafts [Duplan and Legrand, 1971].

Although the results are not decisive, most of them seem to tip the balance in favor of a proliferative advantage for fetal liver cells. One should realize, however, that most of these experiments were carried out under isogeneic transplantation conditions. It is questionable whether the findings apply to histo-incompatible grafting with which one is concerned in man.

Experimental data on the take of fetal liver cell grafts in allogeneic murine recipients are scarce. The titration experiments of Crouch [1959] revealed that the efficiency of fetal liver cells to produce a certain level of survival following lethal irradiation was at least 2–4 times lower than that of bone marrow cells. The author did not specify whether one liver provided enough cells to repopulate an entire host. Since the concentration of stem cells in both types of grafts may differ markedly [e.g. McCulloch and Till, 1963; Micklem, 1966; Silini et al., 1967; Niewisch et al., 1970] and may importantly influence the takeability, it is unfortunate that these values could not be determined in those days. The larger numbers of fetal liver cells required for repopulation are in accord with the take problems encountered in fetal liver cell

grafting in other species, including man [Thomas et al., 1963; Van Putten et al., 1968; Githens et al., 1973].

Giller et al. [1972] observed an enhanced and sustained restoration of the cellularity and of the CFU population in the femoral marrow of allogeneic radiation chimaeras in a follow-up investigation after the transplantation of fetal liver cells. It is questionable whether the promoted repopulation by fetal liver is indeed a direct difference in repopulation capacity, in view of the high numbers of 20×10^6 inoculated cells employed in these investigations. Because GvH reactions are known to produce severe hypoplasia in the lymphoid and haemopoietic organs [Van Bekkum and De Vries, 1967], it might also reflect the reduced GvH reaction in the fetal liver group.

An augmentation of repopulation by fetal liver cells in chimaeras was achieved by replacing a part of the liver cell graft by fetal thymus cells which in themselves had no capacity for haemopoietic reconstitution. As it was shown that adult thymus cells, irrespective of their GvH reactivity, were particularly active in overriding the inhibition of haemopoietic cell proliferation in non-isogeneic recipients [Goodman and Wheeler, 1968; Goodman and Grubbs, 1970; Goodman et al., 1972], the effect of the fetal thymus cells might be attributed to the ability of large numbers of these cells to overcome the allogeneic resistance [McCulloch and Till, 1963; Cudkowicz and Stimpfling, 1964; Hellström and Hellström, 1965]. On the other hand, the fact that extremely low numbers of fetal thymus cells were also effective in promoting such a repopulation [Bortin et al., in press] raises doubts as to the validity of this assumption.

On the whole, our understanding of the factors which govern the take of a fetal liver cell transplant seems to be fragmentary and conflicting. Since many factors such as the number of transfused stem cells, the susceptibility of the cells to genetic repression, and the intrinsic proliferative qualities of the cells are involved in the initial growth of the graft, a comprehensive study in which these factors are integrally evaluated is clearly required.

1.3.3 Fetal liver cell transplantation and GvH disease

In 1958, Uphoff was the first to report on experiments with fetal liver cell transplantation. She found an almost 100% survival following the transfer of fetal liver cells from either parental strain to lethally irradiated F_1 hosts. These values were in sharp contrast to the 10–35% survival of the bone marrow treated group. The lasting beneficial effect of parental fetal liver cells, indistinguishable from a syngeneic transplantation, has stimulated broad interest in fetal liver haemopoietic cell transplantation. Uphoff's publication was followed by numerous others on the same subject. In another study [Uphoff, 1959], the same author demonstrated the superiority of fetal liver cell grafts in three allogeneic mouse strain combinations. Since then, an increasing body of evidence has confirmed the ability of embryonic liver cells to restore irradiated allogeneic recipients and with less severe delayed GvH reactions, although the exact survival data after fetal liver cell transplantation varied from one study

to the other [Uphoff, 1958, 1959; Crouch, 1959; Urso et al., 1959; Lengerova, 1959; Barnes et al., 1961; Bortin and Saltzstein, 1969].

More specifically, Crouch [1959] and Barnes et al. [1961] reported that a marked reduction in GvH mortality was obtained, but that the outcome of fetal liver cell transplantation was not as good as that following the treatment with syngeneic haemopoietic cells. The same investigators established that a low grade GvH disease with typical clinical and pathological findings was the cause of the mortality which occurred in the fetal liver cell infused mice. The survival rate after fetal liver cell allotransplantation was also demonstrated to be dependent on the actual donor-host immunogenetic relationship [Crouch, 1959]. This is probably an important key to the nature of the diverse survival results in the literature.

The ameliorated GvH reaction following fetal liver cell transplantation could not be ascribed to the deficient maturation of immunocompetent lymphocytes. Apparently, a complete repopulation of the lymphoid organs in the host by donor type lymphoid cells took place [Vos et al., 1960]. Within 30–60 days after transplantation, these cells were normally reactive in provoking GvH reactions in secondary genetically different hosts [Tyan, 1964; Van Bekkum, 1965]. Thus, it is conceivable that the decrease in the delayed GvH mortality is due to the fact that the immunocompetent cells which arise from embryonic precursor cells have a greater tendency to develop a specific immunological tolerance to host tissue antigens.

A difficult problem in the interpretation of very late mortality following all types of bone marrow transplantation is the distinction between mild GvH disease and severe immunodeficiency. Some very late mortality after haemopoietic cell transplantation was observed in syngeneic situations, in particular following fetal liver cell transplantation [Barnes et al., 1961]. The fact that the addition of syngeneic lymph node cells prevented this very late mortality in fetal liver cell grafted isogeneic hosts seemed to prove that at least part of this mortality was due to a deficiency in lymphoid proliferation in the irradiated host and not to GvH disease [Barnes et al., 1961]. In recent years, the group of Bortin has returned to the idea that an insufficient immune restoration following fetal liver cell transplantation could be responsible for the late mortality. They demonstrated an improved long-term survival when fetal thymus cells were included in the fetal liver cell graft [Bortin and Saltzstein, 1968; Bortin and Rimm, 1970]. The authors postulated a compensation by lymphoid precursor cells for an immune deficiency. However, the fact that the positive effect of intravenously infused thymus cells could be mimicked by fetal thymus cells enclosed in cell-tight millipore diffusion chambers makes this explanation unlikely and points rather to an effect mediated by thymic factors [Bortin and Rimm, 1970]. Apparently, these results do not distinguish between a thymic influence on the suppression of GvH development and a direct effect on immunological reconstitution.

So far, the majority of experimental data indicate that a GvH reaction is the most plausible explanation for the late mortality in fetal liver cell transplanted mice

[Crouch, 1959; Urso et al., 1959; Lengerova, 1959; Vos et al., 1960; Tyan, 1964]. The immune deficiency which is a regular feature of the delayed GvH syndrome is assumed to be a complication of the GvH reaction itself.

1.3.4 Immunological reconstitution after fetal liver cell transplantation

Although fetal liver tissue is devoid of immunologically reactive cells [Tyan and Cole, 1963; Bortin and Saltzstein, 1968; Tyan et al., 1973], the cells present are apparently fully capable of conferring immunological competence on lethally irradiated *syngeneic* recipients. Embryonic liver cells generate cells which are able to migrate to and repopulate the lymphoid organs [Vos et al., 1960; Taylor, 1964]. Furthermore, the cells to which they give rise are capable of functioning in both humoral and cellular immune reactions, including specific antibody responses [Doria et al., 1962; Tyan et al., 1967] and GvH reactions [Tyan and Cole, 1963, 1964; Tyan, 1964]. The gamma globulins in the serum are rapidly restored to normal levels [Tyan et al., 1967; Tyan and Herzenberg, 1968a, 1968b]. Complete restoration is generally achieved in the syngeneic host and it is similar to that following the transplantation of adult bone marrow cells.

There are many reports to indicate that the immunological recovery in *allogeneic* hosts after haemopoietic cell transplantation is delayed or incomplete [Doria et al., 1962; Gengozian et al., 1965, 1971]. From bone marrow transplantation studies, it is known that allogeneic mouse chimaeras have suppressed levels of IgM and IgG, a reduced cell-mediated immune capacity [Bortin et al., 1971], a lowered primary and secondary antibody response, and a qualitatively abnormal antibody synthesis as evidenced by the absence of a normal conversion from 19S to 7S antibodies [Gengozian and Congdon, 1973]. Although some of these deficiencies may persist, even after the disappearance of GvH symptoms, immunological reconstitution is sufficient to permit long-term survival of the hosts. Similar to the experience with bone marrow transplantation, the rate of immunologic recovery after fetal liver cell transplantation is also depressed in allogeneic hosts [Doria et al., 1962]. However, the time pattern of reconstitution of allogeneic fetal liver cell recipients appears to be more favourable than that of comparable bone marrow recipients [Bortin et al., 1971].

1.3.5 Preclinical fetal liver cell transplantation in dogs and monkeys

Fetal liver cell transplantation has very rarely been subject to preclinical evaluation in large mammals and, to our knowledge, only a few studies have been performed in the dog and the monkey. In dogs, the Seattle group failed to produce consistent takes with liver cell allografts from term or near-term fetuses on five occasions [Thomas et al., 1963]. At best, temporary signs of a take were noted. Since the authors do not comment on the number of cells transfused, a comparison with bone marrow grafts

as regards the takeability cannot be made. Obviously, symptoms of GvH disease were not seen.

A more quantitative study was undertaken by Van Putten et al. [1968] in Rhesus monkeys. These investigators mention problems in establishing takes of fetal liver cell transplants. The total cell yield of a single fetal liver of 100 days gestation did not provide sufficient numbers of cells for engraftment in lethally irradiated histo-incompatible monkeys. Whereas 4×10^8 allogeneic bone marrow cells from adult donors (per kg recipient body weight) produce takes in over 90% of the cases, no takes were obtained with dosages of $2–4 \times 10^8$ fetal liver cells. Therefore pooling of several livers from different embryos was necessary; even then, take problems were observed. With dosages of $5.5–11 \times 10^8$ pooled liver cells per kg, only 4 out of 9 recipients were repopulated. The mean survival time of the four successfully engrafted monkeys of 36 days (range 21–45 days) represents a marked prolongation as compared to that following allogeneic bone marrow transplantation (13.8 days; range 5–25 days) [Dicke, 1970]. The clinical course after transplantation seemed to be rather characteristic of a delayed GvH reaction in all four monkeys. The picture was quite similar to that seen in Rhesus monkeys following grafting with purified bone marrow stem cell fractions [Dicke, 1970; Van Bekkum and Dicke, 1972]. It was concluded that GvH lesions were the direct cause of death in only one animal; severe complicating bacterial infections were diagnosed in the other three.

Thus, the study of Van Putten et al. demonstrated the development of a mild delayed GvH reaction following fetal liver cell transplantation but it also clearly indicated a relatively low takeability of fetal liver cells. The fact that in the dog the liver cell yield per fetus was clearly insufficient, may be related to the multigestational state of this species. Considering that a total cell number of 35×10^8 cells from a dog embryo liver at 6 weeks of pregnancy provides 3×10^8 cells per kg for a 12 kg recipient [Löwenberg and Vriesendorp, 1974], the failures to obtain takes are not surprising in the light of the bone marrow data in this species. A number of 4×10^8 incompatible bone marrow cells per kg was also incapable of repopulating lethally irradiated dogs [Vriesendorp, 1974]. In the monkey, however, the number of fetal liver cells which was administered was in the same range as the number of bone marrow cells which sufficed to achieve a high proportion of takes. This number of fetal liver cells did not produce takes. The latter finding is an indication that different factors are involved in fetal liver cell and bone marrow transplantation which operate to the disadvantage of fetal liver cell take.

1.3.6 Fetal liver cell transplantation in man

The advantage of fetal liver cells with regard to mitigation of GvH disease in animals has led several transplantation groups to employ these grafts in human patients. The very unsatisfactory results of early efforts to treat patients suffering from bone marrow aplasia with fetal haemopoietic cells [Bodley Scott et al., 1961]

is the probable reason why attempts at fetal liver cell grafting in man have mostly been restricted to immunodeficient (CID) infants. Such patients represent ideal recipients from the point of view of graft acceptance, since they possess virtually no immunologically competent cells and have a low body weight. The congenital immunological disorder known as severe CID represents a differentiation anomaly in which the progress of the HSC along the lymphocytic pathway leads to impaired B as well as T-cell-dependent immune functions [De Vries et al., 1968; Hitzig et al., 1971; Van Bekkum, 1972]. Nevertheless, fetal liver cell grafts as a source of normal HSC, have frequently been combined with fetal thymus grafts in the treatment of this disease, on the assumption that the secondary involution of the thymus seen in the advanced stages of the disease [Hitzig et al., 1971] would prevent a rapid differentiation of immunocompetent T cells from grafted HSC.

In a study of Soothill et al. [1971], combined fetal liver cell and fetal thymus tissue therapy was given to a series of 11 CID patients. Restoration of immune functions was not obtained in any of them. When GvH disease incidentally occurred, there was no way to distinguish whether it originated from the thymus or from the liver cell transplant.

Githens et al. [1973] transplanted three patients with CID with liver cells. Fetal liver cells of 12 and 13 week embryonic age were administered to the first two patients; one intravenously and the other intraperitoneally. No signs of either engraftment or of GvH disease were noted. In the third infant, due to repeated failures of take, sequential transplantations were performed with either fetal liver cells, fetal thymus organ grafts, or combined fetal liver cell plus fetal thymus tissue grafts varying in maturation time from 9–22 weeks. Again, no reconstitution was obtained in any of these attempts.

According to our present knowledge, the following factors might have contributed to these failures. Firstly, frozen fetal liver cells were administered in both studies and these may have introduced certain pitfalls. Schaefer et al. [1972a] have shown that the HSC is quite susceptible to damage from the freezing and thawing procedure. Because of these findings, these authors devised a special technique which allows optimal preservation of HSC. In the above mentioned human fetal liver cell transplantation studies, monitoring for stem cell viability [Schaefer et al., 1972a] was not carried out. The fact that in one study [Githens et al., 1973] the eosin resistance of nucleated cells after thawing had fallen to 23–55%, suggests that the preservation procedure might have been responsible for a marked loss of HSC. Secondly, the majority of the grafts in both studies were administered via the i.p. route which is now known to be a relatively inefficient way of establishing haemopoietic cell grafts [Schaefer et al., 1972b]. Taking these losses into account, one can calculate the equivalent of the above cell dosages in terms of fresh fetal liver cells when administered i.v. The number of cells transfused was reported to vary between 10^8–10^9 [Soothill et al., 1971]. Assuming a total body weight of the recipients of 5 kg, an i.v.–i.p. route

efficiency ratio of 3:1 [Schaefer et al., 1972b], and an average loss of 50% of the stem cells following the recovery from freezing [Githens et al., 1973], the above value of 10^8–10^9 cells represents a dosage of $3–30 \times 10^6$ viable fetal liver cells per kg body weight when given i.v. Considering that an i.v. dose of 4×10^8 fresh fetal liver cells did not result in takes in monkeys [Van Putten et al., 1968], it is conceivable that the inoculates employed in these treatments could not provide reconstitutions, even in CID recipients. Making allowance for the fact that the take efficiency of fetal liver cell grafts in the monkey was about 3–4 times less, $3–30 \times 10^6$ fetal liver cells are equivalent to $1–10 \times 10^6$ bone marrow cells. It is also uncertain whether such numbers of unmatched bone marrow cells are sufficient to lead to restoration in CID babies. Although detailed information on minimum bone marrow cell numbers needed for immunologic repair in CID is not available, it is notable that, where full reconstitution has been obtained with matched bone marrow grafts, the cell numbers applied were considerably larger i.e., in the order of 50×10^6 cells per kg (Levey et al., 1971, Van Bekkum, 1972, Yamamura et al., 1972, Gelfand et al., in press).

Two reports appeared recently in which fetal liver cells were successfully used to correct the T and B cell deficiencies in CID infants [Cooper et al., 1973; Döhmann, 1974]. In the first case, 2×10^9 fresh fetal liver cells from a 9 week old embryo were given i.p. and, following a very slow pattern of reconstitution in which the blood lymphocyte count only started to rise at around day 100, complete reconstitution was eventually obtained [Cooper et al., 1973; Keightley et al., 1973]. It is unfortunate that no markers were available to conclusively prove chimaerism. Except for a transient skin rash, no signs of GvH disease were seen. However, the patient developed a nephrotic syndrome from which he died a little more than one year following the transplantation.

In a second case, 2×10^8 fresh fetal liver cells per kg from a 12 week embryo donor were given i.v. This led to a rapid restoration of the immunological deficiency within 4 weeks following transplantation [Döhmann, 1974]. Genetic markers of the embryo were not identified, so that, again, one could not ascertain the true chimaeric state after the recovery. The intestinal tract of this patient, who was kept in strict reverse isolation, had been completely decontaminated.

Relevant factors in both of these successful attempts at immunological repair by fetal liver cell transplantation seem to be that higher cell numbers were applied, the cells were not frozen, and that (at least in the one that showed a rapid reconstitution) the cells were given i.v. Furthermore, it is of prime importance that these transplantations, which were as a matter of fact histo-incompatible, were not associated with obvious GvH disease. They represent the first unmatched haemopoietic cell transplantations which appeared to be of benefit in the treatment of CID. Although one should be careful in drawing too optimistic conclusions (the first child finally died from a possibly immunological complication; the second child is only in the first year following grafting; in neither instance was a take explicitly proven), the observed

reconstitution of CID following fetal liver grafting without GvH disease may have a significant impact on further progress in bone marrow transplantation. As long as histocompatibility selection procedures do not provide full safeguards against the occurrence of serious GvH reactions, fetal liver cell transplants may provide a suitable alternative in haemopoietic cell transplantation. At the same time, this consideration emphasizes the necessity of solving the problem of embryonic liver cell takes. This is of even more importance if one envisages a development in which the application of these transplants is extended to groups of patients with aplasia or leukaemia who lack a matched sibling bone marrow donor.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Experimental animals and irradiation*

Experiments were carried out with mice derived from CBA/Rij, C57BL/KA, and C57BL/Rij inbred strains or with an F_1 hybrid from matings between C57BL/Rij and CBA/Rij strains produced in the Rijswijk colony. The donor mice from which bone marrow was obtained were 6–8 weeks of age.

The recipient mice used for survival studies and spleen colony assays were 12–16 weeks of age. They were irradiated with a supralethal dose of 800 (C57BL) or 900 rad (CBA/Rij and F_1) of X-rays. The physical properties of the X-irradiation were 300 kV; 10 mA; HVL of the beam, 3.0 mm Cu; distance from the focus to the bottom of the mouse cage, 51 cm; dose rate, 55 rad/min; maximum back scatter; (Philips-Müller 300 X-ray machine). Animals were routinely housed five to a cage and given food and water ad libitum.

2.2 *Preparation of cell suspensions*

2.2.1 Mouse bone marrow cell suspensions

The femurs were excised and the proximal ends were cut with scissors. A bent needle placed on a syringe was introduced into the shaft and moved slightly up and down to break up the small bone spiculae. Thereafter, a small amount (approximately 0.2 ml HBSS) was forcibly injected into the shaft to expel the bone marrow. The collected bone marrow was filtered through a nylon gauze sieve and a G_2 sintered glass filter (diameter pores: 30–80 μ).

2.2.2 Spleen and thymus cell suspensions

After excision, spleens and thymuses were cut into small pieces and the resulting tissue fragments were gently squeezed through a nylon gauze sieve consisting of 6 layers to obtain a dispersed cell suspension.

2.2.3 Mouse fetal liver cell suspensions

Mouse embryos were obtained by carefully timed mating. Initially, groups of 20 females were placed with 3 males in a cage overnight. Later in the study, this mating scheme was changed by putting a single breeding pair into a cage for one night. This markedly improved the gestation efficiency. After 16 days (day 0 represents the morning following the night of conception), the pregnant mice were selected from the group by inspection and careful palpation and used as embryo donors.

They were killed by cervical dislocation under ether anaesthesia. Uteri were removed aseptically, placed in Hanks' Balanced Salt Solution (HBSS), and the embryos were dissected free from the uterus. The fetal livers were removed by use of a binocular dissecting microscope; they were pooled and minced with scissors into small cubes of 1–2 mm. The cubes were incubated for 10–15 min in a 0.15% trypsin solution at 37° C in order to disaggregate the tissue. At the end of the incubation, the proteolytic action of the trypsin was terminated by the addition of horse serum to a concentration of 10%. Preparation of the suspension was completed by passing the cells through nylon gauze and G_2 glass (Jena) filters in sequence. The cells were now monocellularly dispersed. When gelatinous clumps were present, they were treated with minute amounts of DNAse. The cell concentration was determined after washing in HBSS.

2.2.4 Human fetal liver cell suspensions

Human fetal livers were obtained from embryos removed by caesarean section. They were received from three sources: The Department of Gynaecology of the University Hospital in Leiden (heads: Prof. Dr. A. Sikkel and Prof. Dr. E. V. van Hall); the Division of Gynaecology of the Elizabeth Gasthuis in Haarlem (heads: Dr. P. E. R. Rhemrev and Dr. W. A. A. van Os) and the Fetal Tissue Bank of the Royal Marsden Hospital in London (head: Dr. S. D. Lawler). The tissues were transferred on ice to Rijswijk and with the exception of the embryonic tissues supplied by the Fetal Tissue Bank in London, usually arrived at the laboratory within 1 h after collection. The organs sent from London by air in ice-cooled containers were processed at approximately 6 h following the operation. After the arrival of the embryos or tissues in the laboratory, the preparation was immediately begun. Human fetal livers were treated by the same procedure as employed for mouse fetal liver cell suspensions. Human fetuses were staged according to measurements of fetal crown-rump length and to duration of pregnancy on the basis of menstrual history. Determination of the age of the tissues sent from London was done at Royal Marsden Hospital.

2.2.5 Cell counting

Nucleated cells were counted in a haemocytometer in Türck solution (0.01% crystal violet and 1% acetic acid in saline). Determination of eosin (0.2%) resistant cells was also routinely performed.

2.3 Identification of mouse chimaeras

After an interval of 30 days following irradiation and transplantation, donor and recipient cells were identified by cytotoxicity typing of cells from peritoneal washings and by haemoglobin electrophoretic typing in red cell lysates.

2.3.1 Cytotoxicity test

Cytotoxic specific isoantisera plus complement were employed for determination of the genotype of the nucleated cells. In this way, peritoneal cells were serologically determined according to the method of Balner [1963]. The cytotoxicity test was performed in Falcon microtitre dishes by exposing a 1 µl of suspension containing approximately 5,000 cells to similar volumes of antiserum and chick complement (the latter sometimes diluted 1:2 or 1:4). The cells were then placed in an incubator at 37° C for 30 minutes. After incubation, 1 µl of trypan blue solution (0.12% trypan blue and 0.3% EDTA in isotonic saline) was added to stain the cells killed by the cytotoxic action. The dishes were left to stand for 10 min at room temperature; 0.5 µl of buffered formalin (30%) was then added to stop any further cellular alterations. Reading of the test was usually done within 5 h following its completion using an inverted microscope. Dead cells were identified by their failure to exclude trypan blue.

Preparation of the antisera

Two antisera were used: a CBA-anti-C57BL and a C57BL-anti-CBA isoantiserum. Isoantisera were obtained from mice given 5 i.p. injections of the allogeneic spleen homogenate (one-fifth spleen per mouse) at weekly intervals. The mice were bled one week after the last injection. The serum was pooled and tested for cytotoxic potency (titres varying between 1:32–1:256).

Collection of peritoneal cells

Peritoneal cells represented the target for the cytotoxicity test and were obtained by i.p. injection of 2 ml HBSS or isotonic saline. After a few minutes of gently massaging the abdomen, the fluid with the cells was reaspirated via a needle. Cells from a normal CBA and a normal C57BL mouse routinely served as controls.

2.3.2 Haemoglobin typing

The haemoglobin type of donor and recipient mice was identified by use of the distinct electrophoretic mobility properties of the haemoglobin from CBA and C57BL/KA mice [Van Bekkum and De Vries, 1967]. The haemoglobin solution was prepared from heparinized blood which was obtained by puncture of the orbital vein at about 4 weeks after transplantation. Repeated washings of the cells in HBSS and subsequent lysis by 30 min treatment with distilled water resulted in the production of a haemoglobin solution in water. Following this, cellulose acetate electrophoresis was performed. The haemoglobin bands of normal CBA and C57BL/KA mice served as references.

2.4 Spleen colony assay (mice)

The spleen colony assay was performed according to Till and McCulloch [1961]. An appropriate number of nucleated haemopoietic cells suspended in HBSS was injected into the tail vein of each of a group of mice within 5 h following lethal

irradiation. The number of cells in 0.5 ml HBSS was adjusted so that the number of colonies arising in the spleen was expected to fall within the range of 5–15. A group of 8–12 mice were injected per experiment. Eight to ten days later, the surviving mice were sacrificed, the spleen dissected out, fixed in Bouin's solution, and the macroscopic colonies on the surface of the spleen counted. The average number of colonies per spleen within a certain group of mice was expressed as the number of colony forming units (CFU-s) per 10^5 cells injected. The arithmetic means of groups of CFU-s values were compared by the Student-T test.

Multiplication curves of CFU-s in spleen or femur were made by estimation of the number of CFU-s in these tissues at various intervals after injection (by assay in secondary recipient mice). The recovery values obtained for CFU-s were expressed as a percentage of the injected number at time zero. The slope of CFU increase was estimated by regression analysis according to Geigy [1970]. Correlation coefficients of the lines all fell in the range of 0.90–0.95; it appeared that omission of the estimates of the beginning and the end of the CFU recovery slope did not increase the correlation coefficient. The regression coefficients of the slopes of CFU increase in spleen or femur were used as an index of the growth rate by the CFU cell population in question.

2.5 Soft agar cultures

The agar culture techniques for clonal haemopoietic cell growth are based on the *in vitro* systems first described by Pluznik and Sachs [1965] and Bradley and Metcalf [1966]. Our culture method, according to a modification adopted by Dicke et al. [1971], consisted of an agar underlayer containing feeder cells and an overlayer with haemopoietic cells. The underlayer containing fibroblast feeder cells served to provide stimulative activity for the growth of haemopoietic colonies in the overlayer. The medium used in all experiments was Eagle's Minimum Essential Medium (MEM) as modified by Dulbecco (Dulbecco's Medium). It was made up in double strength and diluted with equal volumes of agar.

2.5.1 Fibroblast feeder cultures

Two variations of fibroblast feeder culture methods were employed: 1. the original thin agar layer system (TALS) [Dicke et al., 1971] in which the fibroblast feeder cells were spread as a monolayer over the bottom of the petri dish; and 2. a modified TALS in which the fibroblasts were dispersed throughout the underlayer. The latter modification of fibroblast-mixed-underlayers was adopted, for reasons of simplicity, when it turned out that both variants yielded an equivalent colony forming efficiency.

Preparation of underlayers for use in the original TALS: A quantity of 0.5×10^6 fibroblasts in 1 ml medium (Waymouth – 20% calf serum) were pipetted into culture dishes (Greiner 35 mm diameter) (day 1). Adherence of the cells to the bottom of the

dish took place during one day of growth; on the second day (day 2), the cells were irradiated (2000 rad) in order to stop their growth. The preparation of the underlayer was completed by replacing the fluid medium by a standard agar underlayer medium. The underlayer was a mixture of Dulbecco's medium, horse serum (20%), and freshly boiled agar (final concentration 0.5%). The mixture was adjusted to a temperature of 40° C and 1 ml aliquots were subsequently pipetted over the fibroblast layers in the dishes. The medium was left to gel at room temperature for 15–20 min. Plates were then stored in the incubator and used after an interval of 24–48 h (day 3 or 4) for haemopoietic cell-plating. The modified TALS was introduced in our laboratory to permit the preparation of the bottom layer within one day. The procedure was as follows: a mixed suspension of the agar underlayer medium and fibroblasts (pre-irradiated 2000 rad γ-Rays) was prepared and 1 ml was directly poured on the plates (day 1). Fibroblasts were incorporated in the underlayer at a concentration of 0.5×10^6 cells per ml. After solidifying, the dishes were transferred to the incubator. At the time of plating (day 2 or day 3), the overlayer containing the haemopoietic target cells was layered over the bottom layer.

Preparation of overlayers

The composition of the upper layer was similar to that of the underlayer, but with a final agar concentration of 0.25%. The cell number was adjusted so as to grow 30–150 colonies per plate. After solidification of the upper layer, the plates were returned to the incubator for 7–9 days and then scored for colony numbers. The incubation atmosphere was maintained at 37° C, humidified, and constantly flushed with 7.5% CO_2 in air.

Preparation of fibroblast cell lines

The preparation and the maintenance of the mouse fibroblast lines were as follows: Fibroblasts were derived from embryo trunks. The tissues were minced with scissors and the fragments trypsinized in flasks containing a magnetic stirrer for 20 min. The supernatant with cells was pipetted off, centrifuged, and the cell pellet of fibroblasts was suspended in Waymouth medium-calf serum (20%) and dispensed into 10 cm plates. These plates contained the stock of the fibroblasts. The lines were kept for prolonged time in culture; they were trypsinized once weekly and serially passaged for months; the medium was refreshed every fourth day.

2.5.2 Scoring of colonies

The fibroblast cultures were scored after 7–9 days of culture. Colonies, defined as cell aggregates of more than 50 cells, were counted at 25 × magnification using an inverted microscope. Duplicate or triplicate cultures were employed for each estimate. In conformity to common use, the population of cells in the haemopoietic suspension giving rise to colonies was operationally termed "colony forming units in culture"

(CFU-c). The concentration of CFU-c in a suspension was expressed as the number per 10^5 nucleated cells plated.

2.6 *Liquid cultures*

Liquid cultures were performed in siliconized round bottomed glass tubes which contained 1 ml of medium and $1–2 \times 10^6$ bone marrow or fetal liver cells. These cultures were employed to investigate the kinetics of HSC in culture. At various intervals of incubation, cultures were terminated and the number of CFU-s per culture was estimated and expressed as percentage recovery of the number inoculated into culture at the beginning (see fig. 2.1). A second endpoint was concerned with a follow-up of the cycling status of CFU-s during cultivation.

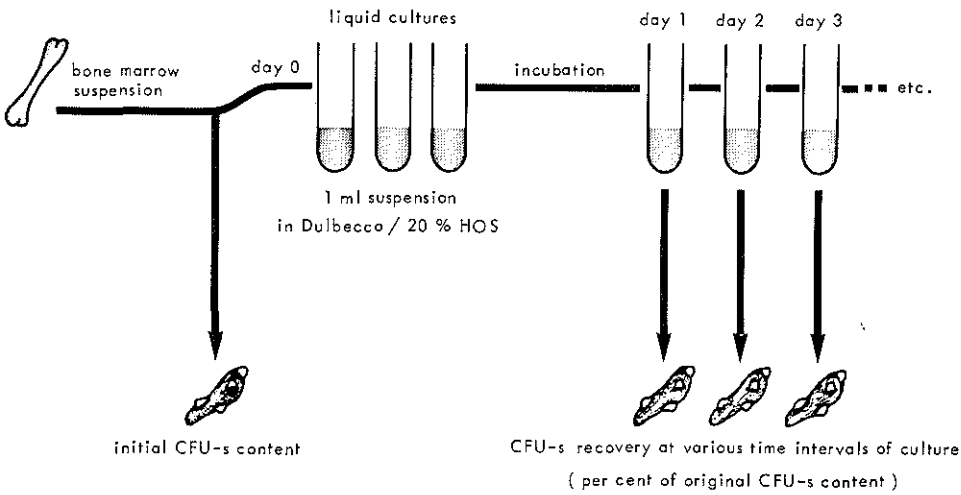

Fig. 2.1 *Haemopoietic cells in liquid culture.* Experimental scheme.

The culture medium was Eagle's MEM as modified by Dulbecco and supplemented with 20% horse serum. Cultures were alternatively supplemented with $0.02–0.05 \times 10^6$ irradiated fibroblasts (2000 rad), 20% fresh fibroblast conditioned medium (FCM), or 5% haemolysate. Nonstimulated cultures were set up at the same time and served as controls. Fibroblast feeder cells were prepared from established lines as described above for soft agar cultures. FCM was obtained as the supernatant medium from the fibroblast stock plates (see under preparation of fibroblast cells) and was freshly added to cultures. Cultures were performed in glass test tubes closed with aluminium foil. All cultures were incubated at 37° C in a humidified incubator which was continuously flushed with a gas mixture of CO_2 (7.5%) and air. At the time of harvest, cultures were taken from the incubator, the cells were suspended by gentle pipetting,

and cell suspensions from at least two tubes were pooled. To assure maximum collection of the cells, the tubes were rinsed twice with 3 ml of HBSS. Following centrifugation (2000 rpm during 10 min), the cells were resuspended in an appropriate volume of HBSS. The cells were used immediately afterwards for *in vitro* thymidine suicide or injection into mice for CFU-s assay.

2.6.1 Preparation of haemolysate

A lysate of red blood cells was prepared according to Bradley et al. [1971, 1972]. In short, blood was obtained aseptically in heparinized syringes by abdominal puncture of the aorta of WAG/Rij rats or by heart puncture of mice. The blood was centrifuged at 2500 rpm and the upper part of the cell pellet including the buffy coat, was removed. The remaining red blood cells were washed three times in phosphate buffered saline (PBS). Following this, a volume of distilled water equal to the volume of the cell pellet was added and mixed with the cells. The cells were allowed to stand for 30 min to permit lysis. After a final sedimentation, the supernatant was collected and used as haemolysate within 7 days of preparation. Haemolysate was added to a concentration of 5% to cultures.

2.6.2 Preparation of mouse serum

Blood was withdrawn from mice by heart puncture and placed into nonheparinized syringes. It was allowed to clot for 2 h; after centrifugation for 10 min at 2500 rpm, the serum was collected.

2.7 In vitro tritiated thymidine suicide technique

The proliferative status of CFU-s was assessed by the tritiated thymidine killing technique as described by Iscove et al. [1970]. The technique is based on the fact that administration of high-specific-activity tritiated thymidine in high doses will kill cells that incorporate it (i.e., cells in S-phase at the time of the isotope exposure).

Samples from each culture under test were exposed to four different incubation regimens:

1. HBSS – no thymidine;
2. 20 μCi/ml of ^{3}HTdR (methyl-^{3}H-thymidine; specific activity 18–22 Ci/mM; Radiochemical Center, Amersham, U.K.);
3. 200 μCi/ml of ^{3}HTdR;
4. ^{3}HTdR (20 μCi/ml) in the presence of excess unlabelled TdR (100 μg/ml).

The inactivation of CFU-s by the isotope in incubations 2 and 3 was determined by comparison with the CFU-s values of the controls (1 and 4). The four tubes containing cells from the same culture were incubated in a water bath for 25 min at 37° C and were repeatedly agitated by hand.

The labelling was terminated by the addition of 10 ml of cold (4° C) HBSS containing 200 mg/l unlabelled TdR. Two subsequent washings with this solution resulted in at least a 300-fold dilution of the isotope. Following centrifugation (2000 rpm for 10 min), the cells were resuspended in a measured volume of HBSS. The cells were used immediately afterwards for injection into irradiated mice (CFU-s assay).

2.8 *Storage of haemopoietic cells*

To permit the transplantation of quantified numbers of stem cells in bone marrow and fetal liver cell grafts, cells were preserved until the numbers of stem cells in the suspensions had been determined following the completion of the CFU-s assay.

Cells were frozen in sterile glass ampoules of 1, 2, or 5 ml volume. The cell concentration was always kept between $10–200 \times 10^6$ cells per ml, according to the recommendations of Schaefer et al. [1972a]. The HBSS medium was supplemented with the cryoprotective agent dimethylsulphoxide (DMSO) (10%) and with calf serum (20%).

Cells were cooled 1° C/min in a controlled-rate freezer to −40° C. The rate of freezing was controlled by a thermocouple inserted into an identical ampoule which was placed in the freezing chamber. When the cooling temperature had fallen to −40° C, the ampoules were stored in a liquid nitrogen container. Upon removal from the liquid nitrogen at the end of the preservation period, the glass ampoules were rapidly thawed in a 40° C waterbath with gentle agitation. The ampoules were opened and the cells transferred to tubes and diluted tenfold with HBSS, so that a final DMSO concentration of 1% was reached. This dilution was performed at an ultraslow stepwise rate, also according to the procedure of Schaefer et al. [1972a]. The cells were washed in HBSS and finally cell counts were performed. Application of this freezing technique yielded complete recovery of stem cell viability as checked by CFU-s or CFU-c assays after thawing.

2.9 *Discontinuous albumin gradient centrifugation*

This cell separation method, as described by Dicke et al. [1969], was used to prepare purified stem cell fractions and to study the physical characteristics of adult and embryonic haemopoietic cells.

The cells were suspended in a 17% bovine serum albumin (BSA) solution and pipetted (as the lightest layer) on top of a gradient consisting of layers with BSA concentrations increasing to the bottom of the tube (layers of 19%, 21%, 23% and sometimes 25% BSA solutions). The tube was centrifuged for 30 min at 10° C in an International model PR-2 centrifuge at 2000 rpm (1000 g at the bottom of the tube). After centrifugation, distinct cell layers were visible at the density interfaces in the gradient. Following this, each fraction was collected with a pipette, washed in HBSS, and the cell

concentration determined in a haemocytometer using Türck solution. The cell fraction between 17% and 19% BSA was designated as fraction 1; the one between 19% and 21% BSA, fraction 2, and so on. Depending on the numbers of cells to be fractionated, a tube was chosen with a diameter of either 0.8 or 1.6 cm, allowing a fractionation of cell numbers up to $100–130 \times 10^6$ and 900×10^6 cells per tube, respectively.

To achieve optimal efficiency in the separation of HSC, adjusted osmolarity of the albumin solution was utilized for each cell suspension. It is known that the osmolarity, representing one of the major physical parameters which influences the distribution of the cells in the gradient, is one of the factors which determines the degree of purification of stem cells [Dicke, 1970]. It appeared that the optimal osmolarity in the gradient for stem cell separation of fetal liver was different from that for bone marrow fractionation: for mouse fetal liver, 320 mOsm/l; and, for mouse bone marrow, 310 mOsm/l. To obtain those differing osmolarities, albumin fraction V powder (Sigma) was dissolved to a concentration of 35% in tris buffer (pH 7.2) of differing osmolarities, namely 260 mOsm/l and 250 mOsm/l respectively (pH 5.3). By diluting the 35% stock solution of the required osmolarity with increasing volumes of sodium chloride-sodium phosphate buffer (N–P buffer; 0.154 m NaCl; 0.01 m Na phosphate buffer; pH 7.2), two-percent-step albumin solutions (25% to 17%) were prepared. The final albumin concentration was checked by refractometry, as previously described by Dicke [1970]. Fraction 1 corresponded to a specific density of 1.0525 ± 0.0012 g/cm^3,
fraction 2 – 1.0575 ± 0.001 g/cm^3
fraction 3 – 1.0637 ± 0.0008 g/cm^3
fraction 4 – 1.0682 ± 0.001 g/cm^3
fraction 5 – 1.0734 ± 0.0012 g/cm^3.

2.10 In vitro stimulation of lymphocytic cells by mitogens

Various haemopoietic cell suspensions or their density subpopulations were tested for their content of lymphocytic cells responding to phytohaemagglutinin (PHA), *E.Coli* endotoxin (LPS, lipopolysaccharide), or concanavalin A (Con A). By measurement of the stimulation of DNA synthesis, cells were distinguished according to the general classification of thymus-derived (T) and bursa-equivalent-derived (B) cells. Cells capable of a mitotic response to PHA and Con A were considered to represent T cells [Janossy and Greaves, 1971; Stobo, 1972; Andersson et al., 1972; Shortman et al., 1973; Stobo and Paul, 1973]. In contrast, *E.Coli* endotoxin was considered a B cell mitogen according to its reported properties of stimulating B target cells exclusively [Gery et al., 1972; Andersson et al., 1972]. Cultures were essentially carried out according to the procedure described by Dicke [Dicke et al., 1969; Dicke, 1970].

2.10.1 *Culture conditions*

Hanks' Eagles Minimal Essential Medium (HE-MEM) supplemented with 20% heat inactivated fetal calf serum (Flow) plus antibiotics (100 units penicillin/ml and 100 μg streptomycin/ml) was used as the culture medium throughout. For each type of haemopoietic cell suspension (e.g. mouse spleen, mouse bone marrow), optimal culture conditions (mitogen dosages; culture time) as defined by maximal DNA synthesis, were established. They are summarized in table 2.1. In general, a fixed

Table 2.1 *Culture conditions for mitogenic stimulation*

haemopoietic cell suspension	nr. of cells $\times 10^6$	volume per culture	dosage of mitogen PHA μl	Con A μg	LPS μg	total cultivation time	amount of isotope added ^{3}H-TdR	^{14}C-TdR
mouse								
bone marrow	1	1 ml	10	30	80	48 h	0.2 μCi[1]	n.d.
spleen	3	1 ml	10	7	50	48 h	0.2 μCi[1]	0.04 μCi[3]
fetal liver	1	1 ml	10	15	100	48 h	0.2 μCi[1]	0.04 μCi[3]
human								
fetal liver	0.2	0.5 ml	10	n.d.	n.d.	96 h	0.25 μCi[2]	0.04 μCi[3]
fetal thymus	0.2	0.5 ml	10	n.d.	n.d.	96 h	0.25 μCi[2]	0.04 μCi[3]

The labelling period was inserted for the last 24 h of the total incubation period.
PHA – phytohaemagglutinin; Con A – concanavalin A; LPS – lipopolysaccharide *E.Coli*
[1] spec. act. 55 mCi/mMol
[2] spec. act. 110 mCi/mMol
[3] spec. act. 13.5 mCi/mMol
n.d. – not done

number of cells was suspended in sterile glass or disposable plastic culture tubes with a round bottom. Six tubes were used per estimate. Three tubes served as test cultures and contained the appropriate concentration of mitogen: the other three, free of the mitogen, were run as control cultures. The mouse cultures were incubated in tightly closed plastic tubes at 37° C in air. The human cultures were performed in glass tubes with tin foil closures, allowing maximal gas exchange; they were placed in a humidified incubator at 37° C with a gas mixture of 5% CO_2 in air. At optimal intervals of incubation (time of maximal DNA synthesis), labelled thymidine was added for a second incubation period of 24 h. In the early experiments ^{3}HTdR was used as the isotope; in later tests, cultures were routinely incubated with ^{14}CTdR. At the end of the 24 h labelling time, the cultures were terminated by placing the tubes in an ice bath. Cells were then treated for estimation of thymidine incorporation into DNA.

2.10.2 *Processing of cultures*

Cells from the ^{3}HTdR labelled cultures were washed in 1 ml of cold isotonic saline

to remove nonspecific radioactivity. Following centrifugation (2000 rpm for 10 min), the supernatant was discarded and the cells were recovered in 2–5 ml of acetic acid (3% in distilled water) in order that lysis of the erythrocytes occurred. Then the cells were centrifuged again. The cell pellet was resuspended in 1 drop of serum (as carrier protein) and 2–5 ml of cold 6.7% TCA (trichloroacetic acid). The tubes were left for at least one hour at 4°C in order to allow complete precipitation. The precipitates were washed twice with 5 ml of 5% TCA and dried for 2–3 h at 37°C (plastic tubes) or 4 h at 80°C (glass tubes). Following this, 0.25 ml of Hyamine was added and the tubes were left overnight at room temperature. The lysed cells were transferred to the counting vials (Packard Instruments, Brussels, Belgium), containing 15 ml of scintillation fluid (4 g PPO and 50 mg POPOP (Merck)) in 1 ltr of toluene (Lamers and Indemans, 's-Hertogenbosch, Holland). The tubes were rinsed with an additional 0.25 ml of Hyamine.

The thymidine uptake in cultures exposed to the ^{14}C isotope was determined by using a filter system: 2 ml of cooled thymidine solution (0.5 mg of unlabelled thymidine per ml in distilled water) was added at the end of cultivation. After a few minutes, the cells were sucked into filters of a manyfold filter system (glass fibre filters; Whatman GA/A; ∅1 cm). The filters were washed once with unlabelled thymidine, and then twice with isotonic saline. After drying at 50–60°C, filters were placed into the counting vials and 5 ml of scintillation fluid was added. The scintillation of the vials was counted in a Mark II Nuclear Chicago Liquid Scintillation Counter.

2.10.3 Expression of responses

Each value reported in the text generally represents the arithmetic mean of triplicate cultures. The mean counts per minute (cpm) were converted into disintegrations per minute after appropriate corrections for quenching (with an external standard). The value for the background estimates of cultures without mitogen were subtracted from the dpm value for cultures with mitogen. The difference (Δ) in dpm was used as the net response of the cells to mitogenic stimulation.

2.11 Morphology

2.11.1 Cell suspensions

A modification of the precipitation method for staining cells as described by Sayk [1960] was used; this was done in order to also adapt the method for the staining of fractionated cell suspensions [Van Bekkum et al., 1971]; 10^5 cells suspended in 20 µl of a buffer solution were mixed with four drops of isotonic saline (supplemented with 20% fetal calf serum) and sedimented onto glass slides for 25 min at room temperature. The cells were fixed in 100% methanol for 3 min. Then the slides were exposed to May Grünwald stain (diluted 1:1 with phosphate citric acid buffer of

pH 5.6) for 1 min and to Giemsa solution (diluted 1:17 with the same buffer) for 15 min. The subsequent immersion of the slides in the pure buffer for 1 min and in glass-distilled water for another min completed the staining procedure. After drying in air, cover glasses were placed on the slides and sealed with Entellan balsam (Merck).

2.11.2 Organs and tissues

Organs to be examined were prepared either as imprint or sectioned preparations. Imprint preparations were treated according to the modified Sayk method described above for cell suspensions. Tissues were fixed in 4% buffered formaldehyde and routinely processed for histopathology at the Pathology Department of the Institute for Experimental Gerontology TNO. A haematoxylin-phloxin-safran stain was used.

CHAPTER 3

A CELLULAR CHARACTERIZATION OF THE FETAL LIVER HAEMOPOIETIC CELL TRANSPLANT

The stem cell is held to play a central role in the outcome of haemopoietic cell transplantation. Thus, any of the observed differences between the effects of fetal liver cell and bone marrow grafts may be determined by differences in the properties of fetal and adult stem cells. In addition to these qualitative aspects, it should be taken into account that differences in the number of stem cells in the transplants may have a significant impact on the fate of the graft.

The investigations described in this chapter were intended to clarify the situation with respect to stem cell numbers in the fetal liver cell graft. They mainly dealt with an enumeration of the HSC population. In view of the role of lymphoid cells in GvH

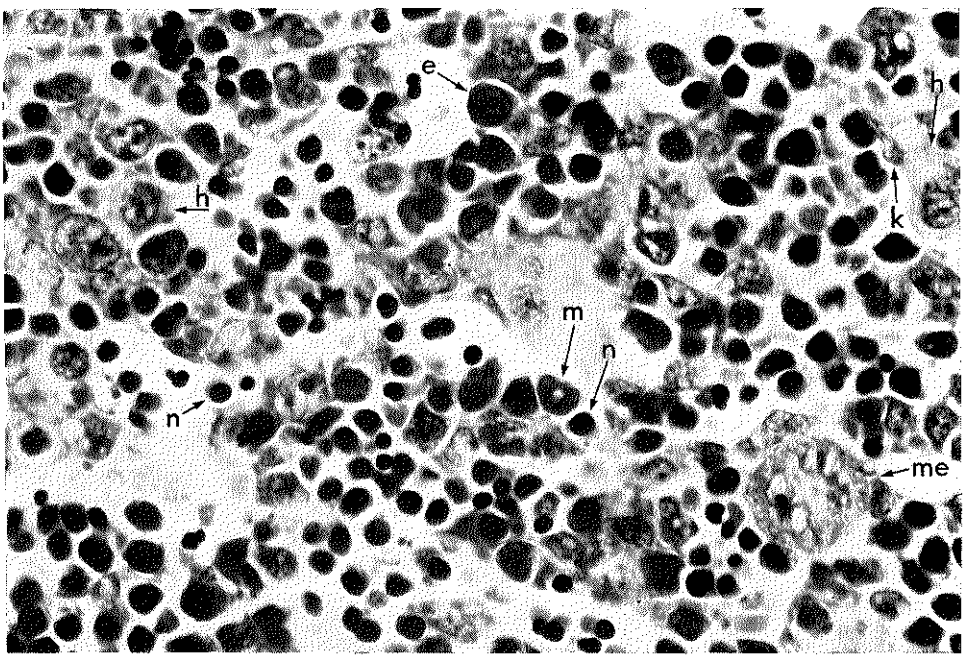

Fig. 3.1 *Section of 16-day fetal liver tissue:* stained with haematoxylin-eosin, ×640.
Arrows indicate the following cells:
h = hepatocyte e = erythroblast
k = Kupffer cell me = megakaryocyte
n = normoblast m = metamyelocyte
Note the abundance of cells of the erythroid series with the dark nuclei.

disease, particular attention was also given to the quantitative estimation of these cells in the transplants.

Additional studies were initiated which were aimed at clarifying the question as to whether adult and fetal HSC exhibit different physical properties. Advantage was taken of the discontinuous albumin gradient fractionation method for exploring density differences between fetal and adult HSC.

3.1 Morphological examination of the fetal liver

Light microscopic examination of the embryonic liver of the mouse reveals little of the adult liver composition. The parenchymatous liver structure does not develop until about 3 days before birth and before that time its histological appearance is characteristic of a full blown haemopoietic tissue with some hepatocytes interdispersed (fig. 3.1 and 3.2). Cross sections and imprint preparations confirm the predominant erythropoietic nature of the organ [Silini et al., 1967] with red cells present at all

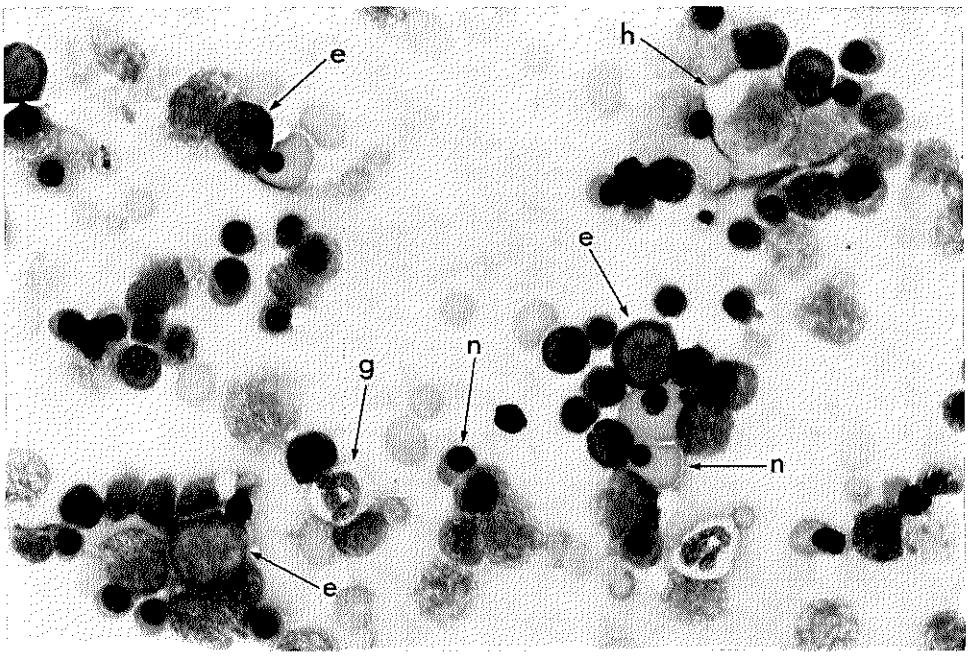

Fig. 3.2 *Imprint preparation of 16-day fetal liver cells:* stained with May Grünwald and Giemsa, ×525.
Arrows indicate the following cells:
h = hepatocyte e = erythroblast
n = normoblast g = granulocyte
Note that two granulocytes are present in this field.

Table 3.1 *Differential morphology of fetal liver and bone marrow cell suspensions*

Cell categories	fetal liver %	bone marrow %
myeloblast	4.5	10.8
promyelocyte	0.4	1.8
metamyelocyte	0.6	0.2
band neutrophil	5.2	31.4
neutrophil	0.2	7.6
eosinophil	–	4.4
total myeloid cells	10.8	56.2
erythroblast	2.8	2.4
normoblast	86.4	36.2
total erythroid cells	89.2	38.6
lymphoid	–	4.8
monocyte	–	0.2
megakaryocyte	–	–

Bone marrow was obtained from 8 week old mice, fetal liver from 16 day embryos.
500 cells were counted in Sayk preparations of both cell suspensions.

stages of differentiation. Megakaryocytes and myeloblasts are rare. Hepatocytes are identified as individual cells or as small aggregates among the haemopoietic cell mass.

Lymphoid cells could not be distinguished in a cytological survey of the various haemopoietic cells in fetal liver cell suspensions (table 3.1). Notably, the relative number of cells belonging to the myeloid series (10.3%) was as much as 5 times lower than that in adult bone marrow (56.2%), whereas the fetal liver erythroid cell numbers outnumbered those of the bone marrow by about 2 times. In other words, the ratio of erythroid-granuloid cells in fetal liver (8.3) greatly exceeded that of 0.7 observed in the marrow.

3.2 Stem cell/progenitor cell compartment

Comparative CFU-s assays [Till and McCulloch, 1961] were conducted in order to determine the number of stem cells in fetal liver and bone marrow. Table 3.2 shows that the CFU-s in fetal liver are present at a low frequency. The CFU-s ratio in the 16 day embryonic organ was found to have an average value of 5.5 CFU-s per 10^5 nucleated cells, which is 5 times less compared to bone marrow. This number of CFU-s seems to increase during the course of ontogenetic development (table 3.3).

The CFU-s values were utilized for the determination of the absolute number of stem cells in the liver cell suspensions by taking into account the spleen colony forming efficiency of the infused CFC i.e., the percentage of all infused CFC that

Table 3.2 *CFU-s concentration in fetal liver and bone marrow cells*

		nr. of CFU-s per 10^5 nucleated cells		
	nr. of exp.	range	mean ± S.E.	$\frac{\text{fetal liver}}{\text{bone marrow}}$ ratio
fetal liver (16 day)	85	1.8–15.7	5.5 ± 0.3	0.20
bone marrow (adult) 8 weeks	26	7.5–44	27.4 ± 1.6	

S.E.: standard error

will form measurable spleen colonies. These so-called efficiency factors were obtained from the CFU growth curve in the spleen. According to the concept of Lahiri et al. [1970] they were represented by the extrapolation points of the CFU growth curves in the spleen, being the fractions of injected CFU that initiate exponential growth and give rise to visible colonies after 7–9 days (see par. 3.6 Addendum). From the experiments carried out to determine the slope of CFU proliferation, it became evident that the extrapolation numbers of fetal liver and marrow CFU multiplication (fig. 3.3) were clearly different, with average values of 2.2% and 4.1%, respectively. In other words, on the assumption that the extrapolation values closely represent the true CFU/CFC ratio, it appears that, of each 100 i.v. administered CFC, the proportion of bone marrow CFC that produces detectable spleen nodules, exceeds that of fetal liver CFC by a factor of 1.9 (4.1/2.2). By correction for their individual CFU/CFC efficiency indices, the total stem cell populations in the fetal liver and bone marrow tissues were calculated to equal values of 250 CFC and 668 CFC per 10^5 nucleated cells, respectively; this represents a difference of a factor of 2.8 (table 3.4).

Table 3.3 *Comparative numbers of CFU-s in haemopoietic tissue at various stages of development*

	nr. of CFU-s per 10^5 cells (mean ± S.E.)	statistical significance of difference between groups
13–15 day fetal liver	5.3 ± 0.5 (14)	$P < 0.01$
18 day fetal liver	11.1 ± 2.2 (3)	$P < 0.001$
8 week adult bone marrow	27.4 ± 1.6 (26)	

The figures in parentheses represent the number of experiments

Table 3.4 *Haemopoietic stem cell numbers in fetal liver and bone marrow cell suspensions*

	nr. of CFU-s per 10^5 cells (mean±S.E.)	spleen colony forming efficiency factor* (f) %	correction for efficiency factor ⟶	nr. of CFC per 10^5 cells
fetal liver (16 day embryo)	5.5±0.3 (85)	2.2	$5.5\times100/2.2$ ⟶	250
f.l.: b.m. ratio	1:5	1:1.9		1:2.8
bone marrow (8 weeks)	27.4±1.6 (26)	4.1	$27.4\times100/4.1$ ⟶	668

* CFU/CFC ratio (%)
The figures in parentheses represent the number of experiments

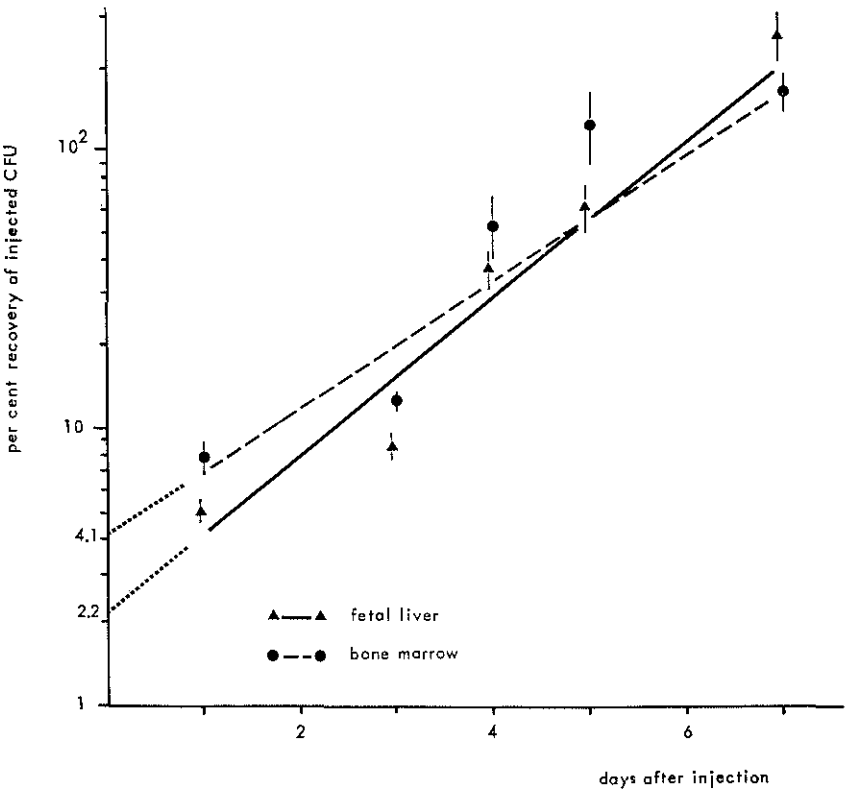

Fig. 3.3 *Increase in fetal liver and bone marrow CFU in the spleen following injection into irradiated syngeneic recipients.*
Each point represents the mean recovery of CFU (±S.E.) from at least 5 separate experiments. The recovery values of CFU in the spleen at various intervals after injection were determined by spleen colony assay in secondary hosts.
Extrapolation points of slopes at time zero: 2.2% (fetal liver) and 4.1% (bone marrow).

Table 3.5 *CFU-c in fetal liver and bone marrow cell suspensions*

	nr. of CFU-c per 10^5 cells (mean±S.E.)	$\frac{\text{fetal liver}}{\text{bone marrow}}$ ratio
fetal liver	25.8± 8.3 (8)	1:4.7
bone marrow	120.9±22.7 (5)	

The figures in parentheses refer to the number of experiments.

Employing the *in vitro* colony assay, it was found that the relative number of another progenitor cell population, the granulocyte-macrophage precursors (CFU-c), is also decreased: 26 CFU-c/10^5 cells in fetal liver versus an average concentration of 121 CFU-c in bone marrow (table 3.5). In this case, the CFC-c cannot be estimated because plating efficiency is not known. The ratio, however, is very close to that of the total number of myeloid cells counted in the cell suspensions (table 3.1).

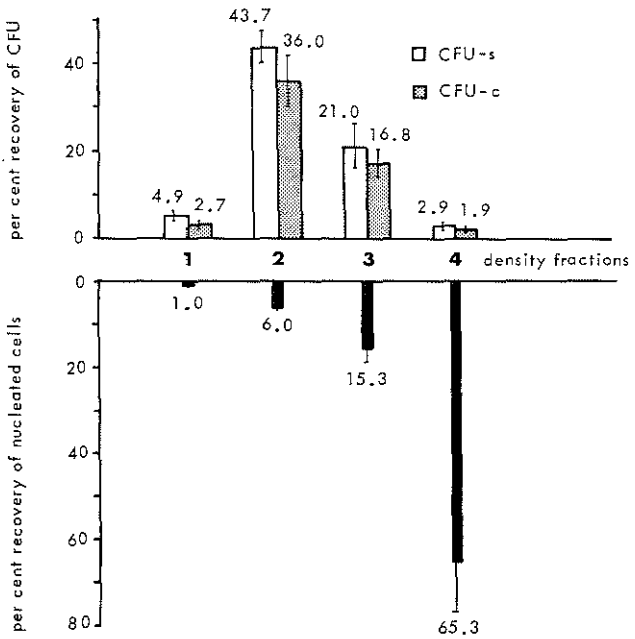

Fig. 3.4 *Density distribution of in vivo and in vitro colony forming cells in 16-day old mouse fetal liver.* Bars represent S.E.

3.3 *Discontinuous albumin density gradient centrifugation of fetal liver cells*

Purified stem cell grafts were employed in the transplantation experiments (to be reported in chapters 4 and 6) in order to assess the role of the non-stem cells in the process of grafting. The technique of discontinuous albumin density gradient centrifugation was applied to separate the stem cells and progenitor cells from the large majority of contaminating cells and to obtain purified stem cell grafts. The distribution pattern of the stem cells (CFU-s) following fractionation is illustrated in fig. 3.4. It appears that the cell class collected in fraction 2 fulfilled the requirement of a purified stem cell suspension; in this fraction, about 40% of the original number of stem cells were recovered in an average concentration of 36 CFU-s per 10^5 cells. This represents an enrichment index of 8.4 × in comparison to the unfractionated suspension (table 3.6). Conversely, in the higher density fractions, the stem cells were diluted to a low concentration due to the accumulation of other cells. It is noteworthy that the CFU-s distribution closely parallels the CFU-c density profile.

Table 3.6 *CFU-s and CFU-c in different density fractions of 16-day old mouse fetal liver*

		CFU-s		CFU-c	
	nr. of CFU-s per 10^5 cells mean ±S.E.	concentration index* of CFU-s mean ±S.E.	% recovery of fractionated CFU-s mean ±S.E.	concentration index* of CFU-c mean ±S.E.	% recovery of fractionated CFU-c mean ±S.E.
unfractionated fetal liver	4.4± 0.2	1		1	
fraction 1	36.7±10.2	9 ±1.8	4.9±0.8	5 ±1.3	2.7±0.6
fraction 2	35.6± 4.8	8.7 ±1.1	43.7±4.4	6.7 ±1.2	36.0±6.3
fraction 3	7.2± 1	2.4 ±0.5	21.0±4.9	1.1 ±0.3	16.8±3.9
fraction 4	0.3± 0.1	0.07±0.01	2.9±0.3	0.06±0.01	1.9±0.5

* Concentration index represents the ratio of CFU-s (or CFU-c) per 10^5 cells in the fraction and CFU-s (or CFU-c) per 10^5 cells in the original suspension.

3.4 *The presence of mitogen reactive lymphocytic cells*

Because of their relationship to GvH disease, special attention was paid to the presence of mature lymphocytes in fetal liver cell grafts. Unfractionated fetal liver cells did not show positive mitogenic responses upon *in vitro* stimulation with the T cell mitogens concanavelin A and phytohaemagglutinin and the B cell activator lipopolysaccharide (table 3.7). The ratio for DNA synthesis in stimulated cells and nonstimulated cells (control) did not exceed a value of 1.3. This contrasted with the high stimulation index of spleen cell suspensions, which exceeded the control incubation by a factor of 7–20 × for the three mitogens. Fetal liver cell responsiveness was

Table 3.7 *In vitro mitogen reactivity of 16-day old mouse fetal liver cells and fetal liver cell density fractions*

	control	Con A	PHA	LPS
fetal liver (total)	9746±700	11170±1546	12761±201	3587±506
	7066±397	5279±184	7211±757	5568±81
fr. 1	2642	1445		
	5828	6495		
fr. 2	6249±585	4454±273	5503±3	5083±120
	15145±1896	17646±1544	18024±1354	13110±1090
fr. 3	6112±174	6542±491	7196±240	2948±9
	12597±254	13875±889	15095±940	5694±1019
fr. 4	6242±361	4708±301	5294±522	2572±209
	1679±185	1120±90	1373±115	1660±127
bone marrow	2174±240	2515±219	3363±475	1607±83
	6680±140	12216±886	6707±719	4531±2747
spleen	1112±78	11284±909	16476±579	7517±833
	2217±54	38167±5387	45958±3081	24287±1984

The figures represent mean dpm ±S.D.
Results of 2 experiments; bone marrow and spleen cells were used for comparison.

more comparable to that of bone marrow. LPS activity of bone marrow cells in culture was also negative and the responses to Con A and PHA were only slightly positive (i.e. maximum stimulation index assessed: 1.8×). Assaying for *in vitro* mitogen responsiveness in fetal liver by incubating the separate density cell fractions with the mitogen also did not reveal clearly positive activity which indicated the absence of subpopulations of T or B lymphocytes in the fetal liver tissue (table 3.7).

3.5 Discussion

A low content of CFU-s was demonstrated in fetal liver tissue, which is in accord with observations of other investigators (table 3.8). A major difficulty in deriving a realistic estimation of the absolute stem cell number from CFU-s values in haemopoietic tissue is the approximation of the fraction, f, of the stem cells which will lodge in the spleen (seeding efficiency) and actually form colonies.

On the basis of the considerations outlined at the end of this chapter (par. 3.6 Addendum), we have used the extrapolation of the slope of multiplication of CFU in the spleen of the irradiated animal to estimate the f factor [Lahiri et al., 1970]. It was found that the spleen colony forming efficiency of fetal liver CFC is on the average 1.9× lower than that of bone marrow CFC.

Inspection of the CFU population recovery curves published by other investigators likewise gives support to the assumption of a relatively low colony forming efficiency

Table 3.8 *CFU-s numbers in mouse fetal liver cell suspensions (as reported in the literature)*

		fetal liver		compared to adult bone marrow
references	strain	fetal age	nr. of CFU-s per 10^5 cells	nr. of CFU-s per 10^5 cells
Till and McCulloch, 1961 McCulloch and Till, 1963	C57BL	near-term	5.5–6.3	8–15.8
Micklem, 1966	–	16 d.	2.2–3.7	
Silini et al., 1967 Silini et al., 1968	$(C3H \times C57BL)F_1$	12–17 d. 17–19 d. adult liver	13.2–7.2 7.2–9.0 0.04	23–46
	Swiss	12–17 d. 17–19 d. adult liver	1.6–3.6 3.6–4.2 2.4	20–40
Duplan, 1968	strain XVII	13–18 d.	6–9	43.5
Vogel et al., 1970	C3H	13 d. 16 d. new born	6.1 ± 1.9 3.8 ± 1.2 3.4 ± 1.3	
Niewisch et al., 1970	C57BL	12 d. 13–15 d. 16–19 d.	9 3.5–2 2–4	
	C3H	12 d. 13–15 d. 16–19 d.	6 5–2 5–11	
Schofield, 1970	$(C3H \times AKR)F_1$	17–18 d.	8.9 ± 0.5	21.8 ± 1.2
Löwenberg, 1974 present study	C57BL	13–15 d. 16 d. 18 d.	5.3 ± 0.5 5.5 ± 0.3 11.1 ± 2.2	27.4 ± 1.6

in the spleen, although no exact data were documented [Schofield, 1970; Vogel et al., 1970]. The 2–3 times reduced Siminovitch factor for fetal liver CFU also corresponds with the lower efficiency of fetal colony forming cells in the formation of spleen colonies [Moore et al., 1970]. This factor representing the recovery of CFU-s at a period of 2 h following infusion is considered to be an index of the efficacy of initial seeding in the spleen [Siminovitch et al., 1963]. In other words, of the two processes – the initial lodging in the spleen and the subsequent loss during the next day (fig. 3.6) – the first one in particular seems to be responsible for the decreased efficiency in colony formation by fetal liver cells. The spleen colony forming efficiency of fetal liver CFC reported by Matioli et al., [1968] was obtained in quite a different manner. Their method is based on the concept that the low efficiency of CFC to grow into countable colonies in the spleen is mainly dependent on the dispersion of the cells

throughout the body following i.v. injection (dilution). It compares the yield of spleen colonies in the i.v. spleen colony assay with that in a local assay (direct injection into the spleen). The so-called dilution factor thus obtained was 3.9% for fetal liver CFC [Vogel et al., 1970] as well as for bone marrow CFC [Matioli et al., 1968]. This is somewhat different from our efficiency figures of 2.2% and 4.1%, respectively. In particular, the equivalence of the dilution factor for fetal liver and bone marrow CFC is surprising, in view of the significantly different seeding factors for both CFU populations [Moore et al., 1970] and the marked differences in CFU recovery in the spleen at 24 h. The validity of this technique has been questioned in that errors may occur due to loss of cells into the circulation [Hendry, 1971] and that some of the colonies formed at the site of injection do not arise from one stem cell but from the cumulative contributions of a number of cellular elements not necessarily being HSC [Till and McCulloch, 1972].

The CFU-s assay indicated a 5 fold difference between fetal liver and bone marrow cells. By adjustment for the CFU/CFC index, the stem cell number (CFC) in a 16-day mouse fetal liver has been calculated at 1/2.8 or 36% of the proportion of stem cells in the bone marrow tissue (table 3.4). It is evident that the lower content of stem cells should be regarded as an important factor in the evaluation of the takeability of fetal liver cell grafts (chapter 4). The low spleen colony forming efficiency of fetal liver CFC results in the CFU-s values overrating the underpopulation of stem cells in fetal liver.

The *in vitro* colony assay was used to assess the size of another population of cells (CFU-c) belonging to the progenitor cell compartment of the liver. The concentration of CFU-c per 10^5 fetal liver nucleated cells was found to be about 1/5 of that of bone marrow (table 3.5). The 5 × decreased values of CFU-c in the fetal liver is consistent with the approximately 5 × reduction in mature granulocytic cells (table 3.1) and suggests that one has to trace the granulopoietic scarcity backwards to before the stage of the CFU-c to a very primitive portion along the differentiation pathway of this cell lineage.

The normal granulopoiesis by fetal liver cells upon transfer to an adult environment [Silini et al., 1967; Moore et al., 1970; Vogel et al., 1970] strongly suggests that the poor myeloid representation in fetal liver is due to local environmental conditions which either exert a selectively inhibitive influence on granulopoiesis or, conversely, a preferential influence on another line of differentiation (erythropoiesis) at the expense of myeloid proliferation.

Purified fetal liver stem cell grafts were obtained by the elimination of the admixed cells using a discontinuous density gradient centrifugation. Fraction 2 had a CFU-s ratio of as many as 36 CFU-s per 10^5 cells which correlates with a mean CFU-s concentration factor of 8.7 (table 3.6). Suppose that, in the projected transplantation studies, X number of stem cells is to be grafted which is mixed in with 100 other cells in the unfractionated fetal liver cell suspension, then the stem cell concentrate-

grafts are minus 88 of the contiguous cells. This purification seems to permit a discrimination between the impact of the more mature embryonic haemopoietic cells and the stem- and progenitor cells on the events observed after transplantation.

From the CFU-s and the CFU-c density distribution values, it appears that the embryonic precursors are of lighter density than are the corresponding cell populations in the adult haemopoietic tissue which routinely appeared in fraction 3 [Dicke, 1970; Dicke et al., 1969, 1973a]. This physical difference between embryonic and adult stem cells is in accordance with investigations of Moore et al. [1970; Metcalf and Moore, 1971] which indicated a sequential appearance of higher density subpopulations of CFU-s and CFU-c during development.

As to the incidence of lymphocytes in fetal liver, no mitogen reactivity was found in the cell suspension. If low-grade activity was present, it was not such that it could be measured in any of the fractions of the gradient. As phytohaemagglutinin (PHA) and concanavalin A (Con A) are considered to be selective T cell stimulators [Janossy and Greaves, 1971; Andersson et al., 1971; Stobo, 1972; Shortman et al., 1973; Stobo and Paul, 1973; Byrd et al., 1973] and the mitogen *Escherichia coli* endotoxin (LPS) a mouse B cell activator [Andersson et al., 1971, 1972; Gery et al., 1972], the absence of lymphocytes (B and T) in the histological preparations is thereby affirmed. The *in vitro* responsiveness of fetal liver cells resembles that of bone marrow to a large extent and indicates a low number of immunocompetent cells in the organ. The question is open as to whether this absence of lymphocytes is specific to the site or to the relatively immature state of embryogenesis. The negative T cell reactivity is compatible with the lack of mature GvH reactive cells [Tyan and Cole, 1963; Bortin and Saltzstein, 1968; Umiel et al., 1968] and of MLC reactive cells in fetal liver [Tyan et al., 1973]. Thus the cells which bring about immunological reconstitution [Doria et al., 1962; Tyan et al., 1967; Tyan and Herzenberg, 1968a, 1968b; Stutman and Good, 1971] and GvH disease following transplantation are most likely to arise from progenitor cells in the fetal liver cell graft. In this respect, it seems that the mouse liver tissue diverges from the human embryonic liver which may be strongly PHA positive at or beyond a gestational time of 16 weeks (table 7.1).

3.6 Addendum

Methodology of estimation of spleen colony forming efficiency

There are several factors which determine the quantification of stem cells in a cell suspension by the spleen colony assay; e.g., possible selective losses during the preparation of the cell suspension, the proportion of the cells which localize in the spleen, and the capacity or failure of these stem cells, for one reason or another (migration, extinction, cell death) to grow into visible colonies in due time [Till and McCulloch, 1972] (fig. 3.5). Loss during preparation must be left out of consideration, since this cannot be estimated. Only about 20% of i.v. injected CFU reaches the

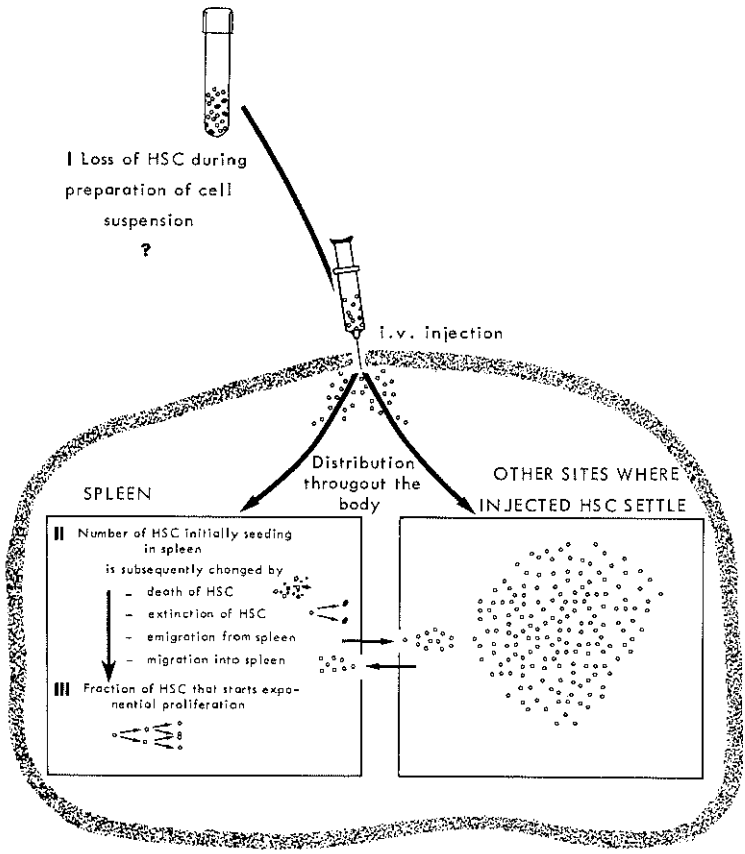

Fig. 3.5 *Factors determining the quantitation of stem cells in a cell suspension by the spleen colony assay. Schematic representation.*
The number of HSC initially seeding in the spleen is approximated by the Siminovitch factor; the number that actually produce colonies, by the Lahiri factor. Effects of death, extinction (differentiation), and migration of HSC leading to a modification of the fraction that gives rise to colonies are difficult to separately assess.

spleen. This can be determined by extracting the cells from the spleen at 2 h after the injection into the host and assaying for the number of recoverable CFU by spleen colony estimation in secondary recipients. By so doing, it was found that, of the CFU which go to the spleen during the first 2 h [Siminovitch factor], an important fraction disappears again during the subsequent 24 h [Lahiri et al., 1970] (fig. 3.6). Following this early drop in CFU in the spleen, an exponential increase of CFU occurs [Lahiri and Van Putten, 1969]. Therefore, the CFU recovery curve can be characterized by a decline of CFU during the first 24 h and an exponential rise thereafter (fig. 3.6). The latter is usually regarded as a function of exponential multipli-

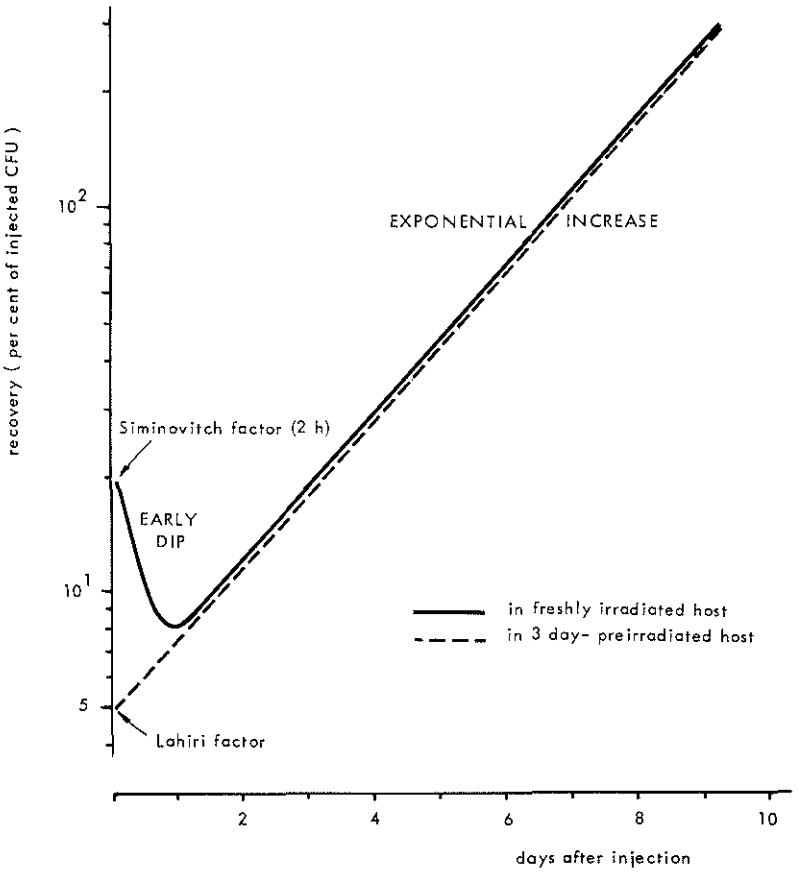

Fig. 3.6 *Curve of per cent recovery of bone marrow CFU in the spleen at varying time intervals after injection into irradiated hosts* (adapted from Lahiri et al., 1969, 1970).
The two sections of the curve are indicated: the initial drop in CFU in the spleen and the subsequent exponential increase. Arrows indicate the Siminovitch factor which is the % recovery of injected CFU at 2 h after injection [Siminovitch et al., 1963] and the Lahiri factor which represents the time-zero-extrapolation point of the exponential slope of CFU [Lahiri et al., 1970]. The curve of CFU recovery in the spleen of the 3-day-preirradiated animal (without early dip) is plotted in the same figure.

cation of CFU, on the assumption that the efficiency in a secondary host does not substantially differ from that in a primary recipient.

The number of injected CFU that seed in the spleen and initiate exponential growth probably represent those CFU that will finally form colonies [Lahiri et al., 1970]. Therefore, the starting point of the exponential component of the CFU recovery curve must be determined to allow enumeration of the portion of injected CFU that give rise to colonies.

However, there is a serious obstacle in directly establishing this starting point of exponential proliferation of CFU in the spleen. This is due to the overlapping of the early decrease in CFU with the beginning of the rise in CFU. In other words, the decline in CFU in the spleen in the first hours after injection probably masks an early proliferation of other CFU [Lahiri et al., 1970].

It is a matter of debate as to whether the appearance of the initial dip in recovered CFU should be attributed to an imbalance in the proliferation possibilities of differentiation and self-reproduction of CFU with differentiation being favoured [Vogel et al., 1968; Lahiri et al., 1970] or to expulsion of CFU from the spleen due to a post-irradiation shrinking of the organ [Testa et al., 1972; Lord and Hendry, 1973]. The fact that the dip is less when CFU which were rapidly self-replicating in the donor [Lahiri et al., 1970], are injected seems to offer support for the first possibility. The first possibility was also favoured by experiments in mice of WW^v genotype which contain haemopoietic cells lacking the capacity to give rise to macroscopic spleen colonies and which can be used as recipients for spleen colony assay without irradiation [Till and McCulloch, 1972]. It was shown that the injection of haemopoietic cells of normal littermates (+/+) into unirradiated WW^v mice and genotypically normal mice lead to the production of the same numbers of spleen colonies. Whether these data may be regarded as valid evidence against the loss of CFU due to splenic shrinkage from irradiation is questionable because they were obtained under pathological conditions and they may not similarly apply to the process of spleen colony formation in normal mice. Certainly, the greater amount of experimental data is in favour of the idea of shrinkage of the spleen causing the early disappearance of CFU from the spleen. Such evidence has come from detailed studies in which correlations were made between the spleen size of the host and CFU recovery values and direct CFU-s assays [Lord and Hendry, 1973]. Furthermore, Lahiri et al. [1970] found that, in the spleens of mice which were irradiated 3 days prior to the injection of haemopoietic cells, the increase in recovery values for CFU did not coincide with an initial decrease and that the exponential growth of CFU commenced as early as 2 h after injection (fig. 3.6). The absence of the dip in the 3-day-preirradiated mice was apparently not the result of an earlier predominance of selfreplication over differentiation; in that case, the slope of CFU increase in the 3-day-preirradiated animal would have been more steep than in the freshly irradiated host. In fact, the angle of the slope and the actual recovery values for CFU in the spleens of pre- and freshly irradiated mice were similar; the only difference being the absence of the initial fall in CFU (fig. 3.6). Therefore, it is most probable that shrinkage of the spleen causes the early disappearance of CFU and that the dip does not occur in the 3-day-preirradiated animals, because the splenic contraction [Lord, 1971] has already taken place before the injection. The identical CFU growth curve, except for the dip, indicates that it makes no difference to the efficiency of the spleen colony assay whether the shrinkage occurs before or after the injection. This is actually supported

by the demonstration by two groups [Shadduck et al., 1971; Lord and Hendry, 1973] that the colony yield in a CFU-s assay is not altered in the preirradiated animal as compared to that in a freshly irradiated mouse. This means in effect that one has a choice of obtaining the efficiency factor of the spleen colony assay either from the previously shrunken spleen or from the spleen which will shrink after the injection of CFU. For practical reasons (i.e., the shorter duration of the assay), we have used the extrapolation value of the CFU growth curve assayed in the spleen of the newly irradiated mouse for f and used this estimate for calculation of the total number of CFC in fetal liver and bone marrow cell suspensions from the CFU-s estimates.

This approach yielded an efficiency number for bone marrow CFU (4.1%) which was roughly of the same order of magnitude as alternative estimations of f by means of very different methods; i.e., the primary transplantation technique, based on the radiation response of CFU, which allowed the calculation of the absolute numbers of endogenous and exogenous splenic CFU and thereby the efficiency of the exogenous assay ($f = 2.7\%$) [Hendry, 1971]; and another first transplantation technique which compared the number of colonies arising from the intrasplenic and the i.v. routes of administration to compute the relative efficiency (dilution) of colony formation of stem cells after injection into the circulation ($f = 3.9\%$) [Matioli et al., 1968].

CHAPTER 4

TAKEABILITY AND REPOPULATION KINETICS OF FETAL LIVER CELLS

In laboratory and clinical practice, the application of fetal liver haemopoietic cell transplants has been associated with a relatively low takeability. Only very rarely has chimaerism resulted from fetal liver cell grafting in man [Bortin, 1970; Soothill et al., 1971; Githens et al., 1973], monkeys or dogs [Thomas et al., 1963; Van Putten et al., 1968]. In the mouse, it has been found that comparatively high numbers of fetal liver cells were required for repopulation [Crouch, 1959]. The cause of the low takeability is largely unknown.

When this property of fetal liver cell grafts was reported for the first time in 1959 [Crouch], two hypothetical reasons were put forward as explanation:

a. the fetal liver tissue contains relatively few proliferative haemopoietic cells or,
b. fetal liver cells encounter important difficulties in adapting to the adult environment.

Experimental haematology had not advanced far enough at that time to permit verification of these possibilities. As investigations into the mechanism of fetal liver cell take have progressed, confusion was added to the situation; several of them indicated enhanced proliferative potencies for fetal-liver-derived cells [Micklem and Loutit, 1966; Micklem and Ford, 1966] and, more specifically, for fetal liver HSC as compared to bone marrow [Schofield, 1970; Micklem, 1972; Metcalf and Moore, 1971e]. These results could not be reconciled with the difficulties experienced in obtaining engraftment. In none of these studies was the proliferation kinetics of the grafted stem cells assessed in direct relation to the criterion of take. A critical difference between the transplantation studies in which one was confronted with difficulties in achieving repopulation, on the one hand, and those indicating a more vigorous fetal liver cell proliferation, on the other, was that the former had mostly been carried out in allogeneic and the latter in isogeneic host-donor combinations. Thus, although a reasonable amount of experimental data on fetal liver cell proliferation has been collected piece by piece, this does not furnish a clear understanding of the basis for the problems of fetal liver cell take.

The following factors are held to influence the take:

1. the number of stem cells grafted;
2. the proliferative capacities of these cells; and
3. the sensitivity of haemopoietic proliferation to allogeneic inhibition.

In order to more adequately explain the failures of fetal liver cells to take, it is

necessary to analyse each of these factors in direct relation to take. Investigations which were concerned with this subject are described in this chapter.

The first series (A) of experiments dealt with the transplantation of fetal liver cells into *syngeneic* recipients. The takeability of fetal liver cells was assessed in comparison to bone marrow grafts in survival studies. Major allowance was made for the number and role of the stem cells in the grafts in these studies. This was done by:

a. measurement of the number of HSC in the grafted cell suspension;
b. employment of purified stem cell suspensions which were free of most of the other fetal liver cells;
c. estimation of the self-renewal of HSC in the spleen and marrow;
d. measurement of the production of differentiated cells by fetal stem cells during the process of haemopoietic recovery following transplantation.

In the second series (B), the repopulating fetal liver and bone marrow grafts were investigated following injection into *allogeneic* recipients. The main problems to be investigated were: How is the growth of the HSC modified in the allogeneic host? Is the allogeneic effect on the proliferative response of bone marrow and fetal liver cells different and, if so, can this difference be explained and quantified in some way? Finally as an approach to enhancing fetal liver cell take, it was investigated whether practical use could be made of thymus cells to promote haemopoietic cell proliferation following grafting in non-isogeneic recipients [Goodman and Shinpock, 1968; Goodman and Grubbs, 1970].

4.1 *Part A: Transplantation of* syngeneic *fetal liver and bone marrow cells*

4.1.1 Survival experiments

In order to measure the takeability of fetal liver and bone marrow cells in syngeneic hosts, groups of lethally irradiated mice were transplanted with grafts containing graded numbers of CFU-s from bone marrow and fetal liver (unfractionated and purified). Repopulation efficiency was evaluated by taking the minimum number of CFU-s which permitted the recipients to survive for at least 30 days (i.e., the time interval covering the critical period of mortality from the irradiation-induced aplasia [Micklem and Loutit, 1966; Van Bekkum and De Vries, 1967]). The survival data are presented in table 4.1. The repopulation efficiency of the cells was expressed in terms of cell numbers required to obtain a level of at least 80% survival. This was done because, even with optimal reconstitution treatment, a small proportion of the animals are lost following supralethal irradiation exposures employed in studies of this kind [Van Bekkum and De Vries, 1967]. It is evident from table 4.1 that 120 CFU-s ($2–3 \times 10^6$ nucleated cells) from fetal liver and 240 CFU-s ($0.8–0.9 \times 10^6$ nucleated cells) from bone marrow led to such a survival rate. The figures correspond

Table 4.1 *Survival of lethally irradiated mice following transplantation of increasing numbers of fetal liver and bone marrow cells*
Syngeneic C57BL → C57BL

fetal liver						bone marrow					
number of cells transplanted				per cent survival		number of cells transplanted				per cent survival	
CFU-s	CFC*	total nucl. cells ($\times 10^6$)	nr. of mice	day 15	day 30	CFU-s	CFC*	total nucl. cells ($\times 10^6$)	nr. of mice	day 15	day 30
7	318	0.1–0.2	10	0	0	7	171	0.02	10	0	0
15	682	0.2–0.4	30	24	21	15	366	0.05	10	0	0
30	1364	0.5–0.7	30	54	46	30	732	0.1	24	0	0
60	2727	1–1.5	30	77	67	60	1463	0.2	20	10	5
120	5454	2–3	28	85	85	120	2926	0.4	25	52	52
240	10908	4–6	30	96	89	240	5854	0.8–1.0	40	85	82
480	22000	8–12	20	100	78	480	11708	1.6–1.9	20	100	88

* Calculated from CFU-s values by correction for spleen colony efficiency factor: 2.2% (fetal liver) and 4.1% (bone marrow) (see chapter 3).
Survival data represent the mean values of 2 separate experiments.

with roughly the same numbers of CFC from both types of grafts (i.e., 5454 fetal liver CFC* and 5854 bone marrow CFC*) and are indicative of equal repopulation potencies of the HSC from both sources. However, the administration of the equivalent number of fetal liver HSC required the infusion of 3 × more nucleated cells as compared to bone marrow. It was investigated as to whether, besides the HSC, these admixed cells (mostly erythroblasts) added to the protective effect of fetal liver. Therefore, the capacity of purified fetal liver HSC to prevent death from the lethal irradiation injury was explored. By comparison of these data (table 4.2) with those of unseparated fetal liver cell grafts (table 4.1, left part), it can be seen that the pattern of survival was independent of purification. The same number of 120 purified CFU-s, now present in only $0.3–0.4 \times 10^6$ nucleated cells, sufficed to provide 80% 30-day survival. Thus, the idea is favoured that the HSC are the cells which determine repopulation and that survival is largely independent of the more mature cells in the graft.

4.1.2 Proliferative capacities of fetal liver stem cells

The proliferative capacities of fetal liver stem cells were also assessed directly. The repopulation rate of CFU was estimated by determining the increase in CFU in the spleen and in the marrow. The curves for accumulation of fetal liver and bone marrow

* Calculated from CFU-s figures after correction for spleen colony efficiency values (according to procedure discussed in Chapter 3).

Table 4.2 *Survival of lethally irradiated mice following transplantation of increasing numbers of cells from purified fetal liver stem cell fractions*
Syngeneic C57BL → C57BL

number of cells transplanted					
CFU-s	CFC*	total nucl. cells ($\times 10^6$)	number of mice	per cent survival day 15	day 30
15	682	0.04	20	5	0
30	1364	0.09	29	31	28
60	2727	0.1–0.2	25	60	52
120	5454	0.3–0.4	30	90	83
240	10908	0.6–0.8	30	93	93
480	22000	1.2–1.6	18	90	83

* Calculated from CFU-s values by correction for spleen colony efficiency: 2.2% (according to data in chapter 3).
Survival results represent the mean values of 2 separate experiments.

CFU are depicted in figure 4.1. The slopes for recovered CFU differed significantly ($P < 0.02$). The mean population doubling time (T_D) of fetal liver CFU was 26 h ($\pm$S.D.: 25 and 27 h), whereas that of bone marrow CFU was 32 h ($\pm$S.D.: 30 and 35 h). The shorter doubling time for the fetal-liver-derived CFU population apparently compensated for the lower fraction, f, of such cells that doubles in the spleen; therefore, in spite of the lower spleen colony forming efficiency of fetal liver CFU, the total number of CFU in the spleen by day 7 was approximately the same as that following infusion of an equal number of bone marrow CFU (fig. 4.1).

It was pointed out in chapter 3 that the slope of increase in CFU as obtained by CFU-s estimations in secondary recipients can be regarded as a true index of proliferation only if the spleen colony forming efficiency during that time is constant. With fetal liver CFU, in particular, it has to be excluded that the embryonic CFU undergo a gradual rise in the f-value to higher, i.e., more adult marrow-like levels following seeding in the sites of the adult organism. A limited number of experiments served to sort out whether the greater accumulation of fetal liver CFU in the spleen as compared to bone marrow CFU should be really regarded as the reflection of more vigorous self-reproduction or instead of increasing spleen colony forming efficiency in the secondary hosts. CFU in fresh fetal liver cell suspensions and CFU derived from the same suspensions after proliferation in irradiated hosts for 7 days were compared for their spleen colony forming efficiency. For practical reasons, the percentage of recoverable CFU in the spleen at 24 h following injection, which closely approximates the real efficiency value, was estimated instead of the entire curve of CFU recovery. From table 4.3, it is evident that the 24 h recovery values for the fetal liver cell suspensions had not changed significantly. Consequently, it can be concluded

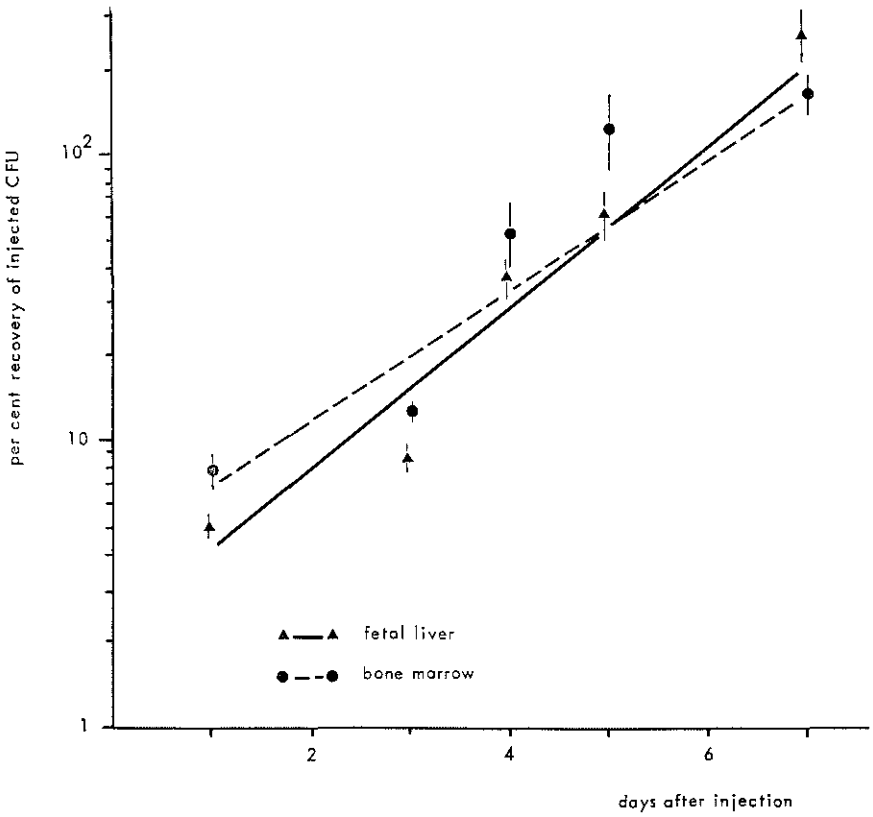

Fig. 4.1 *Recovery of bone marrow and fetal liver CFU-s in spleen following the injection into irradiated syngeneic recipients.*
Recovery is expressed as a percentage of the number of CFU-s injected at time zero. Points represent means (±S.E.) of 6–12 separate experiments.

Table 4.3 *Recovery of fetal liver CFU-s in the spleen at 24 h following injection*
Comparison between primary and secondary cell suspensions

primary suspension % recovery (24 h)	secondary suspension % recovery (24 h)
5.6	6.8
5.7	6.3
5.0	4.0
3.6	3.0
mean 5.0±0.5 (S.E.)	mean 5.0±0.9 (S.E.)

Primary cell suspension: freshly prepared.
Secondary cell suspension: cells recovered from the spleen of lethally irradiated recipients at 7 days after transplantation.

with some confidence that the more rapid increase in fetal liver CFU in the spleen is a true manifestation of a more rapid repopulation by the embryonic CFU.

A limited number of experiments were directed toward measuring recoverable CFU populations in the femur shafts. It was found that the characteristic differences in fetal liver and bone marrow CFU proliferation as established in the spleen also applied to the proliferative events in the marrow (fig. 4.2). Firstly, the embryonic CFU population showed a higher capacity for CFU repopulation of the marrow than did bone marrow CFU. The mean population doubling time of fetal-liver-derived CFU of 29 h (±S.D.: 25 and 33 h) was shorter than that of bone marrow CFU ($T_D = 36$ h; ±S.D.: 32 and 41 h). Secondly, as in the spleen, the fraction of injected fetal liver CFU to lodge in the femur and give rise to daughter CFU was lower than that of bone marrow CFU. The difference between the two slopes was not

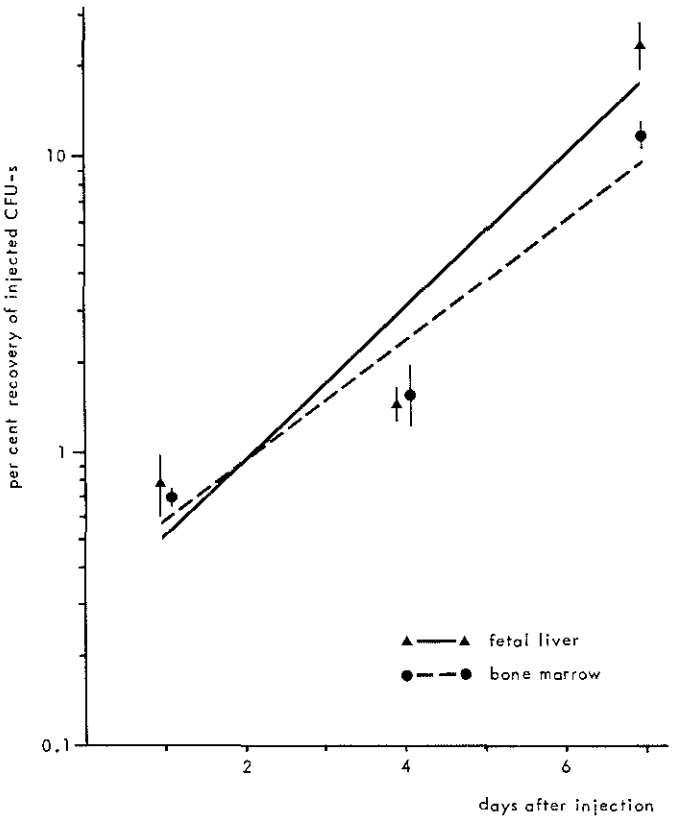

Fig. 4.2 *Recovery of bone marrow and fetal liver CFU-s in femur following injection into irradiated syngeneic recipients.*
Recovery (in one femur shaft) is expressed as a percentage of the number of CFU-s injected at time zero. Points represent means (±S.E.) of 3–4 separate experiments.

statistically significant but it is suggested that, as was the case in the spleen experiments, statistical significance would be acquired if more experiments were performed.

4.1.3 Regeneration of peripheral blood cells

The release of functional end cells into the blood stream is the ultimate step in a series of repopulation events which determines posttransplantation survival by preventing the lethal effects of the thrombocytopenia and the leukopenia of the bone marrow syndrome [Van Bekkum and De Vries, 1967]. In order to compare the capacities of fetal liver and bone marrow HSC in generating these mature cellular elements, lethally irradiated mice which were transplanted with bone marrow or

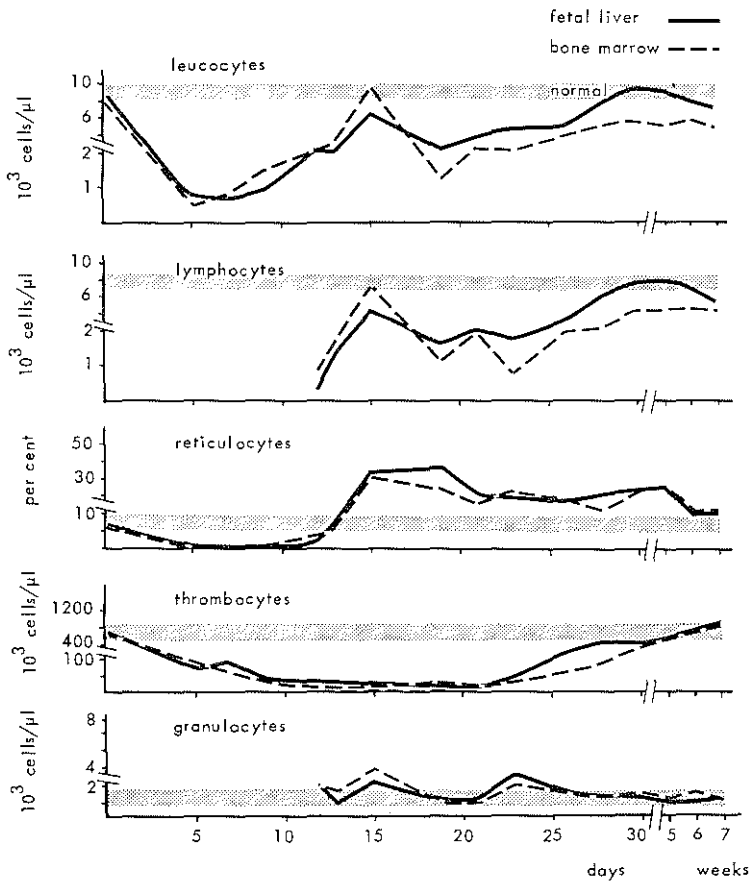

Fig. 4.3 *Blood cell counts of lethally irradiated mice after fetal liver cell and bone marrow transplantation (syngeneic).*

Each estimate is the mean of blood values of 3 animals taken from a group of 20 recipients. On the ordinate the cell counts are plotted, on the abscissa the time (days, weeks) after transplantation.

fetal liver cell grafts providing extended survival (85% and 89%, respectively) were assayed for peripheral blood values throughout the first month. The curves of the blood cell counts are depicted in figure 4.3. The appearance and rise of the mature circulating cells of the varying cell lines after fetal liver cell transplantation occurred parallel to those after bone marrow transplantation. The fact that both grafts were approximately equally effective in restoring the low peripheral cell counts in the blood correlates well with the achievement of the same percentage of survival.

4.1.4 Regeneration of lymphoid cells

Because of the importance of lymphocytic regeneration for survival, and in view of the absence of lymphopoiesis in the fetal liver tissue (chapter 3), information on the extent of lymphoid repopulation after fetal liver cell transplantation is required. Therefore, the responsiveness of recipient spleen cells to stimulation by Con A and LPS was assayed at monthly intervals following the transplantation of 480 bone marrow CFU-s and 240 fetal liver CFU-s (fig. 4.4 and 4.5). These *in vitro* reactions

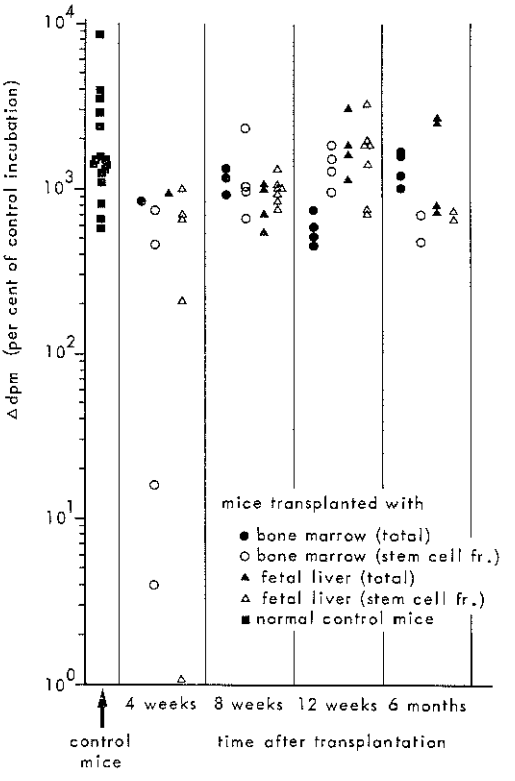

Fig. 4.4 *In vitro LPS responsiveness of spleen cells at various intervals after transplantation (syngeneic).*

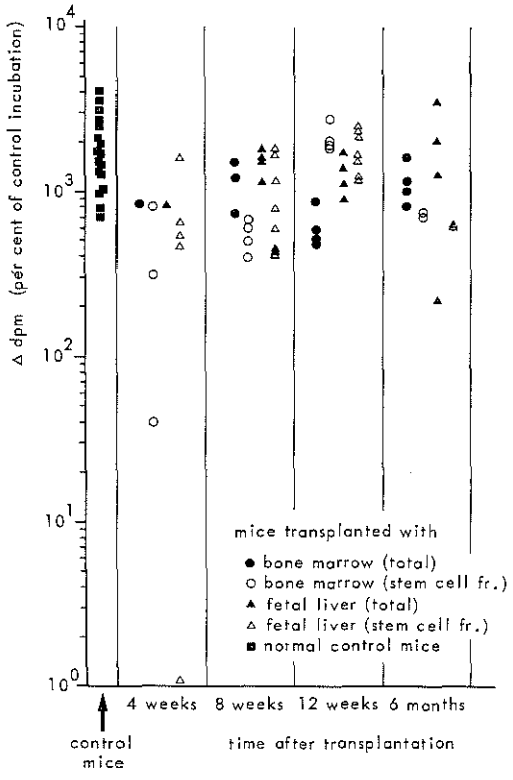

Fig. 4.5 *In vitro Con A responsiveness of spleen cells at various intervals after transplantation (syngeneic).*

were regarded as parameters for the appearance of T and B cells. The reactivity of the spleen cells of some of the animals to the mitogens was still subnormal at one month postgrafting; however, at the second month, all animals which were tested showed normal responses. Sequential analysis for up to 6 months revealed that reconstitution was stable in both groups. From this, it was concluded, that fetal liver and bone marrow cell suspensions are roughly equally capable of conferring lymphocyte reactivity (*in vitro*) on lethally irradiated animals.

4.1.5 Discussion

In experiments based on the restoration of haemopoietic function in lethally irradiated syngeneic mice (bone marrow syndrome), it was revealed that 2–4 times more fetal liver cells ($2–3 \times 10^6$ cells) than bone marrow cells ($0.8–0.9 \times 10^6$ cells) were needed to allow the animals to recover from the effects of radiation. The survival after transplantation with purified stem cell fractions demonstrated that the beneficial effect on 30-day survival could not be attributed to the transfusion of more mature

haemopoietic cells. This supported the concept that HSC represent the principal constituent of the graft which are responsible for reconstitution and it justified the approach to investigate fetal liver cell takeability mainly at the level of HSC. It was found that both types of haemopoietic cell suspensions required approximately equal numbers of HSC for irradiation protection. In other words, the difference in effective cell numbers between fetal liver cell and bone marrow transplants did not reflect a different repopulation proficiency of the two cell populations, but rather the lower frequency of HSC in fetal liver. The equivalent repopulation efficiency of fetal and adult HSC was compatible with their proliferative behaviour:

a. the f-value for i.v. administered fetal CFU in the spleen was approximately 50% lower than that for bone marrow CFU;
b. the more rapid production of fetal-liver-derived CFU in the spleen accounted for the fact that one more fetal liver CFU population doubling had occurred by day 7.

The result of these two processes was that, after about one week, the total number of fetal liver and bone marrow derived HSC in the haemopoietic tissue was the same, if an equal number of HSC from both sources had been grafted at day 0. These findings should be considered in view of the fact that protection against the irradiation-induced aplasia due to the haemopoietic cell grafts was almost completely achieved in the first 14 days following irradiation and that early haemopoietic proliferation determined graft success (survival).

Similarly, a lower seeding efficiency coupled with a shorter doubling time of fetal liver CFU was observed in the femoral sites. The relative importance of CFU proliferation in spleen and marrow for the haemopoietic repopulation as a whole is difficult to judge.

As regards the contribution of haemopoiesis in spleen and marrow, respectively, the following calculation can be made. From the injected number of 5454 fetal liver CFC or 5854 bone marrow CFC in the grafts (sufficient for survival), 120 CFU and 240 CFU, respectively, started to proliferate exponentially in the spleen. Accounting for their doubling times, they produced a total quantity of 10,860 fetal liver CFU (6.4 doublings) and 8,820 bone marrow CFU (5.2 doublings) by day 7. If the proportion of injected fetal CFU and bone marrow CFU that reproduced in the marrow is represented by the extrapolation numbers of the CFU recovery curve, i.e., 0.28% and 0.37%, respectively, if it is further assumed that one femur represents 1/60* of the total marrow haemopoietic tissue of the organism and, finally, if the femoral marrow is accepted as being representative also for the other marrow sites, it follows that, out of 5454 injected fetal liver CFC or 5854 bone marrow CFC, 912 fetal and

* According to Van Bekkum (1969), the total marrow content of an adult mouse can be taken as 6×10^8 cells. If we consider that one femur shaft contains in the order of 10×10^6 cells, it represents 1/60 of the total marrow cellularity of the animal. Others have calculated the size of the bone marrow cell population in the mouse at $3–5 \times 10^8$ cells [Lajtha, 1965] and 8×10^8 cells [Pegg, 1962].

1,260 bone marrow CFC initiated proliferation in the marrow. Considering their respective population doubling times (in the femur) of 29 h and 36 h, these cells gave rise to 50,760 (5.8 doublings) fetal-liver-derived CFU and 32,760 (4.7 doublings) bone-marrow-derived CFU during 7 days of growth. If we add this to the number of CFU generated in the spleen, the total yields of CFU from the fetal liver and bone marrow grafts were $10{,}860 + 50{,}760 = 61{,}620$ CFU and $8{,}820 + 32{,}760 = 41{,}580$ CFU, respectively.

Thus, the more rapid doubling rate of fetal liver CFU did not just counterbalance the lower fraction, f, of injected CFU that was engaged in regeneration; it even over-compensated for the lower f-value when compared with bone-marrow-derived repopulation. The difference in production of CFU in the organism by a factor of 1.5 between both grafts seems too small to be detected in a transplantation study based on titration of cell numbers. Therefore these numbers of CFU could well agree with the observed equivalent protective efficiency. However, if one accepts the experimental evidence in the literature [Vogel et al., 1970; Schofield, 1970] which indicates that the exponential increase in CFU continues for another 3 days, the total yield at day 10 becomes 415×10^3 fetal-liver-derived CFU and 186×10^3 bone-marrow-derived CFU. This difference in production of CFU is too large to disregard, in particular in view of the fact that the more efficient generation of new CFU by fetal liver cells does not lead to better survival; or, stated otherwise, why do fetal liver HSC, though having an equivalent efficiency in terms of affording survival, require a larger net production of HSC in the host? The most logical explanation is that fetal liver cell repopulation involves at the same time a proportionally smaller propensity for cell differentiation or a greater tendency for differentiating cells to die. Such an interpretation would be in good agreement with our finding that, in spite of the more rapid repopulation of the haemopoietic tissues with fetal-liver-derived CFU, the rise in peripheral blood cells was virtually similar following fetal liver cell and bone marrow transplantation. This could also fit in with a general concept that fetal cells have a higher propensity for proliferation and a smaller tendency towards differentiation than do adult cells. Others have also suggested that an increased self-replication capacity of early embryonic CFU is related to impaired differentiation [Metcalf and Moore, 1971b]. As long as we have no methods to separately measure replication, differentiation, and death of cells on the basis of HSC injected, one cannot compare the relative involvement of embryonic and adult HSC in any of these processes and verify such a concept.

Evidence was obtained for a nearly identical time pattern of lymphoid reconstitution after fetal liver cell and bone marrow transplantation. Because of the poor lymphopoiesis in the fetal liver (chapter 3), it is indicated that the lymphocytes which appeared were products of differentiation from stem cells. This is further evidence for equivalent repopulation capacities for both stem cell populations.

The more rapidly ascending growth curves for fetal CFU as compared to bone

marrow CFU were observed in the spleen as well as in the femoral marrow. This is an indication of different intrinsic growth characteristics of CFU specific to the source of the cells. In the spleen, such differences have previously been noticed by Schofield [1970]. The fetal liver CFU population generation time of 16 h assessed by this investigator was markedly shorter than our value of 26 h. Although no definite explanation can be given, it is possible that differences in animal strains employed or in the embryonic age of the fetal liver donors account for this dissimilarity. Other workers have established that T_D values of fetal liver CFU markedly vary (between 18–30 h), depending on embryonic maturation [Vogel et al., 1970].

In chapter 3, the low spleen colony forming efficiency of fetal liver CFU was extensively discussed. It has been shown in this chapter that this low figure was probably not due to a different distribution of fetal CFU, in the sense that more CFU settled in the marrow, and, accordingly, fewer fetal liver CFU reached the spleen. From the results set out in this chapter, it was suggested that the colony forming efficiency of fetal liver CFU was also lower in the marrow of the femur shaft than that of bone marrow CFU. Thus, one is left with the question as to why the efficiency of colony formation by fetal liver CFU is consistently lower. The most likely possibility is that fetal liver CFU encounter greater difficulties in adjusting to the new environment and that a higher proportion of injected fetal liver CFU is "inactivated" after the abrupt transfer from the embryonic to the adult environment. If this is so, the two explanations formerly offered by Crouch [1959] for the higher number of fetal liver cells needed for transplantation, i.e., the low number of proliferating cells in fetal liver and the difficulties associated with the change to a different microenvironment, would both be valid.

4.2 *Part B: Transplantation of* allogeneic *fetal liver and bone marrow cells*

4.2.1 Survival experiments

Experiments very similar to those described in part A, but in allogeneic recipients, were carried out. In table 4.4, the survival data are compiled for groups of CBA mice receiving graded numbers of CFU-s* of fetal liver and bone marrow cell suspensions obtained from C57BL donor mice. It appeared that the numbers of fetal liver and bone marrow CFU-s which were necessary for at least 80% survival were the same for both cell suspensions. However, the cell inocula from the two sources contained quite different numbers of HSC (i.e., CFC) and of nucleated cells.

The number of nucleated cells in the transplants was $4–5 \times 10^6$ bone marrow cells or $20–30 \times 10^6$ fetal liver cells per recipient. In order to determine whether or not the

* The number of CFU-s was determined as usual by injection of haemopoietic cells into lethally irradiated *syngeneic* mice.

Table 4.4 *Survival of lethally irradiated mice following transplantation of increasing numbers of fetal liver and bone marrow cells*
Allogeneic C57BL → CBA

fetal liver						bone marrow					
number of cells transplanted				per cent survival		number of cells transplanted				per cent survival	
CFU-s	CFC*	total nucl. cells ($\times 10^6$)	nr. of mice	day 15	day 30	CFU-s	CFC*	total nucl. cells ($\times 10^6$)	nr. of mice	day 15	day 30
15	682	0.2–0.4	10	20	0	15	366	0.05	10	0	0
30	1364	0.5–0.7	26	54	23	30	732	0.1	20	35	0
60	2727	1.0–1.5	30	67	3	60	1463	0.2	35	26	0
120	5454	2 –3	30	87	60	120	2926	0.4	30	77	10
240	10908	4 –6	30	87	60	240	5854	0.8–1.0	30	77	37
580	26364	10 –14.5	30	100	78	580	14146	1.9–2.4	32	87	69
1200	55000	20 –30	41	98	80	1200	29300	4–5	39	99	99
2000	86000	35 –50	72	94	92	2000	46300	6–8	47	98	94

* Calculated from CFU-s values by correction for spleen colony efficiency: 2.2% (fetal liver) and 4.1% (bone marrow) (according to data in chapter 3).
Survival data represent the mean values of 2 separate experiments.

protective effect of the fetal liver cell transplant was substantially dependent on the much higher number of more differentiated nucleated cells (6 × that of bone marrow),

Table 4.5 *Survival of lethally irradiated mice following transplantation of increasing numbers of cells from purified fetal liver stem cell fractions*
Allogeneic C57BL → CBA

number of cells transplanted					
CFU-s	CFC*	total nucl. cells ($\times 10^6$)	number of mice	per cent survival day 15	day 30
15	682	0.04	20	25	0
30	1364	0.09	38	79	24
60	2727	0.1–0.2	40	88	35
120	5454	0.3–0.4	40	88	40
240	10908	0.6–0.8	29	86	62
580	26364	1.4–1.8	40	98	77
1200	55000	3–4	41	100	95
2000	86000	5–7	38	100	95

* Calculated from CFU-s values by correction for spleen colony efficiency: 2.2% (according to data in chapter 3).
Survival results represent the mean values of 2 separate experiments.

the efficiency of purified stem cell grafts was also investigated. From these data, it became evident that fetal liver stem cell fractions exhibited about the same repopulation potential as did the unseparated fetal liver cell suspensions (compare table 4.5 with table 4.4, left section). The number of CFU-s (1200) and CFC (55,000) in the fetal liver stem cell concentrate which produced 80% survivors was the same as for the unfractionated embryonic liver cell transplant, although the absolute cell number had been reduced to as low as $3–4 \times 10^6$ cells. This finding reinforces the notion that HSC represent the cell population largely responsible for graft success, also in allogeneic recipients.

The number of HSC in the fetal liver cell graft (55,000 CFC) which was calculated from the CFU-s figures by correction for the spleen colony forming efficiency (chapter 3) was nearly double that of CFC present in the bone marrow transplant (29,270 CFC) (table 4.4). These findings show that, whereas fetal liver and bone

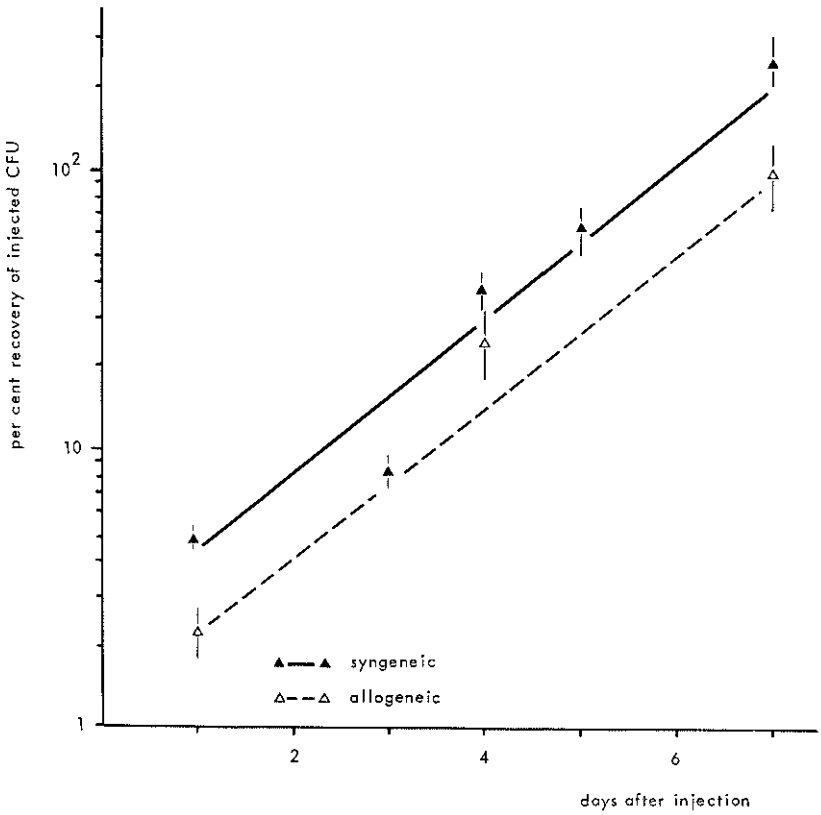

Fig. 4.6 *Recovery of fetal liver CFU-s in spleen following injection into irradiated syngeneic and allogeneic recipients.*
Recovery is expressed as a percentage of the number of CFU-s injected at time zero. Points represent means (±S.E.) of 4–12 separate experiments.

marrow HSC were found to be equally capable of repopulating a lethally irradiated animal in syngeneic transplantation, fetal liver HSC were approximately half as effective in the allogeneic situation.

4.2.2 Proliferation of fetal liver stem cells following allotransplantation

Experiments were conducted in order to discover the basis for the stronger allogeneic barrier inhibiting repopulation by HSC from fetal liver. Grafts of bone marrow and fetal liver cells were injected into lethally irradiated mice and the variation in the recoverable fraction of injected CFU in the spleen with time after transplantation was determined.* The growth curve of the embryonic CFU population in the allo-

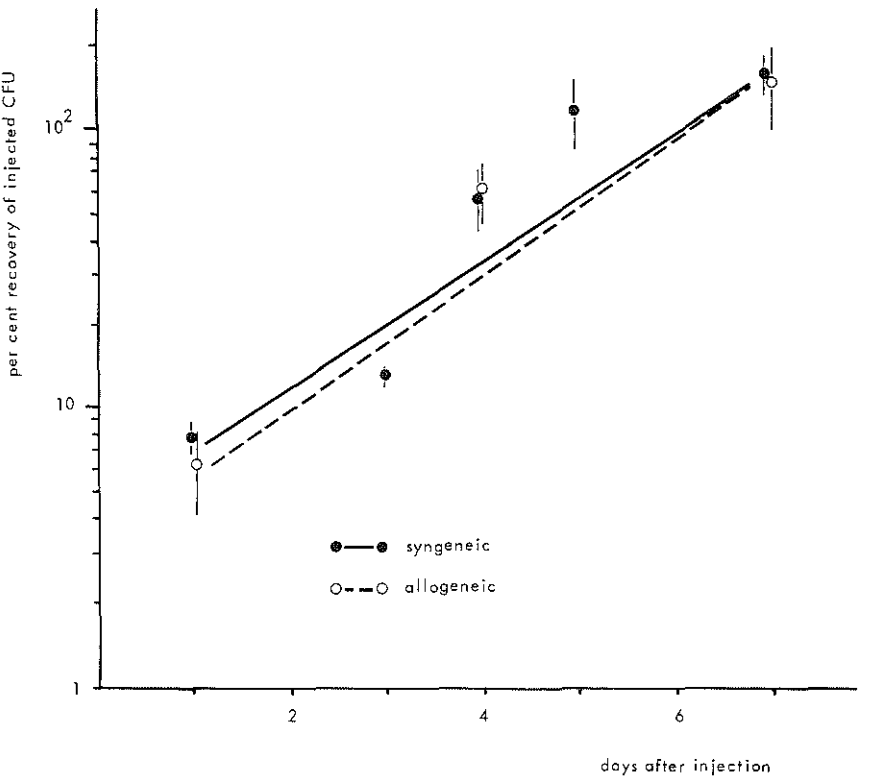

Fig. 4.7 *Recovery of bone marrow CFU-s in spleen following injection into irradiated syngeneic and allogeneic recipients.*

Recovery is expressed as a percentage of the number of CFU-s injected at time zero. Points represent means (±S.E.) of 4–12 separate experiments.

* This was done by injecting the cells collected from both syngeneic and allogeneic chimaeras into *syngeneic* lethally irradiated mice for regular CFU-s assay.

geneic spleen (plotted in fig. 4.6) shows that the production of fetal liver CFU in the allogeneic animal is delayed in comparison to its syngeneic counterpart. The delay in multiplication of fetal liver CFU corresponds with a reduction by a factor of 0.5 in the f-value in the allogeneic spleen (stat. sign. of diff. $P < 0.001$). By contrast, the rate of doubling of bone marrow CFU following injection into the allogeneic recipient appeared nearly identical to that in the syngeneic spleen (fig. 4.7). It is remarkable that the respective slopes of CFU regeneration in allogeneic were similar to those in syngeneic spleens; mean population doubling times of fetal liver CFU of 26 h (±S.D.: 24 and 30 h) and of bone marrow CFU 30 h (±S.D.: 25 and 37 h) were nearly equal to those reported in part A. The fact that the f-factor for bone marrow CFU was not reduced, while that for fetal liver CFU was decreased by a factor of 0.5 in allo-

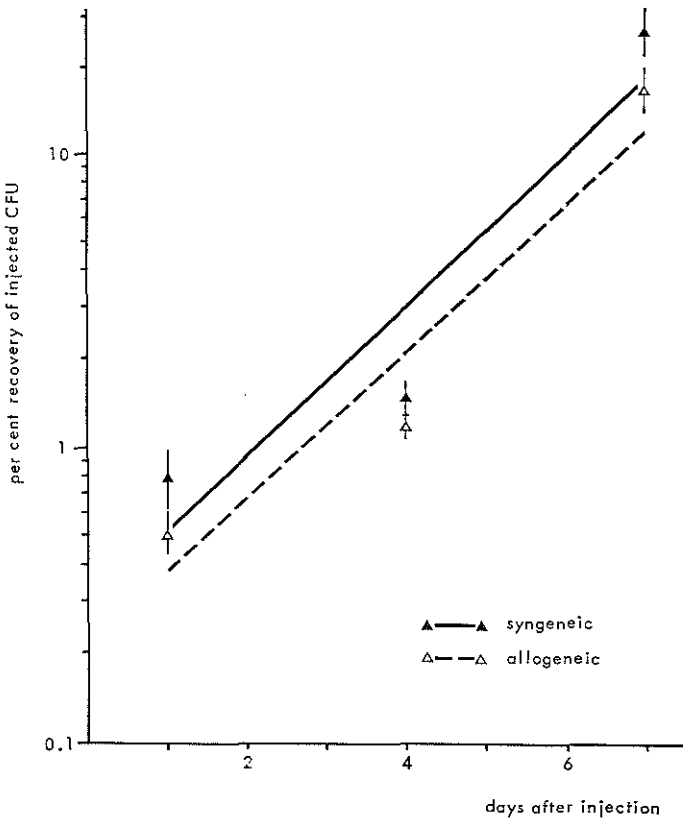

Fig. 4.8 *Recovery of fetal liver CFU-s in femur following injection into irradiated syngeneic and allogeneic recipients.*
Recovery (in one femur shaft) is expressed as a percentage of the number of CFU-s injected at time zero. Points represent means (±S.E.) of 3–4 separate experiments.

geneic situations may offer a satisfactory explanation for the 2-fold difference in fetal liver and bone marrow cell numbers needed for allogeneic repopulation.

Repopulation of the femoral marrow exhibited essentially the same aspects of CFU proliferation as seen in the spleen (fig. 4.8 and 4.9):

a. the rate of proliferation (slope) of the CFU populations of fetal and adult origin did not differ from those in the syngeneic femur;
b. the fraction, f, of fetal liver CFU was reduced in the allogeneic femur to a larger degree than *f* for bone marrow CFU, as compared to the comparative syngeneic f-values.

These data seem to provide insight into the causes for the difference between the repopulation capacity of fetal liver cell and bone marrow grafts in allogeneic irradiated recipients. However, they do not provide an explanation for the large difference in cell numbers needed for syngeneic versus allogeneic repopulation by the two types of

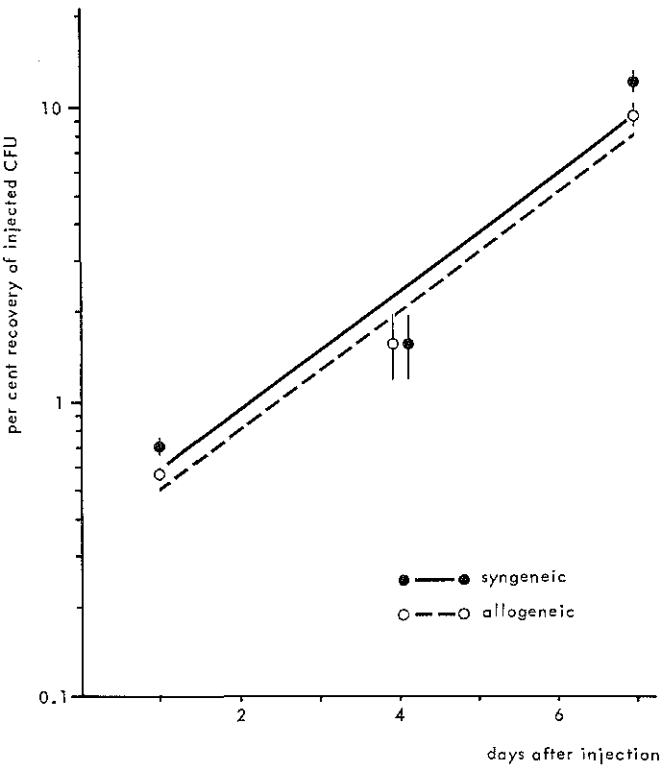

Fig. 4.9 *Recovery of bone marrow CFU-s in femur following injection into irradiated syngeneic and allogeneic recipients.*
Recovery (in one femur shaft) is expressed as a percentage of the number of CFU-s injected at time zero. Points represent means (±S.E.) of 3 separate experiments.

grafts. Why does allogeneic haemopoietic cell transplantation require 5 × more bone marrow cells and 10 × more fetal liver cells than does syngeneic grafting? This is a pertinent question in that CFU repopulation at any time interval after allotransplantation was identical to that after syngeneic transplantation when marrow grafts were used, while they differed by a factor of 2 for fetal liver cell grafts as a result of a lower f-value. Therefore the possibility must be considered that inhibition of the differentiation capacity of HSC was largely responsible for the iso-allo difference in cell numbers. Experiments were designed to ascertain whether the iso-allo difference in cell numbers could be associated with reduced cellular differentiation. This was done by performing spleen colony assays in allogeneic hosts. Spleen colony formation is essentially understood to be the product of replication of HSC and the subsequent production of differentiated progeny; it is the size of the latter population which determines whether a colony becomes visible or not. Since the number of HSC which reaches the spleen to start proliferation as well as the doubling rate of this population have been determined, the degree of suppression of colony numbers in allogeneic hosts which is due to an impaired production of the more mature progeny can be derived from these data and the difference in colony counts. Direct comparisons were made with colony number estimations in syngeneic animals and allogeneic inhibition was expressed as an iso-allo ratio of spleen colony forming ability

$$\left(= \frac{\text{number of macroscopic spleen colonies in allogeneic host}}{\text{number of macroscopic spleen colonies in syngeneic host}} \times 100\%\right).$$

From the results (table 4.6), it appeared that spleen colony formation by bone marrow CFU in allogeneic recipients was suppressed to 60% of the syngeneic values. Because in allogeneic hosts repopulation of bone marrow CFU has been shown not to be decreased, this 0.6 × depression may be entirely attributed to less differentiation in the colonies. Depression of the spleen colony forming ability of fetal liver cells in non-isogeneic hosts was much more pronounced; i.e., to about 30% of syngeneic figures. If one allows for the 50% reduced f-factor of fetal liver CFU in the allogeneic

Table 4.6 *Spleen colony formation in isogeneic and allogeneic recipients*

	number of colonies per 10^5 cells injected		
type of graft	isogeneic C57BL→C57BL	allogeneic C57BL→CBA	$\frac{\text{allogeneic}}{\text{isogeneic}}$ (%)*
bone marrow	28.3±3.4 (10)	17.4±1.9	63.7±6.4
fetal liver	8.2±0.6 (12)	2.5±0.2	33.6±3.4

* Repression ratio in per cent
Mean ±S.E.
The figures in parentheses represent the number of experiments.

spleen, one may calculate that a percentage of 60 of the remaining CFU numbers is suppressed in terms of differentiation in the colonies.

Because the supply of functionally mature peripheral blood cells is a very close parameter for efficient repopulation following transplantation, separate experiments were directed toward estimating the rate of recovery of peripheral blood cell counts. The results indicated that, after the transplantation of marginal numbers of haemopoietic cells, the increase in cells in the peripheral blood was delayed to the disadvantage of the allogeneic grafts (fig. 4.10 and 4.11). In particular, recoveries of the erythroid and the lymphoid cell lines occurred more slowly in the allogeneic recipient; patterns of recovery of platelets were the same in the isogeneic and allogeneic animals

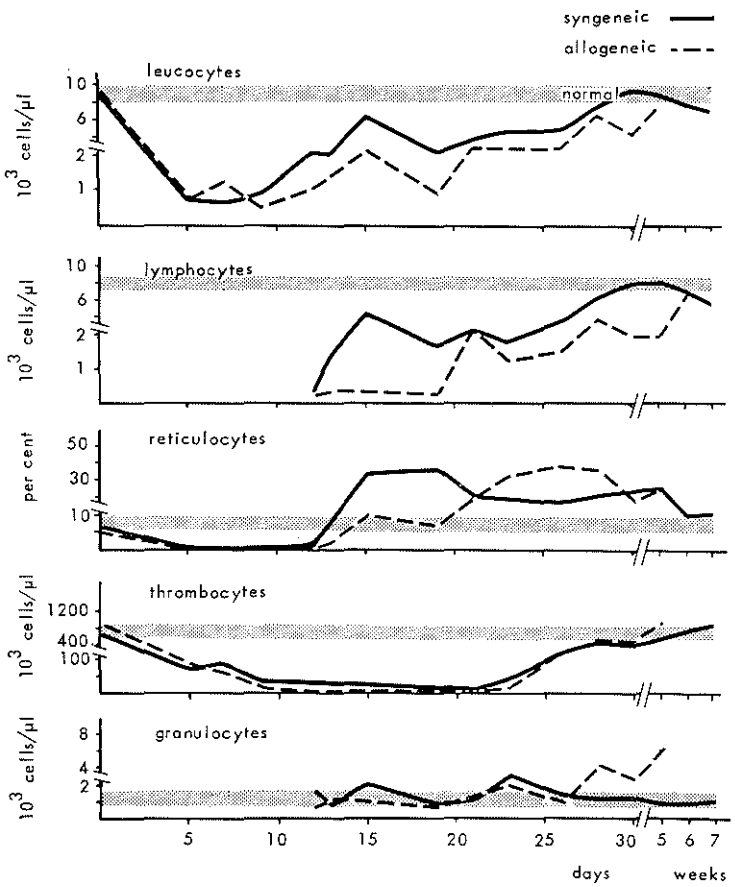

Fig. 4.10 *Blood cell counts of lethally irradiated mice after transplantation of syngeneic and allogeneic* fetal liver *cells.*

Each point is the mean of blood values of 3 animals taken from a group of 20 recipients. On the ordinate the cell counts are plotted, on the abscissa the time (days, weeks) after transplantation.

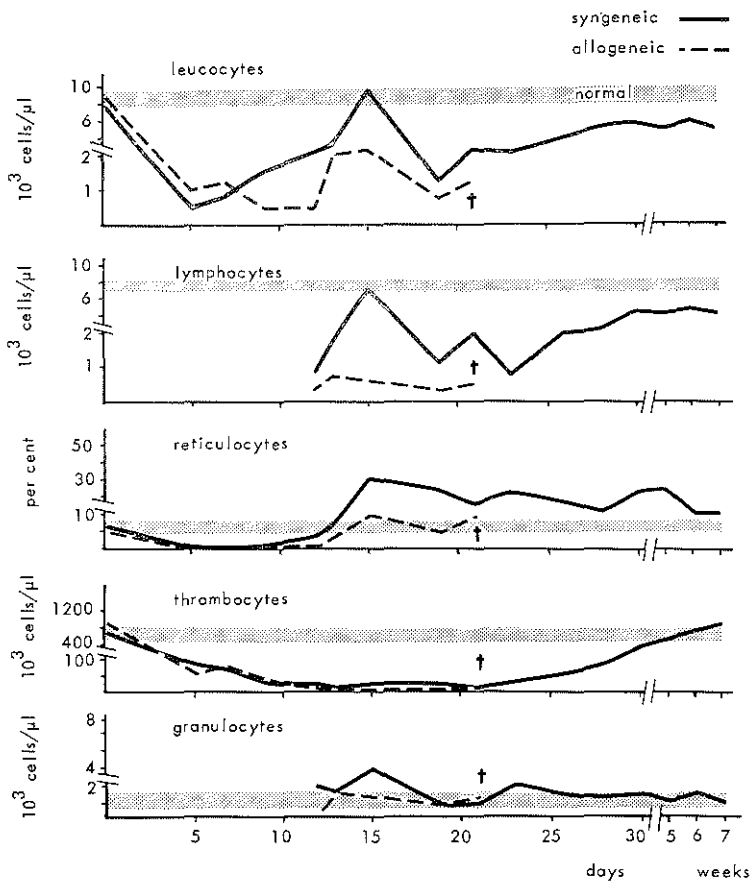

Fig. 4.11 *Blood cell counts of lethally irradiated mice after transplantation of syngeneic and allogeneic* bone marrow *cells.*
Each point is the mean of blood values of 3 animals taken from a group of 20 recipients. On the ordinate the cell counts are plotted, on the abscissa the time (days, weeks) after transplantation. In the group of allogeneic recipients there were no survivors beyond day 21.

at the transplanted cell dosages used. However, these data on repression of differentiation do not seem to entirely explain the 5-fold difference in cell numbers required for isogeneic and allogeneic repopulation.

4.2.3 Nature of allogeneic repression

Some experiments were devoted to clarifying the delayed haemopoietic cell repopulation in the allografts. Attention was paid to distinguishing between the two main groups of causative factors which are believed to influence non-isogeneic grafting: immunological and environmental (hybrid resistance). Because hybrid resistance is

the most outstanding example of the latter category, it was investigated whether the features of allogeneic suppression resembled the hybrid resistance phenomenon. Therefore, fetal liver and bone marrow CFU (parental) were grafted into lethally irradiated F_1 hybrid mice and assayed for colony forming capacity in the spleen. It was found that hybrid resistance acted quite strongly and suppressed colony numbers to 17% (bone marrow) and 5% (fetal liver) of the isogeneic values (table 4.7). It has been reported in the literature that hybrid resistance, at least against bone marrow cells, can be overcome by adding large numbers of thymocytes from adult animals

Table 4.7 *Spleen colony formation in isogeneic and hybrid recipients*

	number of colonies per 10^5 cells injected		
type of graft	isogeneic C57BL→C57BL	hybrid C57BL→F_1 (C57BL×CBA)	$\frac{\text{hybrid}}{\text{isogeneic}}$ (%)*
bone marrow	21.0±1.6 (8)	3.5±0.3	17.2±1.9
fetal liver	4.6±0.7 (5)	0.3±0.1	5.1±1.4

* Repression ratio in per cent.
Mean ±S.E.
The figures in parentheses represent the number of experiments.

(isogeneic to the haemopoietic cell graft) to the transplant [Goodman and Wheeler, 1968; Goodman and Grubbs, 1970]. An attempt was made to find out whether such abrogation could also be obtained in the case of hybrid resistance against fetal liver cells. These experiments revealed that both fetal liver and bone marrow CFU were susceptible to the effect of simultaneously injected thymic cells (tables 4.8 and 4.9). P→F_1 repression could be entirely abolished when the thymus cell inoculum contained in the order of 100×10^6 cells. Following this, the allogeneic variant of CFU suppression was tested for its sensitivity to be abrogated by injected thymocytes. It was found that the allogeneic inhibition could be overcome similarly to that in P→F_1 transplantation (tables 4.10 and 4.11). However, the allogeneic repression was less pronounced than hybrid suppression and abolition of resistance could be achieved by lower numbers of thymocytes – approximately 30×10^6 cells. The fact that allogeneic inhibition was totally abolished by the action of the thymocytes may be regarded as an indication that allogeneic inhibition and hybrid resistance had the same basis.

It is noteworthy that modification of growth in the allogeneic host due to the addition of thymus cells not only led to an increase in colony numbers up to the syngeneic level, but even to an overshoot of colony counts when compared to syngeneic values. In the case of fetal liver CFU, this resulted in a near doubling of isogeneic numbers. The increase over syngeneic values suggests that, also in a syngeneic recipient, spleen

Table 4.8 *Effect of* adult *thymocytes on spleen colony formation by fetal liver cells in* hybrid *hosts C57BL→F$_1$(C57BL×CBA)*

nr. of thym. injected ($\times 10^8$)	number of CFU-s per 10^5 fetal liver cells exp. 1	exp. 2	exp. 3	exp. 4	exp. 5	% of syngeneic colony numbers mean ±S.E.
0	0.5	0.1	0.2	0.2	0.2	5±1
0.5	0.4	0.1	0.2	0.3	–	5±1
5	0.6	0.1	0.2	0.2	–	6±1
10	1.0	0.2	0.3	0.2	0.7	10±3
30	1.9	0.5	0.9	1.0	–	22±4
100	–	3.6	3.7	4.1	–	110±26
control fetal liver C57BL→C57BL	5.4	2.3	3.6	6	5.8	100

100×10^6 thymocytes: 0.6±0.2 CFU-s.

colony formation does not reach maximal expression without supplementation with thymocytes. This hypothesis was tested by adding thymocytes to the cells in a syngeneic spleen colony set-up. Indeed, the results showed that, when the grafts were supplemented with thymus cells, the CFU values increased above normal values (table 4.12). Most striking was the elevation of colony numbers of syngeneic fetal liver cells which showed a nearly 2-fold increase over the controls. One could argue whether the increase in colonies in the presence of thymocytes was really fetal-liver-derived and not of thymus cell origin. The latter possibility was unlikely because numbers of 100×10^6 thymocytes produced only 0.6 spleen colonies per recipient on

Table 4.9 *Effect of* adult *thymocytes on spleen colony formation by bone marrow cells in* hybrid *hosts C57BL→F$_1$(C57BL×CBA)*

nr. of thym. injected ($\times 10^6$)	number of CFU-s per 10^5 bone marrow cells exp. 1	exp. 2	exp. 3	exp. 4	exp. 5	% of syngeneic colony numbers mean ±S.E.
0	3.0	4.7	4.5	2.4	4.0	17±2
0.5	3.5	6.7	7.8	–	–	27±5
5	3.3	7.8	9.3	–	–	31±8
10	8.4	9.6	14.7	–	–	49±5
30	9.8	12.4	16.6	9.7	–	58±6
100	24.6	28.7	35.4	–	27.6	147±15
control bone marrow C57BL→C57BL	21.5	19.6	25.6	15.8	14.7	100

100×10^6 thymocytes: 0.6±0.2 CFU-s.

Table 4.10 *Effect of* adult *thymocytes on spleen colony formation by fetal liver cells in* allogeneic *hosts*
C57BL→CBA

nr. of thym. injected ($\times 10^6$)	number of CFU-s per 10^5 fetal liver cells exp. 1	exp. 2	exp. 3	exp. 4	exp. 5	% of syngeneic colony numbers mean ±S.E.
0	2.6	1.6	2.8	2.8	2.9	35±4
0.5	2.0	1.0	–	–	–	21±9
5	1.9	2.6	–	4.5	3.5	42±7
10	4.8	2.5	8.3	2.6	2.3	42±10
30	18.6	13.8	13.0	–	7.8	187±36
control fetal liver C57BL→C57BL	6.6	8	6.9	8	7.3	100

100×10^6 thymocytes: 0.6±0.2 CFU-s.

the average. Furthermore, this possibility was minimized by the results of experiments listed in table 4.13, from which it became evident that: a. colony formation did not occur when the fetal liver cells had been exposed to a high dose of irradiation *in vitro* (2000 rad γ) prior to the injection; this is argument in favor of the idea that the colony forming cells had a fetal liver origin and against the possibility that the synergistic activity had a reverse direction, i.e., the fetal liver cells unmasked colony forming activity in the thymus cell suspension; b. the same *in vitro* preirradiation of the thymocytes did not reduce the colony forming ability of fetal liver cells or fetal liver cells plus thymus cells. These findings indicate that colony forming ability, in

Table 4.11 *Effect of* adult *thymocytes on spleen colony formation by bone marrow cells in* allogeneic *hosts*
C57BL→CBA

nr. of thym. injected ($\times 10^6$)	number of CFU-s per 10^5 bone marrow cells exp. 1	exp. 2	exp. 3	exp. 4	% of syngeneic colony numbers mean ±S.E.
0	15.0	23.3	17.8	18.6	77±7
0.5	18.7	14.6	14.3	–	65±9
5	17.5	20.6	24.4	20.0	84±3
10	23.3	26.6	26.1	21.6	100±3
30	22.5	25.5	30.0	–	104±3
control bone marrow C57BL→C57BL	22.5	24.7	27.5	22.8	100

100×10^6 thymocytes: 0.6±0.2 CFU-s.

Table 4.12 *Effect of* adult *thymocytes on spleen colony formation by fetal liver and bone marrow cells in* syngeneic *hosts*
C57BL→C57BL

	number of CFU-s per 10^5 cells								% of control without thymocytes
	exp. 1	exp. 2	exp. 3	exp. 4	exp. 5	exp. 6	exp. 7	exp. 8	mean ± S.E.
fetal liver									
−thymocytes (control)	6.1	6.6	8.0	6.9	8.0	7.3	10.0	7.5	100
$+30\times10^6$ thymocytes	11.4			13.1	11.0	7.2	14.4	9.4	139±15
$+100\times10^6$ thymocytes		16.0	14.0	17.2	11.5	8.8	12.0		175±24
bone marrow									
−thymocytes (control)	27.8	17.2	16.7	14.0	5.7	30.8	13.3	15.8	100
$+30\times10^6$ thymocytes						35.2	17.0	16.7	116±6
$+100\times10^6$ thymocytes	32.0	36.3	28.0	16.3	5.0	35.2			135±19

100×10^6 thymocytes: 0.6±0.2 CFU-s.

particular of fetal liver cells, can be markedly enhanced in isogeneic conditions. It could mean that the thymocytes remove not only allogeneic but also syngeneic barriers against transferred fetal haemopoietic cell growth.

4.2.4 Potentiation of take by thymocytes

Faced with the relatively low takeability of fetal liver cell allografts and the findings that thymic cells markedly stimulated fetal-liver-derived haemopoiesis in genetically different hosts, it was investigated whether supplementation of the transplant with thymus cells could be of practical use to promote fetal liver cell allo-engraftment. In a number of experiments in which varying dosages of C57BL fetal liver cells were injected into CBA mice, the effect of administration of thymus cells was measured by the criterion of irradiation protection.

Experiments were carried out with 3 dosages of adult thymus cells; namely, 0.1×10^6, 5×10^6, and 100×10^6 cells per recipient, thus covering a rather broad

Table 4.13 *Effect of* adult *thymocytes on the ability of fetal liver cells to form spleen colonies in* syngeneic *hosts*

nr. of cells injected			CFU-s per 10^5 fetal liver cells	
fetal liver	+	thymus	exp. 1	exp. 2
2×10^5	+	−	6.3	8.5
1×10^5	+	50×10^6	9.5	14.3
1×10^5	+	50×10^6 (2000 rad γ)	12.0	12.9
2×10^5 (2000 rad γ)	+	50×10^6	0	0

Table 4.14 *Effect of injection of* adult *thymocytes on survival after* allogeneic *fetal liver cell transplantation*
C57BL→CBA

nr. of fetal liver cells ($\times 10^6$)	−adult thymocytes			+adult thymocytes			
	nr. of animals	% survival day 30	day 100	nr. of thymocytes ($\times 10^6$)	nr. of animals	% survival day 30	day 100
0.1–0.2	20	0		100	15	0	
1	13	46	0	0.1	15	13	0
				5	13	0	
	9	0		5	8	25	0
				100	7	0	
2	9	33	11	100	9	0	
30	15	100	27	0.1	14	86	23
	12	58	8	5	13	23	0
				100	13	0	

range. The results (table 4.14) were by no means indicative of an increased early survival rate. In most instances, the combined fetal liver/thymus cell grafts had an adverse, detrimental, effect. The large number of 100×10^6 cells induced a severe acute GvH reaction and led to 100% mortality within 30 days, irrespective of the number of fetal liver cells transplanted. Neither did the two other thymus cell dosages support better early survival. With the exception of a somewhat prolonged survival

Table 4.15 *Effect of injection of* fetal *thymocytes on survival following* allogeneic *fetal liver cell transplantation*
C57BL→CBA

nr. of fetal liver cells ($\times 10^6$)	−fetal thymocytes			+fetal thymocytes			
	nr. of animals	% survival day 30	day 100	nr. of thymocytes ($\times 10^6$)	nr. of animals	% survival day 30	day 100
0.03–0.2	39	0		0.001–0.007	44	11	5
0.4	78	9	2	0.01	85	35	14
	25	8	0	0.1	25	36	8
1	44	39	9	0.03	41	51	12
	47	38	4	0.1	53	26	4
	22	28	0	5	27	18	0
30	15	100	27	0.1	14	28	23
				1	15	80	13
	27	81	19	5	25	84	15

in one of the test groups, only negative influences on the rate of 30-day survival were noted.

Because fetal thymocytes involve fewer risks for GvH disease and because low numbers of these cells have been claimed to enhance fetal liver cell take [Bortin et al., 1973], the capacity of these cells to enhance survival was also examined. The results are presented in table 4.15. While dosages of $0.03–0.4 \times 10^6$ fetal liver cells alone were incapable of supporting significant survival, addition of small numbers of fetal thymus cells caused substantial improvement. Such a beneficial effect of the thymus cell inoculum could not be reproduced when supplemented to higher numbers of fetal liver cells which alone gave about 30% survivors at 30 days.

4.2.5 Discussion

A summary of cell numbers which were required to achieve a minimum survival of 80% is presented in table 4.16. These data demonstrate that:

a. Whereas the same number of fetal liver and bone marrow HSC had to be given for repopulation in the syngeneic combinations (part A), the number of embryonic HSC required in the allogeneic host was double that of bone marrow HSC. Apparently fetal liver HSC encounter a comparatively greater barrier in repopulating allogeneic recipients.
b. This greater barrier could be identified with a decrease in the production of fetal liver HSC in the allogeneic sites to half of the syngeneic values as a result of a decrease in the f-value, whilst, under the same conditions, production of bone marrow HSC was not reduced.
c. The 2 × higher stem cell number needed for fetal liver cell take, coupled with the 3 × lower frequency of HSC in this tissue, accounted for a 6 × larger number of nucleated cells needed for survival after fetal liver cell allotransplantation as compared to bone marrow allografting. Thus, the difference in cell numbers between effective fetal liver and bone marrow cell grafts was more pronounced

Table 4.16 *Number of injected haemopoietic cells required for 80% survival of lethally irradiated recipients at 30 days*

	syngeneic C57BL→C57BL	allogeneic C57BL→CBA
bone marrow transplant	240 CFU-s 5800 CFC $0.8–1.0 \times 10^6$ nucl. cells	1200 CFU-s 29300 CFC $4–5 \times 10^6$ nucl. cells
fetal liver transplant	120 CFU-s 5400 CFC	1200 CFU-s 55000 CFC
unfractionated	2–3 $\times 10^6$ nucl. cells	$20–30 \times 10^6$ nucl. cells
stem cell rich fraction	$0.3–0.4 \times 10^6$ nucl. cells	3–4 $\times 10^6$ nucl. cells

Summarized results from tables 4.1, 4.2, 4.4 and 4.5.
nucl. cells = nucleated cells.

than in the syngeneic experiments in which the requirement for more cells just corresponded with the 3 × lower concentration of HSC in fetal liver.

d. These numbers of fetal liver cells required to restore lethally irradiated allogeneic mice (80% survival by $20–30 \times 10^6$ cells; 50% survival by $2–3 \times 10^6$ cells) are in accord with the protective activity of nucleated cells from fetal liver as reported by other investigators [Crouch, 1959; Bortin et al., 1973]. Because survival was independent of removal of a great number of the cells (discontinuous albumin density gradient centrifugation), it appeared that the HSC were responsible for survival following fetal liver cell transplantation and not the other admixed cells. It was found that one mouse embryonic liver of 16 days' maturation yielded 28×10^6 cells on the average. Thus, it is evident that cells from one organ may suffice to successfully engraft an allogeneic recipient in this species.

Very characteristically, the allogeneic spleen and femur sites were shown to allow, without modification, the growth rates of bone marrow CFU as well as fetal liver CFU, which were specific of either type of cells in syngeneic hosts. The essential difference between the allogeneic curve of repopulating fetal liver CFU and the syngeneic curve was that the fraction, f, was depressed. This indicated that allogeneic suppression of fetal liver CFU recovery values was produced during the first 24 h of exponential multiplication. Theoretically, this could be the result of either a low seeding efficiency of fetal CFU to the allogeneic spleen and marrow or an inactivation of a proportion of the CFU after they settled in those sites. The latter possibility is favoured. In so far as allogeneic inhibition of spleen colony formation was due to impaired differentiation within the colonies, this was not the product of a suppressed seeding rate but of local suppression of growth. The fact that the entire suppression could be completely eliminated (by injection of thymocytes) suggests that the decreased f-factor had a common origin with the reduced differentiation effectivity.

In considering mechanisms for the very early repressive influence upon the fraction, f, of embryonic HSC, and for repression of differentiation in the spleen colonies formed by allogeneic CFU, it should be taken into account that the initial take of haemopoietic cell transplants in non-isogeneic hosts may depend on a number of genetically determined factors [Goodman and Wheeler, 1968]. Some of them have traditionally been classified as immunological. Primary attention should be paid to the immunological response of the host which leads to rejection of the grafted cells [e.g., Van Bekkum and De Vries, 1967]. Furthermore, it should be considered that a GvH reaction, if present, may have a negative influence on repopulation [Boranic et al., 1973]. Finally, in recent years it has become clear that haemopoietic cell growth is also governed by nonimmunological parameters, including the so-called hybrid resistance factors [Cudkowicz and Stimpfling, 1964; Cudkowicz and Bennett, 1971]. The influence of these latter physiological factors has been interpreted in terms of a requirement of the grafted cells for a series of environmental conditions which direct or

control cell proliferation [Cudkowicz and Bennett, 1971; Trentin, 1971]. In this sense, it is conceivable that hybrid resistance is the result of unsuccessful adaptation of the grafted cells to the foreign local environment [McCulloch et al., 1973]. Indirect evidence has suggested that this effect depends on short range influences by macrophage cells in spleen and marrow [Cudkowicz and Bennett, 1971; Lotzova et al., 1974 in press]. It is not known whether the activity is achieved by cell-cell contact or by substances diffusing over short distances, although there are indications that at least some short range interactions are mediated through specific cell surface components that can be characterized genetically [McCulloch et al., 1973]. Whereas experimental data have established many of the characteristics and the mode of action of immunological reactivity, the properties and mechanism of hybrid resistance are as yet only vaguely defined. The question as to whether the reduced repopulation of transplanted allogeneic CFU in the reported experiments was the result of an immunological process or of environmental resistance to these cells cannot be answered with certainty. However, in our opinion, a nonimmunological activity of host against graft offers the most satisfactory explanation for the aspects of repression of CFU proliferation and spleen colony formation observed; notably, for the following reasons:

Firstly, the possibility of a GvH reaction interfering with repopulation early after transplantation should be rejected because the haemopoietic fetal liver tissue contained few, if any, immunocompetent cells (chapter 3).

Secondly, the possible operation of an immunological host-versus-graft response may also be discounted because: a. CFU repression was less marked when C57BL cells were injected into CBA hosts than when administered to F_1 (CBA × C57BL). Since the grafted cells might be expected to meet a strong histocompatibility barrier in the former case and in the latter none, these results make the operation of an immunological host-versus-graft reaction improbable; b. the large dose of irradiation given to the recipients may be expected to have abolished any immunological reaction of the host, at least during the 7–9 days period of assaying for CFU proliferative activity [Smith and Vos, 1963; Cudkowicz and Bennett, 1971]; c. the rapid onset of the repressive effect on the f-value of fetal liver CFU within 24 h after transplantation renders an immunological reaction improbable, since this usually involves an induction and effector phase (including cell proliferative events) of more than one day's duration.

Thirdly, the kind of repression observed shared some characteristic features of hybrid resistance: Hybrid resistance has been considered a clearcut example of environmental resistance, because basic immunogenetic principles founded on observations concerning organ and tissue transplantation indicate the absence of immunological host-anti-donor activity. This opinion was further substantiated by findings that hybrid resistance was different from the usual immunological response in several other aspects (e.g., not modified by neonatal thymectomy) [Cudkowicz and Bennett, 1971]. The inhibition of fetal liver and bone marrow-derived haemopoiesis in the

allogeneic recipient showed a remarkable resemblance to the kind of repression observed in the hybrid host: 1. the growth kinetics of CFU in the allogeneic spleen were similar to those previously reported in the hybrid spleen (decreased f-value of CFU proliferation in the presence of unchanged doubling time) [McCulloch et al., 1973; Buurman et al., 1974 in press] and 2. hybrid resistance against haemopoietic cell growth can be overcome by injection of very large doses of donor type thymus cells [Goodman and Wheeler, 1968; Goodman and Shinpock, 1968; Goodman and Grubbs, 1970; Trentin, 1971]. We have shown that repression of spleen colony formation by bone marrow as well as fetal liver CFU, in both allogeneic and F_1 hybrid mice, could be overcome by the same means.

On the other hand, the demonstration that, beyond the first day, the growth rate of CFU was as rapid as in syngeneic conditions in all situations tested, seems contradictory to the claim that the function of environmental resistance is relatively radioresistant (not depending on direct cell proliferation) [Cudkowicz and Bennett, 1971].

While there was almost no additional mortality after the second week following the cell injection in syngeneic transplantation, considerable mortality was still encountered in the 3rd and 4th weeks in the allogeneic experiments. This indicated that the repopulation events which occurred beyond the first 10-day period following grafting were still relevant to the efficiency of survival in allotransplantation. It cannot be excluded that immunological activities (e.g., some recovery of homograft response) also come into effect at these later time intervals. Neither do the experiments give assurance that the effects on stem cell proliferation which occur at later stages involved the same quantitative relationships as those observed during the first week. This places some restrictions on the value of these findings on initial haemopoietic repair in furnishing a generally adequate insight into the engraftment process as a whole.

It has been pointed out that the number of stem cells which were capable of repopulating lethally irradiated allogeneic mice exceeded those in syngeneic transplantation by factors of 5 (bone marrow) and of 10 (fetal liver) (table 4.16). These iso-allo differences in requirement for cell numbers were not consistent with the degree of reduction in CFU repopulation of the tissues. If we consider that the iso-allo ratio of required bone marrow cell numbers was 5, it is surprising that production of CFU in spleen as well as in femur was almost not depressed. As has been stressed earlier, the 10-fold cell requirement in allogeneic versus isogeneic fetal liver cell transplantation cannot be explained on the basis of CFU production either, the latter being only 50% less in allogeneic conditions. As in the case of bone marrow transplantation, therefore, a factor of 5 cannot be accounted for. In addition, it was demonstrated that a reduced generation of mature end cells in allogeneic conditions interfered with early repopulation. This was evidenced by the decreased regeneration of end cells in the blood following allotransplantation as compared to that after syngeneic grafting. Furthermore, spleen colony formation was reduced, which could only be ascribed in

part to a lower f-value of CFU. As far as differentiation impairment was involved in reduced spleen colony formation in allogeneic animals, this was calculated to account for a repression by factors of 0.6–0.7. Although this leaves little room for doubt that the allogeneic interference with differentiation counteracts graft success and leads to the demand for larger cell numbers to be grafted, this degree of suppression can only account in part for the 5 × impaired takeability. The remainder of the iso-allo difference in cell numbers, still unexplained, is thus 3–3.5 ×. One is therefore tempted to postulate that repopulation during the 10 days of our observation period does not fully predict the eventual haemopoietic recovery required for 30 day survival; e.g., developing GvH activity or homograft responsiveness of the host could both disturb the kinetic relationships which are involved in an earlier phase. Whatever the explanation, the extension of the observation period of the kinetics of repopulation to later time intervals seems clearly required.

Measurement of the recovery of cell counts in the blood throughout the first month after transplantation gave evidence that cells of the varying cell lines exhibited dissimilar susceptibility to repression. The recovery of thrombocytes was slightly repressed and that of erythroid and lymphocytic cells markedly. The delayed restoration of the lymphocytic cells in the blood could be indicative of a comparatively prolonged state of immunological incompetence of the allogeneic host. This would be consistent with results described in chapter 6 which demonstrated that mitogen responsiveness (*in vitro*) of lymphoid cells in the spleen in a proportion of the allogeneic chimaeras remained subnormal for several months. The cause of the lymphopenia may, at least partly, be related to involvement of these cells in GvH reactions, e.g., due to their migration from the blood to sites of immunological activity. The postulate that the GvH disease is at the root of the suppressed lymphocyte counts, and also of decreased lymphocyte reactivity, would fit in with other findings indicating that the decreased immunological reactivity in allogeneic chimaeras is not a primary repopulation defect but is instead a secondary phenomenon of GvH disease (chapter 6).

It has been reported that thymus cells do not augment haemopoietic cell growth following transplantation in syngeneic hosts [Goodman and Shinpock, 1968]. This conclusion was based on experiments with bone marrow cells in which the rate of Fe^{59} incorporation was the endpoint for measurement of haemopoietic activity. Our results were at variance with these findings and indicated a slight increase in isogeneic spleen colony numbers following addition of thymus cells to the bone marrow graft. More impressive was the nearly 2-fold increase in fetal-liver-derived colony numbers upon combined fetal liver cell/thymus cell injection. To our knowledge, similar studies on thymus cell influences on syngeneic fetal liver cell derived haemopoiesis have not been previously published. The meaning of these findings could be that, by analogy with the allogeneic system, even in a syngeneic fetal liver cell transfer system not all requirements for growth are met without the addition of

thymocytes. The thymus cell effect on fetal liver CFU is particularly marked. A possibility which comes to mind to explain this finding is that a kind of resistance due to the inappropriate adaptation of the fetal cells to the adult environment (ontogenetic resistance) is overcome. It is clear that, for more definitive interpretation, the experiments need extension.

Attempts at engraftment of haemopoietic cells in non-isogeneic hosts usually rely on suitable conditioning of the recipient and the infusion of proper cell numbers. A new approach would be provided by the modification of the cellular constitution of the transplant as a means to promote take. In this respect, the attempts which took advantage of the potentiating effect of thymocytes on fetal liver haemopoiesis were not very promising. Although it is known that thymocytes are 10–100 × less GvH reactive on a cell-for-cell basis than spleen cells and lymph node cells [Vos et al., 1959; Mathé et al., 1964; Blomgren, 1973; Tigelaar and Asofsky, 1973], the injection of larger numbers of adult thymocytes entails the danger of inducing very severe acute GvH disease. All animals in the group receiving the largest thymus cell dose succumbed from GvH disease, which was consistent with previous experience [Goodman and Shinpock, 1968]. When testing these cells at lower concentrations, antagonistic effects on survival were also noted in several instances. Although the lowest number of thymus cells inoculated did not elicit a detectable GvH reaction, it also did not lead to improved survival. It seems of great interest to test irradiated thymocytes which have lost GvH potencies for their capacity to enhance take in the allogeneic system. This is particularly so, in view of the fact that irradiated thymocytes were shown to retain their stimulatory activity on fetal liver haemopoiesis in syngeneic splenic colonies. However, the fact that adult thymus cells of the same immunogenetic constitution are obviously not available in man, rules out a possible practical value of this procedure for clinical fetal liver cell transplantation. Therefore one can only consider the feasibility of using fetal thymocytes to promote take of fetal liver cells.

Results obtained by the inoculation of fetal thymocytes in mice were also somewhat more favourable than that of adult thymocytes, in that no acute GvH reaction appeared following their administration and under certain conditions somewhat better survival was obtained than with fetal liver cells alone. Colony assays with fetal thymus cells were not performed and it is therefore not certain whether the effect of the small numbers of these cells is comparable with that of the large numbers of adult thymocytes. Additional experiments may clarify the question as to whether they exert their action in overcoming environmental resistance. For the time being, one can conclude that the degree of improvement in early survival provided by these cells was moderate (at best survival increased from 8 to 35%). These results were in the same range as those described by Bortin et al. [1974]. Furthermore, the enhancing effect of the fetal thymus cells was confined to a rather narrow dose range of fetal liver cell numbers. Because one can expect only a limited effect from the employment of these cells, their usefulness in human practice must be doubted.

In the above considerations, fetal thymocytes of man were taken to be the equivalent of the fetal thymocyte population of the mouse, but in view of the positive PHA-reactivity of the former cell population (table 7.1) and differences in maturation of the ontogenetic stages of both species [Rugh, 1958] it is possible that they are more comparable to thymus cells from adult mouse donors. Therefore, when applying fetal thymocytes in humans, preventive measures against the development of GvH disease may be indicated. It is not known whether irradiated thymus cells are still capable of enhancing take. In view of this, extension of the experiments for testing the effect of *irradiated* adult thymus cells on take following fetal liver cell allotransplantation in the mouse deserves intensified interest. There is a second factor which is uncertain in the application of fetal thymus cells in man: does the cell number dependency of adult thymus cells for a stimulatory effect on haemopoiesis in the mouse warrant application in the human being? One should be aware that it will not be feasible to obtain the cell number requirements in man which are involved in the mouse, i.e., $30–100 \times 10^6$ adult thymus cells per murine recipient of 20 g; this corresponds with the unrealistic fetal thymus cell yield of $8–25 \times 10^9$ cells* from one human embryo for a CID patient of 5 kg. However, it is not certain whether these quantitative relationships can be extrapolated to transplantation in man without modifications. Therefore, it seems particularly worthwhile to carry out investigations in primates (monkeys) aimed at testing the validity of the procedure under the quantitative conditions which are obtainable in man.

The investigations reported have disclosed some of the peculiar repopulation features of fetal liver stem cells which could underlie the low takeability of fetal liver cell transplants. In the syngeneic host, the repopulation capacities of fetal liver and bone marrow stem cells were shown to be approximately equivalent and this was attributed to the sum of the effects of a reduced f-factor and a more rapid proliferation rate and a lower differentiation capacity of fetal liver stem cells. A further decrease in the f-value of fetal liver stem cells was induced in the allogeneic host. From these findings, in conjunction with the observation of the low content of HSC in fetal liver, the fact that fetal liver cell grafting required many more cells than bone marrow grafting for efficient repopulation could be understood. If one extrapolates the quantitative relationships involved in fetal liver cell takeability to the human species, the great number of failures in fetal liver cell transplantation in man seem to be satisfactorily explained (paragraph 7.4). This is even more so if one takes into account the application of a poor cell freezing technique and the low efficiency of the i.p. route of administration of the cells usually employed in human transplantation (paragraph 1.3.6). According to the experimental results reported in this chapter, there is every reason to

* The yield of one human embryonic thymus did not exceed 0.5×10^9 cells in our series of fetal material.

conclude, in line with Crouch [1959], that fetal liver cell take suffers the drawbacks of the low content of proliferative cells (i.e. stem cells) and of a lower adaptability of the cells to the allogeneic environment (i.e., reduced f-factor) and maybe even to the adult syngeneic environment.

CHAPTER 5

STEM CELL MULTIPLICATION IN VITRO

The take problem encountered in fetal liver cell grafting involves the requirement for comparatively larger numbers of cells for repopulation as well as the insufficient cell yield from one embryonic liver for complete restoration of the recipient. It has been shown in mice that this barrier can be overcome by increasing the cell number, e.g., by pooling cells from more than one fetal liver per transplant. Such an approach presents no problems in inbred mouse strains; in man, however, the pooling of livers necessarily leads to a graft of a heterogeneous immunogenetic origin. This has significant disadvantages in terms of takeability and logistics [Van Bekkum, 1969a]. One of the hypothetical solutions to this problem is to increase the number of stem cells from one donor by inducing multiplication of HSC in tissue culture prior to grafting.

There are culture techniques which allow the growth of clones of haemopoietic cells, e.g., macrophages, granulocytes, and erythrocytes [Pluznik and Sachs, 1965; Bradley and Metcalf, 1966; Stephenson et al., 1971]. However, evidence has been provided that these colonies are not formed by HSC but by precursor cells of the committed cell lines [Metcalf and Moore, 1971a; Gregory et al., 1973]. One possible exception is the thin agar layer system (TALS) in which irradiated fibroblasts are employed as feeder cells. In this culture system, developed in our laboratory, involvement of the pluripotent HSC in the process of colony formation has been suggested by the demonstration of an increase in CFU-s in the colonies [Dicke et al., 1971, 1974]. The basis for the proliferation of the HSC in this *in vitro* system is not known. Elucidation of the factors which influence stem cell proliferation might open the possibility for a further increase in the number of stem cells in culture, so that such a system might eventually be used for preparative and transplantation purposes. One of the first requirements for the understanding of this system is an analysis of the kinetics of proliferation *in vitro*. Unfortunately, such investigations have been impossible so far because of the solidified agar in the TALS.

In this chapter, attempts to obtain proliferation of murine HSC by using the constituents of fibroblast cultures in a liquid system as an analogue to the TALS will be discussed. The experiments were concerned with an analysis of the *in vitro* kinetics of HSC in this system. It was particularly examined whether an actual increase in the initial number of HSC could be obtained. In order to determine whether a humoral factor caused stem cell proliferation in the fibroblast-enriched culture, the effect of fibroblast conditioned medium was investigated. Certain other stimuli which are known to influence haemopoietic cell growth were also tested for stem cell

activation. In the first series of the experiments (part A) bone marrow of adult mice was used as a source of HSC. In the second part of this chapter (part B), results on the behaviour of fetal liver HSC in liquid culture will be reported.

5.1 *Part A: The kinetics of* bone marrow *stem cells in liquid culture*

5.1.1 Effect of fibroblast feeder cells on maintenance of bone marrow CFU-s in culture

Bone marrow cells were incubated in liquid culture and the cultures were discontinued

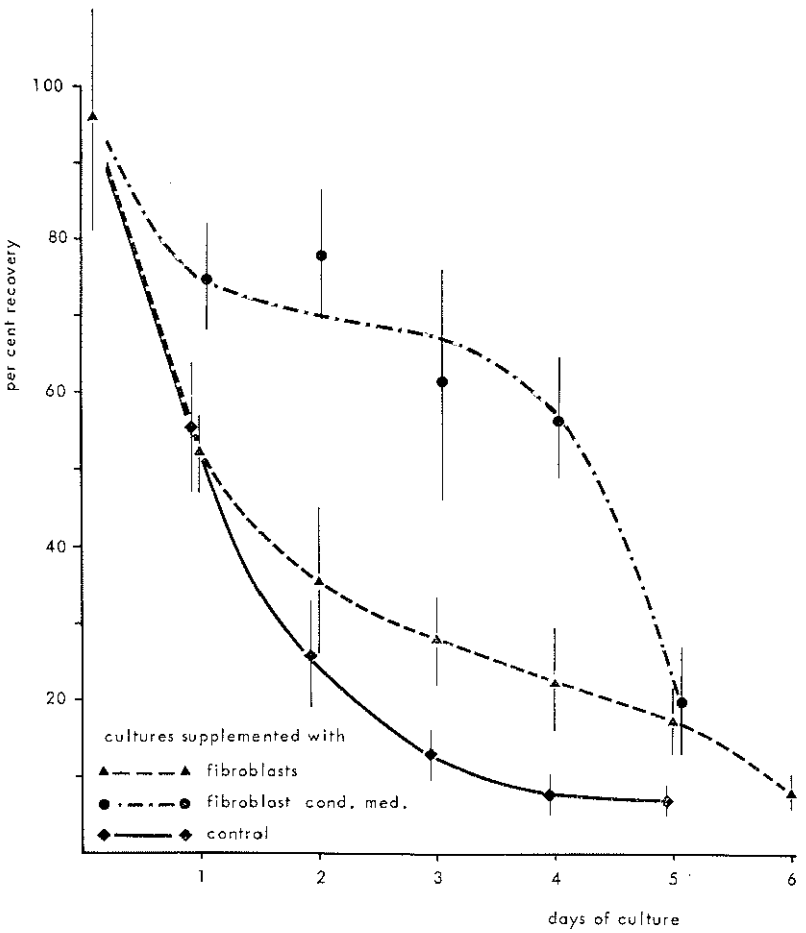

Fig. 5.1 *Effect of irradiated fibroblast feeder cells and fibroblast-conditioned medium on maintenance of bone marrow CFU-s in short term culture.*
The percentage recovery of the initial number of CFU-s (bone marrow) placed in culture was determined at different intervals. Each point represents the mean (±S.E.) of 4–6 separate experiments

at daily intervals for assaying of CFU-s numbers as a function of time. The number of CFU-s harvested per culture was expressed as the per cent recovery of the initial number seeded. The survival rate of HSC from bone marrow in fibroblast cultures was investigated in this manner during the course of a 5–6 day culture period. In fig. 5.1, the results of estimations performed at 1-day intervals in a limited series of experiments are shown. In the control cultures without fibroblasts, CFU-s decrease exponentially with a half-time of 24 h, resulting in a recovery of 10% of the initially seeded number of CFU-s at 3 days. In the presence of irradiated fibroblasts, the initial decline is very similar, but the rate of disappearance is less between 2 and 4 days (fig. 5.1). All estimates which were performed in these and other experiments on day 3 of culture are collected in table 5.1. The results show a statistically significant difference in CFU-s values between cultures with and without fibroblasts ($P < 0.0005$).

Table 5.1 *The effect of fibroblasts and FCM on maintenance of bone marrow CFU-s in vitro (determined at day 3)*

additive to culture	nr. of exp.	day 3 recovery (%)* mean ±S.E.	significance of difference with control
fibroblasts (2000 rad)	19	30.7± 3.7	$P < 0.0005$
FCM	4	61.5±15.7	$P < 0.0005$
none (control)	10	9.6± 2.1	

* CFU recovery is expressed as a percentage of the initial number of CFU present at time zero of cultivation.

If the improved maintenance of CFU-s numbers in fibroblast-supplemented cultures is due to factors released by the feeder cells into the medium, the early precipitous decline in CFU-s might be caused by an insufficient level of these factors in the medium during the first day. To test this, the effect of the addition of fibroblast-conditioned medium (FCM) at time zero was investigated. It is shown in fig. 5.1 that the resulting CFU-s values on day 1 were significantly higher ($P < 0.005$) than those in cultures containing fibroblasts. The increased yield on day 1 was followed by increased recovery values during the next 3–4 days. After day 4, a fall in the plateau values of CFU-s occurred in the FCM culture, which is compatible with the assumption that the active principle of FCM becomes depleted at that time. These findings suggest that the effect of fibroblasts on stem cell survival is mediated by a factor released by these cells.

It is known that FCM contains colony stimulating factor (CSF) which has the same biological activity as CSF from pregnant mouse uterus extract [Van den Engh, 1974]. It is of interest to know whether the factor affecting stem cells in culture

Table 5.2 *The effect of "fresh" and "stored" FCM added to the cultures on maintenance of bone marrow CFU-s in vitro*

FCM	nr. of exp.	% recovery of CFU-s* (day 3) mean ±S.E.	significance of difference
fresh	4	61.5±15.7	
stored**	5	23.4± 4.8	$P < 0.05$

* CFU recovery is expressed as a percentage of the original number of CFU inoculated into the culture.

** Storage for approximately 4–6 weeks at 4°C.

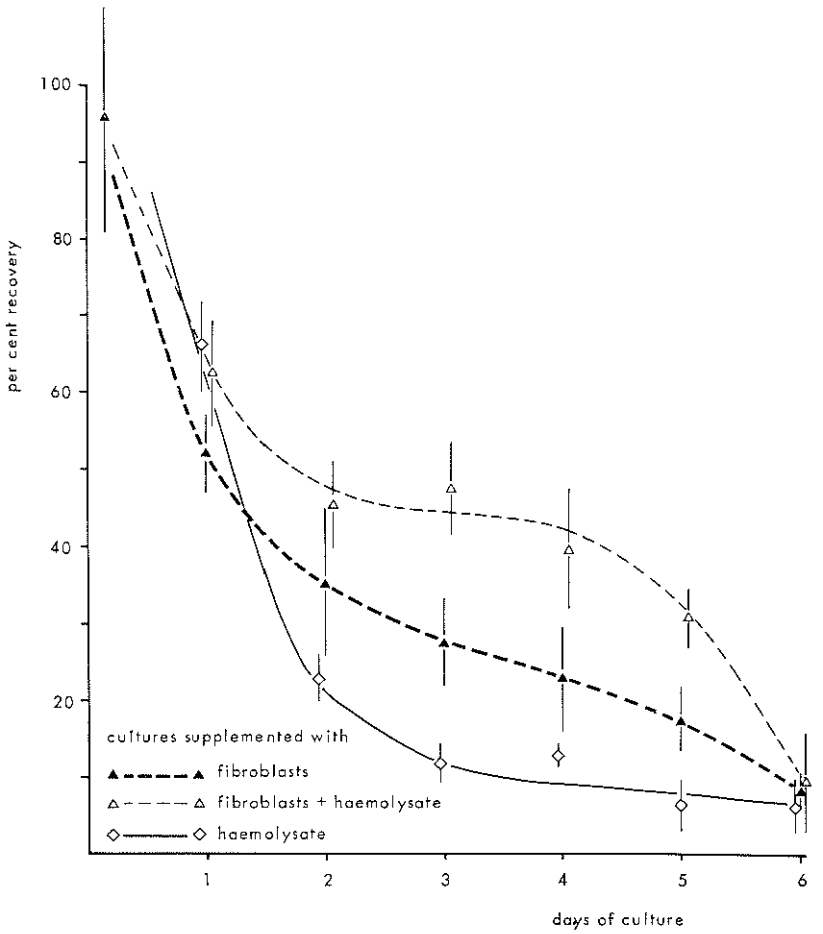

Fig. 5.2 *Effect of haemolysate on maintenance of bone marrow CFU-s in short term cultures containing irradiated fibroblast cells.*

The percentage recovery of the initial number of CFU-s (bone marrow) placed in culture was determined at different intervals. Each point represents the mean (±S.E.) of 4–6 separate experiments.

is identical to CSF. This seems not to be the case, because the addition of purified CSF from pregnant mouse uterus did not result in increased CFU-s recovery. Another difference between the two factors is that storage of FCM at 4°C for 4–6 weeks significantly reduced its capacity for maintaining stem cell numbers (table 5.2), while it is known that CSF can be stored for such periods without loss of activity. This suggests that the activity exerted by FCM on HSC resides in a material distinct from CSF.

5.1.2 Effect of other substances on maintenance of bone marrow CFU-s in culture

Bradley et al. [1972] and Testa and Lajtha [1973] reported that the addition of haemolysate or red blood cells to agar culture systems results in an increase in the number and size of macrophage-granulocytic colonies and in the improved maintenance of CFU-s numbers in culture. Therefore it seemed of interest to investigate the effect of haemolysate in combination with fibroblasts or FCM on persistence of CFU-s in liquid culture. The result is shown in fig. 5.2. In contrast to Testa's findings with red blood cells in agar cultures, haemolysate alone does not maintain the CFU-s numbers in the liquid system. However, in the presence of fibroblasts, haemolysate gives rise to an increased CFU-s recovery which is statistically significant for the day 3 values.

Note that the initial loss of CFU-s in the first 24 h is not influenced by the addition of haemolysate. The effect of haemolysate on FCM-containing cultures was also examined. Figure 5.3 shows that the presence of haemolysate does not significantly change the CFU-s values in the first 4 days of culture. However, the sudden decline in CFU-s numbers in FCM cultures on day 5 does not occur in the presence of haemolysate.

Table 5.3 *The effect of serum from pre-irradiated mice on maintenance of bone marrow CFU-s in culture*

	% recovery of CFU-s*		
additive to culture	day 1	day 3	day 6
serum from pre-irr. mice (20%)	65.6	7.8	2.2
serum from pre-irr. mice (30%)	50	15.6	0.6
no serum (control)	55.4	12.9	n.d.
serum from non-irr. mice (30%)	n.d.	4.9	n.d.

Mean values of 2 experiments.
Serum was obtained from adult mice which had been irradiated 3 days in advance with a lethal dose of X-rays. The serum was pooled and freshly added to cultures in two concentrations (20% and 30%). These results may also be compared with the data on CFU-s maintenance in non-supplemented cultures plotted in fig. 5.1.
* CFU recovery is expressed as a mean percentage of the original number of CFU inoculated into culture.

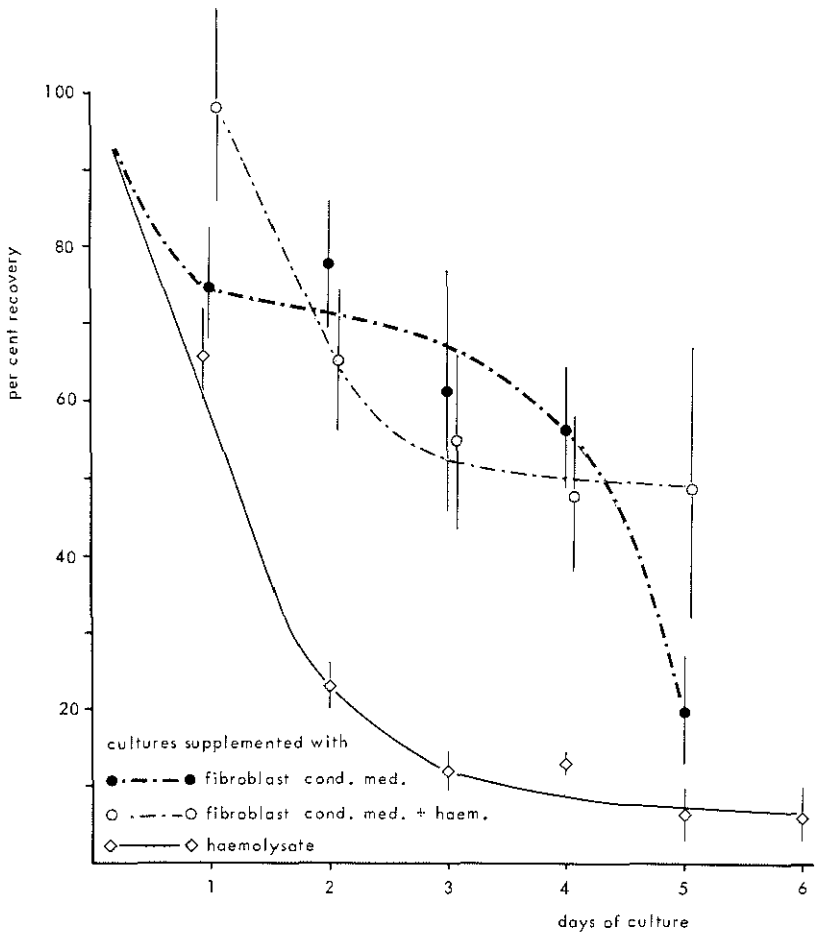

Fig. 5.3 *Effect of haemolysate on maintenance of bone marrow CFU-s in short term culture containing fibroblast-conditioned medium.*
The percentage recovery of the initial number of CFU-s (bone marrow) placed in culture was determined at different intervals. Each point represents the mean (±S.E.) of 4–8 separate experiments.

Because irradiated mice provide an *in vivo* environment in which stem cells proliferate rapidly (see e.g., paragraph 4.1.2), we have also investigated the effect of freshly-obtained serum from 3 day-pre-irradiated mice (with a lethal dose of X-rays) on the maintenance of stem cells in culture. The results may be compared with the data plotted in fig. 5.1; they indicate that no significant stem cell maintaining activity could be demonstrated in the serum (table 5.3).

5.1.3 Proliferative status of bone marrow CFU-s in culture

When considering the mechanism underlying the maintenance of HSC numbers in

bone marrow cultures exposed to irradiated fibroblasts, it seemed of particular interest to establish whether this was the result of:

a. preservation of HSC originally present in suspension; or
b. stimulation of HSC proliferation and concomitant disappearance of HSC.

Since the second process implies an active cycling of stem cells, thymidine suicide experiments were carried out during the first 3 days of culture.

As the *in vitro* thymidine suicide technique was considered insufficiently sensitive to distinguish small differences in cell proliferation, the data were utilized mainly

Table 5.4 *In vitro thymidine suicide of bone marrow CFU-s cultivated for 1, 2, or 3 days*

type of culture	thymidine exposures (per ml)		% of CFU-s surviving thymidine incubation (mean) at day 1		at day 2		at day 3	
fibroblasts	1	HBSS	100 (3)		100 (1)		100 (3)	
	2	20 μCi ^{3}HTdR	93	−	65	+	40	+
	3	200 μCi ^{3}HTdR	87		45		56	
	4	20 μCi ^{3}HTdR+100 μg TdR	100		109		87	
FCM	1	HBSS	100 (2)		100 (2)		100 (4)	
	2	20 μCi ^{3}HTdR	79	+	68	+	75	+
	3	200 μCi ^{3}HTdR	78		72		77	
	4	20 μCi ^{3}HTdR+100 μg TdR	107		100		102	
fibroblasts+ haemolysate	1	HBSS	100 (1)		100 (1)		100 (3)	
	2	20 μCi ^{3}HTdR	88	−	87	−	82	−
	3	200 μCi ^{3}HTdR	81		87		87	
	4	20 μCi ^{3}HTdR+100 μg TdR	93		101		97	
FCM+ haemolysate	1	HBSS	100 (3)		100 (1)		100 (3)	
	2	20 μCi ^{3}HTdR	96	−	71	+	87	−
	3	200 μCi ^{3}HTdR	85		79		77	
	4	20 μCi ^{3}HTdR+100 μg TdR	104		91		92	
haemolysate	1	HBSS	100 (2)		100 (1)		100 (2)	
	2	20 μCi ^{3}HTdR	99	−	110	−	91	−
	3	200 μCi ^{3}HTdR	100		112		103	
	4	20 μCi ^{3}HTdR+100 μg TdR	98		102		103	
control (medium)	1	HBSS	100 (1)		100 (1)		100 (3)	
	2	20 μCi ^{3}HTdR	81	−	98	−	111	−
	3	200 μCi ^{3}HTdR	88		110		101	
	4	20 μCi ^{3}HTdR+100 μg TdR	97		103		103	

The number of CFU-s estimated following incubation in HBSS (control) was set at 100%. The other CFU-s exposure values are expressed relative to the control.
−: kill of CFU-s by isotope exposure $< 20\%$ – resting
+: kill of CFU-s by isotope exposure $\geqslant 20\%$ – cycling
The figures in parentheses represent the number of separate experiments.

to define the cell population as cycling or non-cycling. A suicide percentage of less than 20% was arbitrarily taken to characterize a largely resting population of HSC, while killing percentages exceeding 20% were taken to indicate cycling populations.

According to these criteria, CFU-s were found to remain predominantly in the resting state during a 3-day culture period in control cultures (table 5.4). In contrast, a majority of CFU-s cultured in the presence of fibroblasts or FCM were found to move into S-phase during the first 3 days of culture, although with a lag period of 1 day in the cultures containing fibroblasts. The results also suggest that the cell cycle activation of the HSC in the latter cultures was reduced by the addition of haemolysate. The lysate itself did not induce proliferation of CFU-s in any of the experiments.

5.1.4 Discussion

From the survival kinetics presented for CFU-s, it can be concluded that the recovery of CFU-s is markedly improved in the presence of irradiated fibroblasts. This maintenance of CFU-s numbers is associated with a substantial activation of the cells into cycling. The fact that CFU-s numbers are better maintained with a larger fraction of the cells proliferating can only be explained by self-replication of at least a proportion of the cycling cells. If all proliferating stem cells in fibroblast cultures were to differentiate into more mature progeny, the loss of CFU-s should be more rapid; on the assumption that the mean duration of the S-phase is shorter than 24 h, or, in other words, that the 50% fraction of CFU-s which is found in DNA synthesis on days 2 and 3, do not represent the same cells. If we interpret the 50% S-phase killing as representing the entire CFU population which is in proliferation, if we further attribute all proliferation to self-renewal activity of CFU, and if we finally assume that the mean cycling time, as *in vivo*, is in the order of 32 h (chapter 4), the CFU population in culture should double nearly every day. The observation that the self-reproduction is not paralleled by a net increase, but by a slow fall in CFU-s numbers in the cultures, suggests that some other process (either death or differentiation of CFU-s) counteracts the increase due to proliferation. These findings are compatible with the observations that stem cell replication occurred in colonies in the TALS without resulting in a net increase over the whole culture-dish [Dicke et al., 1971].

It is possible that the reported activity of fibroblasts is shared by cells from other sources such as renal tubules. Whereas the latter feeders have been shown to result in improved recovery values of CFU-s [McCulloch and Till, 1971], the actual survival of CFU-s was somewhat lower (day 3 recovery about 15%) than the values described here for fibroblast-enriched cultures (on day 3: 30% CFU-s recovery). These investigators also performed ^{3}HTdR uptake experiments and noted an increased suicide of CFU-s in cultures containing kidney tubules. However, these data are not directly comparable to our suicide figures, because a prolonged isotope exposure was employed

in the former study (the isotope was incorporated for 2 days into the bone marrow cultures).

The similar effect of fibroblasts and FCM on the maintenance of CFU-s numbers and on their cycling status suggests that the effects of fibroblasts are mediated by a factor elaborated by the cells into the medium. Cultures to which FCM has been added, did not show the delay seen in fibroblast supplemented cultures during the first 24 h, which may indicate that this lag period is due to a gradual build up of an effective level of the factor in the medium. It is difficult to explain why Testa and Lajtha [1973] using a FCM supplemented culture obtained strikingly lower CFU-s survival profiles; by day 4, only 10% of the original number of CFU-s could be detected. Since these authors do not explicitly mention the necessity of using freshly harvested medium, the disagreement with our data may depend on their use of aged medium.

Since it has been confirmed that promotion of stem cell maintenance in culture cannot be achieved by CSF [Summer et al., 1972; Van den Engh, 1974], a factor other than CSF should be considered as activating stem cells in cultures containing fibroblasts. The factor differs from CSF in that it cannot withstand a storage period of 4–6 weeks at 4 °C. It is certainly of interest to investigate whether there is a relationship between the fibroblast-derived factors and other stem cell cycle activating substances, e.g., cAMP and various neurotransmitters [Byron, 1971].

Among the multiple effects of red blood cells and their products on *in vitro* haemopoiesis [Bradley et al., 1972], a favourable influence on the survival of stem cells in FCM containing cultures has been reported [Testa and Lajtha, 1973]. Our results in liquid culture indicate that the lysate alone is unable to promote an improved CFU-s maintenance as do fibroblasts and their products. The additional rise in absolute CFU numbers in fibroblast cultures supplemented with haemolysate goes parallel with a substantial reduction in the sensitivity of these cells to ^{3}HTdR killing. These phenomena suggest that the enhanced persistence of HSC by fibroblasts and by haemolysate may not be due to the same mechanism. Whereas a process of active stem cell proliferation is induced by fibroblasts, indications are that the lysate when combined with the fibroblasts may operate by preventing resting stem cells from differentiating or dying.

On the basis of the available results, it is not possible to speculate whether the stem-cell-activating mediators released by fibroblast feeder cells have physiological relevance. Though stem cells replicate rapidly in irradiated mice, the failure to demonstrate *in vitro* stem cell maintenance activity in the serum of these mice may be due to interference by inhibitors rather than to the actual absence of *in vivo* stem cell stimulating regulators in the serum.

5.2 *Part B: The kinetics of fetal liver stem cells in liquid culture*

5.2.1 *Maintenance of fetal liver CFU-s in culture*

The survival of fetal liver HSC in culture was determined in the same way as in the experiments with bone marrow cells (fig. 5.4). On comparing figs. 5.1 and 5.4, a striking difference between the maintenance of bone marrow and fetal liver CFU-s can be seen; the persistence of fetal liver CFU-s in unstimulated cultures was markedly better. Whereas only 10% of the initial number of bone marrow CFU-s could be recovered in control cultures after 3 days, a significantly higher proportion of 36% (± 5.5) of seeded CFU-s was found in fetal liver cell cultures ($P < 0.05$). As fetal liver CFU-s are in a rapidly cycling state *in vivo* [Becker et al., 1965; Duplan, 1970],

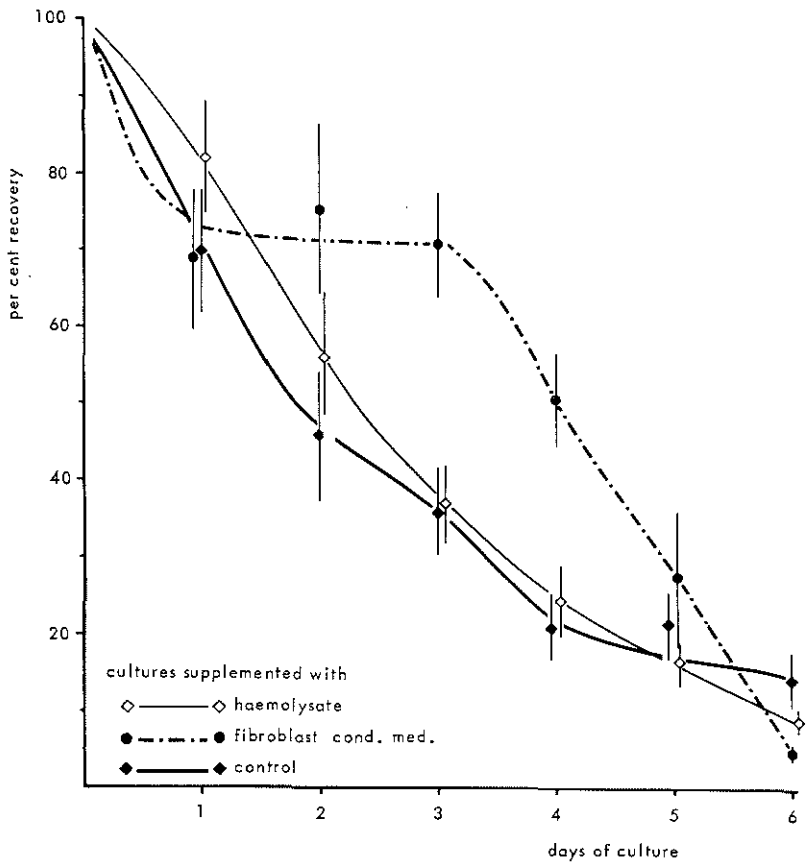

Fig. 5.4 *Effect of fibroblast conditioned medium and haemolysate on maintenance of fetal liver CFU-s in short term culture.*
The percentage recovery of the initial number of CFU-s placed in culture was determined at different intervals. Each point represents the mean ($\pm$S.E.) of 6–8 separate experiments.

it is assumed that the increased fetal liver CFU values are due to a continuation of the proliferative activity following the transfer to culture.

Thymidine suicide experiments confirmed that fetal liver CFU-s proceed to proliferate following their seeding in non-supplemented cultures (table 5.5). A high proportion of the cells is still found in cycle on day 2; only by day 3 do the cells appear to shift to a predominantly resting state.

Table 5.5 *In vitro thymidine suicide of fetal liver CFU-s in non-supplemented cultures*

thymidine exposures (per ml)		% of CFU-s surviving thymidine incubation (mean) at day 2		at day 3	
1	HBSS	100 (2)		100 (3)	
2	20 μCi ^{3}HTdR	78	+	89	−
3	200 μCi ^{3}HTdR	66		82	
4	20 μCi ^{3}HTdR + 100 μg TdR	93		108	

The number of CFU-s estimated following incubation in HBSS (control) was set at 100%. The other CFU-s exposure values are expressed relative to the control.
−: kill of CFU-s by isotope exposure $< 20\%$ – resting
+: kill of CFU-s by isotope exposure $\geqslant 20\%$ – cycling
The figures in parentheses represent the number of separate experiments.

5.2.2 *Effect of FCM on maintenance of fetal liver CFU-s in culture*

Because the best maintenance of bone marrow CFU-s numbers was observed in FCM cultures, the influence of FCM on fetal liver CFU-s was also studied. The numbers of fetal liver CFU-s were found to persist at a plateau of about 70% during the first 3 days (fig. 5.4). Comparison of these values with the control figures shows a clear difference (stat. sign. at day 3: $P < 0.001$). Following day 3, a decline in CFU-s in culture takes place. In contrast to the CFU-s in the control cultures which are no longer proliferating on day 3 (table 5.5), a large proportion continues in S-phase during this time interval in the cultures containing FCM (table 5.6).

5.2.3 *Effect of haemolysate on maintenance of fetal liver CFU-s in culture*

Neither haemolysate alone (fig. 5.4) nor haemolysate in combination with FCM (fig. 5.5) promotes the increased survival of CFU-s. The failure of the lysate to influence cycling of fetal liver CFU-s is compatible with the impression gained in the bone marrow experiments that the lysate selectively preserves a resting population of CFU-s. It was also found that the proliferative status is not significantly altered in the presence of the red blood cell preparation (table 5.6).

Table 5.6 *In vitro thymidine suicide of fetal liver CFU-s following 3 days of culture*

type of culture	thymidine exposures (per ml)		% of CFU-s surviving thymidine incubation (mean)	
FCM	1	HBSS	100 (2)	
	2	20 μCi ^{3}HTdR	75	+
	3	200 μCi ^{3}HTdR	69	
	4	20 μCi ^{3}HTdR+100 μg TdR	92	
FCM+haemolysate	1	HBSS	100 (1)	
	2	20 μCi ^{3}HTdR	81	+
	3	200 μCi ^{3}HTdR	75	
	4	20 μCi ^{3}HTdR+100 μg TdR	100	
haemolysate	1	HBSS	100 (4)	
	2	20 μCi ^{3}HTdR	100	−
	3	200 μCi ^{3}HTdR	91	
	4	20 μCi ^{3}HTdR+100 μg TdR	89	
medium (control)	1	HBSS	100 (3)	
	2	20 μCi ^{3}HTdR	89	−
	3	200 μCi ^{3}HTdR	82	
	4	20 μCi ^{3}HTdR+100 μg TdR	108	

The number of CFU-s estimated following incubation in HBSS (control) was set at 100%.
The other CFU-s exposure values are expressed relative to the control.
−: kill of CFU-s by isotope exposure $< 20\%$ – resting
+: kill of CFU-s by isotope exposure $\geqslant 20\%$ – cycling
The figures in parentheses represent the number of separate experiments.

5.2.4 Discussion

In vitro survival studies and thymidine suicide experiments indicate that the improved maintenance of fetal liver CFU-s in nonsupplemented cultures results from a continuation of *in vivo* cycling of CFU-s. This is consistent with the finding that bone marrow CFU-s which are rapidly proliferating in the donor also survive in greater numbers in culture [Testa and Lajtha, 1973]. It was found that the fetal liver CFU-s in the control cultures cease active cell cycling by day 3. The finding that CFU-s showed prolonged survival and continued to be in a proliferative state for a longer period in the presence of FCM, suggests that the increase in survival of fetal liver CFU-s in FCM supplemented cultures depended on self-replication of CFU-s due to action of the stem cell activating factor (SAF) in the FCM. The level of recoverable fetal liver HSC in FCM supplemented culture appeared to be of the same order of magnitude as for bone marrow cells, i.e., at about 70% at day 3. The fact that SAF did not produce an actual increase in the initial number of fetal liver HSC or bone marrow HSC indicates that the fibroblast system as described above does not yet fulfil the need for stem cell multiplication for clinical bone marrow transplantation.

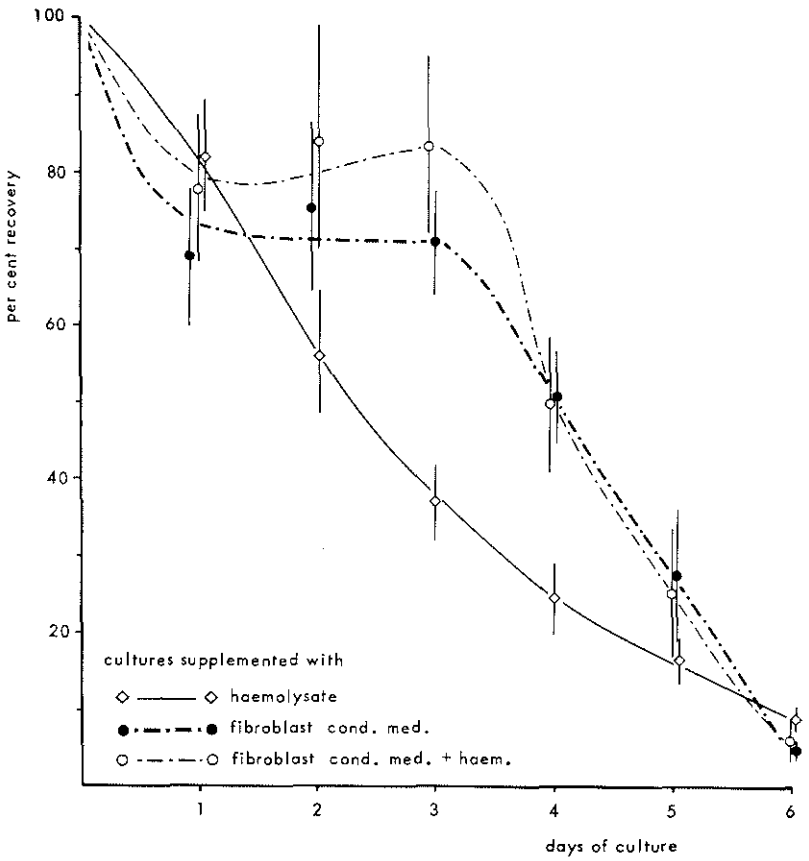

Fig. 5.5 *Effect of haemolysate on maintenance of fetal liver CFU-s in short term cultures containing fibroblast conditioned medium.*
The percentage recovery of the initial number of CFU-s placed in culture was determined at different intervals. Each point represents the mean (±S.E.) of 6–8 separate experiments.

As has been suggested above, an explanation for the absence of a net increase in HSC numbers during culture may be the simultaneous operation of differentiation stimuli at the level of HSC. This would be compatible with the indications that the HSC is the parent cell of at least some of the colonies of mature cells which grow in the fibroblast culture systems. [Dicke et al., 1971]. If this is the case, the separation of SAF from such associated stimulative substances in FCM may be of crucial importance for further development of the manipulation of stem cell reproduction.

CHAPTER 6

DELAYED GRAFT VERSUS HOST REACTIVITY OF FETAL LIVER CELLS

The literature contains extensive experimental data indicating that the delayed GvH disease following allogeneic fetal liver cell transplantation is less severe than that following bone marrow transplantation [Uphoff, 1958; 1959; Urso et al., 1959; Crouch, 1959; Lengerova, 1959; Barnes et al., 1961; Bortin and Saltzstein, 1969]. It is not certain as to which cellular element in the liver cell graft is responsible for the weak GvH reaction. It could be due to the progeny of lymphocytes which may be present in the graft in very small numbers or to immunocompetent cells originating from the HSC during the first month after transplantation. The answer to this question has direct practical implications because, if small numbers of lymphocytes cause the weak GvH reaction, these might be removed by density fractionation of fetal liver cell suspensions. Our finding that mature lymphocytes could not be detected in fetal liver neither by a morphological survey nor by mitogen stimulation, even in density subfractions (paragraph 3.4), seems to provide an answer. The indication is that lymphocytes newly matured from the HSC after grafting give rise to the GvH reaction.

With the consideration that lymphocytes differentiating from HSC following the cell transfer play a principal role in the occurrence of delayed GvH disease, the question arises as to whether the reduced GvH mortality could be due to a failure of the fetal HSC to generate functionally competent lymphocytes. This is quite unlikely, because the lymphoid cells arising from fetal liver grafted cells have been found to be capable of eliciting GvH reactions in third party hosts within 30–60 days after transplantation [Tyan, 1964; Van Bekkum, 1965]. It is also not likely in view of the same rate of recovery of mitogen responsiveness in spleen cells following the inoculation of fetal liver and bone marrow cells (paragraph 4.1.4). Thus it seems that the decreased GvH mortality following fetal liver cell transplantation is not the consequence of a deficient production of immunocompetent cells by the grafted embryonic HSC. It suggests rather that the lymphocytes arising from fetal liver HSC in the host tissue environment differ from those originating from bone marrow HSC in that they more easily attain a state of specific immunological tolerance.

The available data do not provide evidence on whether the characteristics of the lymphocytes developing from the fetal liver cell transplant differ from those being generated by the bone marrow as the result of:

a. the low number of HSC in the liver leading to a very gradual appearance of immunocompetent cells with a resulting tolerance; or

b. qualitative differences which already reside in the fetal liver HSC and which are transmitted to the lymphocytic offspring.

Clarification of this situation is of some practical importance, because, if the first factor accounts for the mild outcome of GvH disease, the use of smaller numbers of HSC can be considered as a means of preventing the delayed type of GvH disease, as has been advocated by Van Bekkum [1972] for treating CID infants (sneak-in).

The experiments described in this chapter were concerned with this issue.

6.1 *Survival experiments after transplantation of fetal liver and bone marrow cells*

The severity of GvH disease was determined following the infusion of equal numbers of bone marrow and fetal liver CFU-s, in order to exclude quantitative differences in the numbers of grafted HSC as the reason for the different patterns of GvH disease. Moreover, to rule out the influence of cells other than HSC, the effect of purified HSC grafts from both sources on delayed GvH disease was also studied.

The survival data after fetal liver cell transplantation are collected in table 6.1 (left section). It is evident from these results that the start of the mortality followed an effective protection during the first 30 days; mortality continued to occur over the second and the third months. The final 100-day survival varied between 32–42%. The progressive mortality was, according to the time of appearance, typical of a delayed GvH syndrome. This survival pattern was in clear contrast with the comparable bone marrow experiments (table 6.2, left section) in which, after a similarly effective initial repopulation, almost total mortality had occurred at 100 days after transplantation. It should be noted that this mortality was strictly confined to the second month in contrast to the fetal liver experiments.

Transplantation of purified stem cell fractions containing the same number of

Table 6.1 *Survival following allogeneic fetal liver cell transplantation (C57BL→CBA)*

graft size for groups I and II		*group I* unfractionated fetal liver					*group II* stem cell concentrates (fetal liver)				
nr. of CFU-s	nr. of CFC*	nr. of nucl. cells $\times 10^6$	nr. of mice	% survival day 30	day 60	day 100	nr. of nucl. cells $\times 10^6$	nr. of mice	% survival day 30	day 60	day 100
1200	56000	20–30	41	80	49	32	3–4	41	95	59	37
1900	86000	35–50	72	92	56	42	5–7	38	95	47	32
		pooled data	113	88	53	39		79	95	53	35

* Calculated by correction for spleen colony efficiency factor (2.2%).

Table 6.2 *Survival following allogeneic bone marrow transplantation (C57BL→CBA)*

graft size for groups I and II		*group I* unfractionated bone marrow					*group II* stem cell concentrates (bone marrow)				
		nr. of		% survival			nr. of		% survival		
nr. of CFU-s	nr. of CFC*	nucl. cells $\times 10^6$	nr. of mice	day 30	day 60	day 100	nucl. cells $\times 10^6$	nr. of mice	day 30	day 60	day 100
1200	30000	4–5	39	99	5	3	0.7	27	74	15	4
1900	46000	6–8	47	94	6	4	1 –1.2	37	86	5	0
3500	86000	12–15	81	84	1	0	1.8–2.2	33	91	3	0
		pooled data	167	80	4	2		97	85	7	1

* Calculated by correction for spleen colony efficiency factor (4.1%).

stem cells as were present in the unseparated haemopoietic cell transplants failed to modify the survival rate in the fetal liver or in the bone marrow infused hosts (tables 6.1 and 6.2, right sections). The unaltered GvH mortality pattern following the inoculation of stem cell concentrates (from which as much as 80–90% of the other

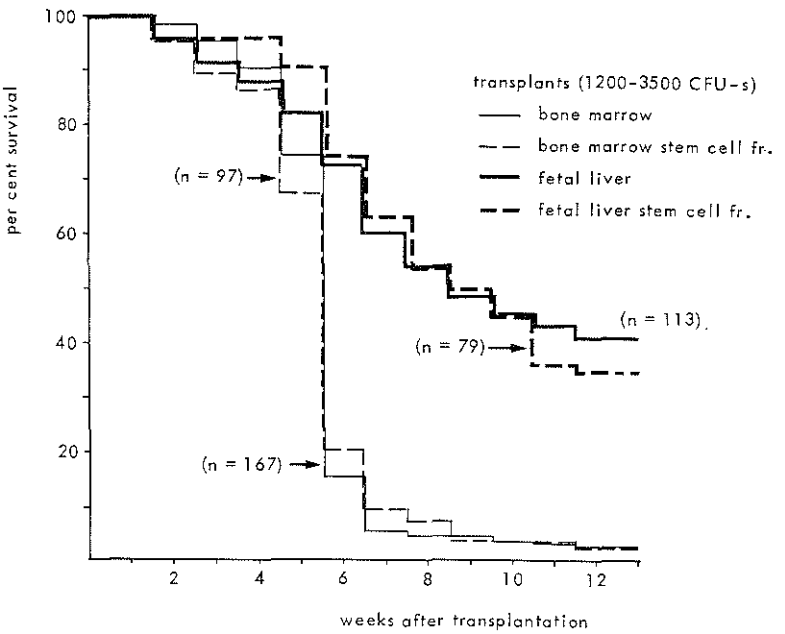

Fig. 6.1 *Survival of lethally irradiated mice following allotransplantation of fetal liver and bone marrow cells (C57BL→CBA)*
Pooled data from tables 6.1 and 6.2.

cells had been selectively removed (chapter 3)) indicates that the role of cells other than stem cells in the grafted cell suspension can be neglected in the pathogenesis of the delayed GvH reaction. This is consistent with the idea that the delayed GvH mortality after both fetal liver cell and bone marrow transplantation is initiated by cells derived from stem cells in the tissue. It is also apparent that the degree of delayed mortality among the subgroups receiving varying numbers of CFU-s of fetal liver or bone marrow did not change significantly (tables 6.1 and 6.2). In view of this, it is likely that the distinct mortality patterns are conditioned by qualitative properties of the embryonic and adult stem cells rather than by quantitative parameters.

The survival profiles following fetal liver cell and bone marrow transplantation give an overall picture of the characteristic difference between the two tissues (fig. 6.1). Despite the lower incidence of death following fetal liver cell grafting, the proportional mortality is still of considerable magnitude, namely, about 60%.

The literature [Barnes et al., 1962; Bortin and Saltzstein, 1969] contains indications that a primary incomplete immune restoration rather than a secondary immunodeficiency due to GvH disease is responsible for the delayed mortality following fetal liver cell treatment. A precise distinction between the two possible etiologies is made difficult by the fact that GvH reactions are regularly accompanied by immuno-

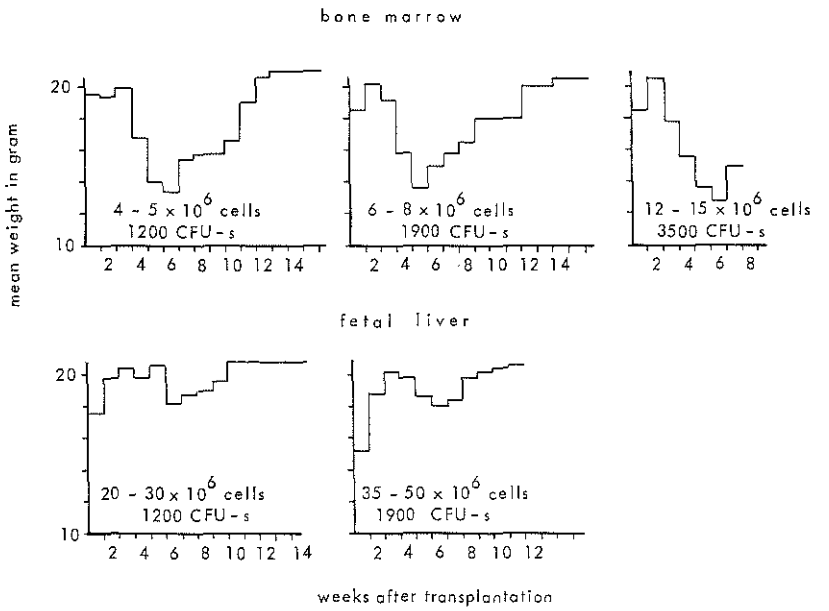

Fig. 6.2 *Weight curves of mice following allotransplantation with unfractionated bone marrow and fetal liver cells.*
The numbers of mice which were used for the follow-up measurements of body weight varied between 36–41 per group.

Table 6.3 *Pathology of allogeneic chimaeras (C57BL → CBA)*

	fetal liver		bone marrow	
lesions	nr. of animals*	degree of GvH disease	nr. of animals*	degree of GvH disease
duodenum				
– no abnormalities	3/10	–	6/10	–
– dead cells or cellular debris	7/10	±	4/10	±
ileum				
– no abnormalities	2/10	–	7/10	–
– dead cells or cellular debris	8/10	±	3/10	±
colon				
– no abnormalities	4/10	–	2/10	–
– dead cells; submucous oedema	2/10	±	0/10	
– cellular debris, dead cells, submucous oedema, infiltration of l.propria, hyperplastic crypts	4/10	+	8/10	+/++
liver				
– no abnormalities	0/10		5/10	–
– dead cells; granulocytic infiltration	10/10	±/+	5/10	+/++
spleen				
– no abnormalities	8/10	–	10/10	–
– hypotrophy of lymphoid cells	2/10	+	0/10	
lymph node				
– normal repopulation (follicles with germ centers)	6/10	–	2/10	–
– atrophy	4/10	+	8/10	+
bone marrow				
– normal repopulation	10/10	–	10/10	–
skin				
– normal	0/10		0/10	
– dyskeratotic cells; acanthosis	10/10	±/+	10/10	±/+
grading of GvH pathology per individual recipient	6/10	minimal	3/10	minimal
	4/10	moderate	6/10	moderate
			1/10	severe

Ten mice from each group of recipients treated with fetal liver cell or bone marrow grafts containing 1200 CFU-s were killed at 5 weeks after transplantation for histopathological examination of the organs. The degree of GvH reaction has been scored according to Van Bekkum and De Vries [1967]: – no GvH lesions; ± dubious; + slight; ++ moderate; +++ severe lesions. Grading of GvH pathology per individual host was done according to the criteria of ACS/NIH Bone Marrow Transplant Registry (1973).

* The proportion refers to the fraction of the total number of mice (10) which manifested the histological changes concerned.

deficiency [Simonsen, 1962; Van Bekkum and De Vries, 1967; Barchilon and Gershon, 1970; Zaleski and Milgrom, 1973].

In an attempt to make such a distinction, the histological changes in various organs have been studied by sacrificing mice at 5 weeks after the cell infusion. Microscopic examination revealed lesions typical of delayed GvH disease [Van Bekkum and De Vries, 1967]; the changes observed were colitis (necrosis, infiltration, submucous oedema), lymph node atrophy, cellular necrosis in the liver, and slight abnormalities of the skin (acanthosis, dyskeratosis) (table 6.3). The marrow was always found to be fully repopulated. These microscopic observations coincided with the characteristic clinical symptoms of diarrhoea and weight loss (fig. 6.2). On the whole, the macroscopic and microscopic abnormalities in the bone-marrow-treated recipients were similar but somewhat more pronounced. A high frequency of infectious complications was established. The incidence of positive blood cultures following death was in the order of 50–80% and involved mostly Gram negative

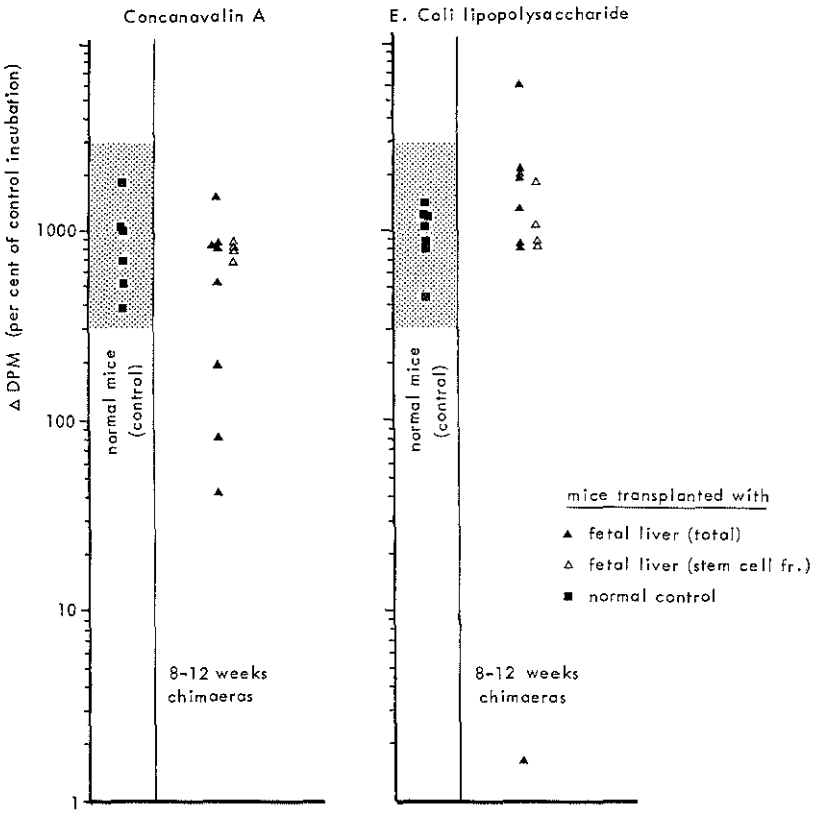

Fig. 6.3 *In vitro mitogen responsiveness of spleen cells from allogeneic fetal liver cell chimaeras (C57BL→CBA).*

bacteria. These findings are consistent with the classical picture of a delayed GvH syndrome [Van Bekkum and De Vries, 1967].

In order to verify whether an immunodeficient state plays a role in the mortality of the fetal liver cell recipients, as found after allogeneic bone marrow transplantation [Van Bekkum and De Vries, 1967; Van Bekkum et al., 1974], the mitogen responsiveness of the spleen cells was estimated. It was found that, during the period of 8–12 weeks after grafting, the spleen cells of the test animals displayed a subnormal *in vitro* lymphocyte reactivity to Con A (fig. 6.3). It had been demonstrated in chapter 4 that the mitogen reactivity in the syngeneic situation had returned to normal levels by this time. The cells of the animals gave normal responses to LPS, with one exception, which indicates that the recovery of the cell mediated immunity may be specifically impaired. If the decreased lymphocyte activity represents a primary immunodeficiency, the addition of lymphocyte precursors to the graft might compensate for the deficiency. On the basis of the findings of Bortin and co-workers [Bortin and Saltzstein, 1969; Bortin and Rimm, 1970], the effect of injection of fetal thymocytes as regards their ability to promote survival after fetal liver cell transplantation was investigated.

The results (table 6.4) show that the combination of *fetal* thymus lymphocyte precursor cells with the fetal liver cells did not have a beneficial effect on 100 day survival. Low numbers of *adult* thymocytes also had no positive effect, whereas higher numbers induced acute GvH disease. The finding that the immunological incompetence could not be overcome by treatment with lymphoid precursor cells, suggests that it represents a secondary manifestation of GvH disease.

Table 6.4 *Effect of injection of fetal and adult thymocytes on survival following unfractionated fetal liver cell and bone marrow transplantation (C57BL→CBA)*

	nr. of mice	per cent survival		
		day 30	day 60	day 100
fetal liver cells 28×10^6 nucl. cells – 2000 CFU-s				
no thymocytes	27	81	44	22
$+0.1$–1×10^6 fetal thymocytes	29	83	28	17
$+5\times10^6$ fetal thymocytes	28	89	25	18
$+0.1\times10^6$ adult thymocytes	14	86	29	21
$+5\times10^6$ adult thymocytes	13	23	8	0
bone marrow cells 6.4×10^6 nucl. cells – 2000 CFU-s				
no thymocytes	19	95	5	5
$+5\times10^6$ fetal thymocytes	20	95	5	5
$+5\times10^6$ adult thymocytes	20	20	0	0

The fetal and adult thymus cells were added to the haemopoietic cell transplant.

6.2 *Discussion*

The data presented in this chapter show improved survival of recipients receiving fetal liver cells as compared to animals grafted with adult allogeneic marrow. This is in accordance with the consensus in the literature on the favourable effect of fetal liver cells in reducing delayed mortality (table 6.5, summary of results). The difference in the survival pattern following fetal liver cell and bone marrow grafting has been shown to be due to differences in the severity of delayed GvH disease provoked by the two tissues.

Table 6.5 *Survival following non-isogeneic fetal liver cell transplantation (mouse) (collected data)*

		fetal liver				*bone marrow*	
						(when included as reference in same study)	
investigator	strain combination	cell dose $\times 10^6$ [1]	nr. of mice	gestational age (donor) in days	% survival 90–100 d	nr. of mice	% survival 90–100 d
Uphoff, 1958	C57BL→F_1 (C57BL × DBA)	3.7–15	40	14–16	92	104	11
	DBA→F_1	3.7	10	14–16	90	80	34
Crouch, 1959	C57BL→CBA	12.5–50	60	14–16	14 [2]	34	36 [2]
		70–135	18	14–16	59 [2]		
	CBA→C57BL	12.5–50	49	14–16	87 [2]	40	40 [2]
Urso et al., 1959	F_1(101 × C3H)→ F_1(C57BL × A)	50–65	47	12–19	71	88	15
Lengerova, 1959	(non inbred)	20	104	2–3 prenatal	62.5		
Barnes et al., 1961	C57BL→CBA	3–12	?	15–18	80–60	?	10
Bortin et al., 1969	A/J→CBA/J	20	160	13	42	50	0
		20	30	18	58		
Löwenberg, 1974	C57BL→CBA	1200 CFU-s [3]	41	16	32	39	3
		86000 CFC [4]	76	16	42	81	0

[1] per recipient (of bone marrow and fetal liver)
[2] 60 day survival
[3] 1200 CFU-s – 4–5 $\times 10^6$ bone marrow or 20–30 $\times 10^6$ fetal liver nucl. cells
[4] 86000 CFC – 12–15 $\times 10^6$ bone marrow or 35–50 $\times 10^6$ fetal liver nucl. cells

It was considered unlikely that the mortality after transplantation of fetal liver cells was due to a primary immunological insufficiency. This conclusion was based on the following findings:

1. The histological picture of the recipient animals was consistent with characteristic GvH disease.
2. The absence of a beneficial effect on survival by the supplementation of the graft with fetal thymic cells.

The marked lymphoid tissue atrophy, the *in vitro* signs of reduced mitogen activity, and the frequent occurrence of overt infections are suggestive that the immunodeficiency, though associated with and most likely caused by GvH disease, is the main causative factor in the mortality after fetal liver cell transplantation. The results of our experiments with the fetal thymus, do not agree with the data of Bortin and co-workers, who observed a positive effect of these cells on survival and concluded that a primary immunodeficiency was the major cause of late mortality after allogeneic fetal liver cell infusions [Bortin and Saltzstein, 1969; Bortin and Rimm, 1970]. The reason for this disagreement is unclear; perhaps differences in the mouse strain combinations are involved.

It has been demonstrated that the usefulness of gradient centrifugation is restricted to the elimination of cells responsible for eliciting the acute GvH reaction [Dicke et al., 1968, 1969]. This procedure apparently does not abrogate the delayed GvH reactions arising from bone marrow or from fetal liver cells.

The delayed GvH reactions occurring after bone marrow and fetal liver cell transplantation are presumed to be evoked by immunocompetent cells originating from the grafted HSC population. It cannot be excluded that lymphocytes derived from committed lymphoid precursor cells present in the graft take part in the delayed GvH reaction. Information with regard to the involvement of this cell population in the production of GvH disease can be obtained only when detection methods for committed lymphoid precursor cells become available. The results of our experiments in which purified stem cell suspensions were employed as grafts are in accordance with the concept that differentiation of HSC into immunocompetent cells is the cause of the delayed GvH reaction. The fact that the delayed GvH disease appeared to be unaffected by the removal of roughly 90% of the more differentiated cells in the grafts is compatible with the above concept.

These results are in support of inherent differences in fetal liver HSC as compared to bone marrow HSC. The possibility that numerical differences in the HSC populations in fetal liver cell and bone marrow grafts were responsible for the different degrees of delayed GvH disease was dismissed on the basis of survival data of groups of mice which received varying dosages of CFC and CFU-s. The bone marrow and fetal liver subgroups of recipients grafted with the largest cell inoculum received approximately equal quantities of HSC (calculated at about 86,000 CFC); yet the profiles of GvH mortality were consistently characteristic of the source of the haemopoietic cells transplanted. The possibility that a different distribution of injected HSC to spleen and marrow accounted for the divergent type of GvH syndrome also

was unlikely because the distinct survival patterns after fetal liver cell and bone marrow grafting remained unchanged when the numbers of CFU-s were matched instead of the CFC numbers. The data therefore favour the idea that the fetal liver type of GvH disease is brought about by specific attributes of embryonic stem cells.

This leads to the conclusion that the development of acute and delayed GvH disease is determined by the content of mature immunocompetent cells in the grafts [Van Bekkum and De Vries, 1967; Dicke et al., 1969] and the type of the HSC grafted will determine whether a severe or a mild delayed GvH reaction will appear. It is plausible to relate the special stem cell properties of fetal liver cells to the immature stage of development. Since embryonic HSC are the precursors of the adult stem cell population in normal ontogenesis [Metcalf and Moore, 1971f], it would have been more logical to expect that a bone marrow pattern of GvH mortality would occur after fetal liver cell grafting, be it with a certain delay. This would also have been in keeping with the finding that fetal liver stem cells undergo normal ontogenetic cellular changes after transfer to an adult environment [Metcalf and Moore, 1971f]. In view of the observation that lymphocyte recovery after fetal liver cell and bone marrow transplantation occurred at a similar rate (chapter 4), there is no real indication suggestive of a delayed development of immunologically competent cells, which would in turn favour the induction of tolerance in the fetal liver chimaeras. As the less severe GvH reaction has to be attributed to an enhanced tendency of the newly generated lymphoid cells to develop immunological tolerance to the antigens of the allogeneic hosts [Vos et al., 1960; Tyan, 1964], this tendency has to be regarded as the expression of intrinsic differentiation properties specific of fetal liver stem cells. It seems to imply that the bone marrow stem cell offspring originating from grafted fetal liver stem cells in the allogeneic host is not identical with a bone marrow stem cell population which has descended from embryonic liver cells in normal maturation, i.e., noninterrupted and in a syngeneic environment.

Although the survival rate following fetal liver cell transplantation proved to be superior to bone marrow grafting in the present investigation, there is still a considerable incidence of mortality when no additional treatment is applied. The demonstration of a high incidence of terminal septicaemia in mice expiring from fetal liver GvH disease suggests that bacterial infections are a decisive step in the sequence of pathological events leading to death similar to the situation in GvH disease following bone marrow grafting. As it has been shown that the delayed GvH mortality after bone marrow transplantation is not due to direct immunological damage to organs and tissues but rather to complicating infections [Van Bekkum et al., 1974], it is suggested that the differences between bone marrow and fetal liver GvH mortality can be explained as a reduced susceptibility to infections due to minimized GvH reactions. It is to be expected that, similar to bone marrow grafting, the prevention of superimposed infections by inducing and maintaining the gnotobiotic state for the time of the immunosuppressive condition may prevent the mortality during that period.

CHAPTER 7

GENERAL DISCUSSION. IMPLICATIONS AND RECOMMENDATIONS FOR FETAL LIVER CELL TRANSPLANTATION IN HUMANS

7.1 The position of fetal liver cell transplants

Since methods have become available in recent years, which are effective in the prevention of acute GvH disease in allogeneic bone marrow transplantation [Dicke et al., 1968, 1970], it is the delayed GvH reaction that poses the major problem today. In order that haemopoietic cell transplantation may become a reliable treatment regimen for patients with a defective stem cell compartment (aplasia, CID), attempts to prevent or control GvH disease are of primary importance. The GvH reaction is difficult to treat once it has become manifest; therefore, attention has been increasingly directed towards possible modes of prevention. In this connection, fetal liver cell transplants are of great interest, firstly, because they do not elicit acute GvH reactions and, secondly, because they have been shown to induce less severe delayed GvH reactions than allogeneic bone marrow cells. This procedure deserves special consideration, because the effect of measures which are being developed for the control of GvH disease following allogeneic bone marrow grafting is expected to be limited.

In the first place, progress in donor selection is not likely to fulfil the needs of a generally preventive method because a. the approach of selecting the non-GvH inducing subclass among the histocompatible sibling donors, if successful, will tend to confine bone marrow therapy to an even more select group of patients; b. one cannot be very optimistic about the successful prevention of GvH disease by using MLC/HLA compatible donors outside the family. This latter opinion is based on the fact that even bone marrow grafts from siblings who are full-house identical for the major histocompatibility complex induce GvH reactions in as much as 50% of the cases in the treatment of aplasia [Storb et al., 1974]. It is anticipated therefore that progress in histocompatibility testing will at best provide only a partial solution to the problem of delayed GvH disease.

Secondly, the procedure of bacteriological decontamination and isolation is not expected to completely solve the problem. This procedure was highly promising in mice in that delayed GvH mortality could be completely prevented while allowing restoration of the immune system [Van Bekkum et al., 1974]. Very recent data of Hendriks et al. [1974] in the monkey confirmed that decontamination significantly mitigated the lesions characteristic of delayed type GvH disease. However, this was associated with a prolonged state of immuno-deficiency during which the animals

died from overwhelming viral infections. Although the method had a satisfactory effect in postponing or preventing the onset of delayed GvH mortality following allogeneic bone marrow transplantation, it did not offer a complete solution.

Other current approaches (e.g., immunological enhancement) aimed at the control of delayed GvH reactions cannot be considered for the time being because they are not at a stage of development which meets the conditions for practical application.

Considering this, there are obvious needs for other methods to avoid severe delayed GvH disease. On the basis of the existing experimental and clinical evidence, it is envisaged that fetal liver cell grafts may, at least for a certain group of patients, satisfy these needs. Thus, for the time being, fetal liver cell grafts do seem to have practical applicability, although the category of potential recipients is as yet limited.

7.2 Graft versus Host disease after fetal liver cell transplantation

The experimental data collected in this study have shown that the moderate character of the delayed GvH reaction produced by fetal liver cells is due to special properties intrinsic to the embryonic HSC and not to numerical differences between the HSC in the fetal liver and bone marrow cell populations. It was demonstrated that stem cell purification of the fetal graft prior to transplantation (devised particularly for the elimination of acute GvH reactive cells from the graft) did not modify the incidence of GvH mortality in the mouse. It is not certain that a similar conclusion applies to human fetal liver cell grafts, in view of the observations on the PHA reactivity of fetal liver cells (table 7.1). In our limited series, strongly positive PHA reactivity was found at a gestational age of 16 weeks or later.

These findings, considered in connection with previous results of Dicke et al.

Table 7.1 *PHA responsiveness of human fetal liver and fetal thymus cells*

	fetal liver		fetal thymus	
embryonic age (wks)	control dpm	PHA response Δdpm	control dpm	PHA response Δdpm
8	288	+ 25	29	+ 1162
12	3182	− 182		
16	55	+ 106	22	+ 2806
16	783	+ 62		
16	5431	+1619		
17	1756	− 305		
17	120	+ 87		
18	780	− 80	95	+14560
18	2228	+ 826		
18	15	+ 2	11	+ 1028
18	21	+ 334	12	+ 530
18	82	+ 196	10	+ 1948

Table 7.2 *PHA responsiveness of adult and fetal haemopoietic cell grafts in mouse and man*

	mouse	man
bone marrow	−	+*
fetal liver	−	− or +

* According to Dicke et al. [1969, 1970].

[1968, 1970], reveal a species difference between human beings and rodents as regards the proportion of PHA responsive lymphocytes in both fetal liver and bone marrow: these cells being considerably more abundant in the human tissues than in the analogous haemopoietic tissues of mice (table 7.2). Therefore, one should be aware of the possibility of inducing acute GvH disease with fetal liver cell transplantation in humans. This raises the question as to whether special preventive measures to avoid these complications should be taken (see paragraph 7.4).

7.3 Considerations on the take of fetal liver cell grafts

It was outlined in chapter 1 that failures of take represent a serious bottleneck in fetal liver cell transplantation in man. The uncertainty as to the source of the difficulties prompted the present investigation into the biological basis of the take of fetal liver cell grafts. In the course of these studies, two factors were recognized to play a major role in determining takes following fetal liver cell allografting: a. the low number of stem cells in fetal liver; and b. the comparatively high susceptibility of the fraction of fetal liver HSC which initiate repopulation to allogeneic repression.

Titration studies of purified and nonpurified grafts also established the fundamental rule that the takeability problem is relative, in that it can be overcome by increasing the number of HSC in the graft. This can be achieved in mice simply by the pooling of fetal livers from the same inbred strain. In man, pooling is not practical, because it leads to a miscellaneous immunogenetic composition of the graft which suppresses the takeability and therefore counteracts the effect of increasing the number of stem cells [Van Bekkum, 1969a]. This led us to search for methods by which fetal liver HSC of a single immunogenetic make-up could be reproduced in larger numbers. In the culture system which was chosen for these studies, a net production of HSC has not been achieved so far, although self-replication of this cell population could be significantly augmented. Apparently, proliferation in this system was counteracted by a marked loss of HSC due to either death or differentiation. Further investigations, e.g., isolation of the so-called stem-cell-activating factor (SAF), should prove whether *in vitro* production of HSC by means of simple culture methods can be obtained. At the same time, this poses the question as to whether these HSC retain their normal proliferation capacities following *in vitro* multiplication.

Another line of investigation concerned with the promotion of the take of the

haemopoietic cell transplant was the addition of fetal thymocytes to the liver cell graft. These cells did show a stimulating effect on early repopulation, which is in agreement with the findings of Bortin et al. [1973] that *fetal* thymus cells are capable of enhancing take. However, this course of action carries the risk of inducing a severe GvH reaction in man. At all fetal ages tested, strongly PHA positive responses were scored throughout our fetal thymus series, the earliest sample being from a fetus of 8 weeks gestation (table 7.1). The concern for possible GvH hazards following the application of these cells in humans is not contradicted by the experience that i.m. implantation of small fragments of fetal thymus (up to 16 weeks of fetal age) does not induce GvH disease, because the introduction of GvH aggressive cells as organ pieces via the i.m. or s.c. route is far less efficient than the i.v. administration of cell suspensions [Van Bekkum, 1972]. If one accepts that the strict correlation between PHA responsiveness (*in vitro*) and GvH reactivity [Dicke et al., 1968; Dicke, 1970] also applies to human embryonic cell suspensions, one should either follow a very conservative policy as regards the use of fetal thymocytes in clinical practice, or employ irradiated thymus cells. However, no data on the effectivity if irradiated thymus cells in stimulating take of fetal liver cell allografts are available. It was shown in our experiments that irradiated adult thymocytes are similarly active as are non-irradiated thymus cells in stimulating haemopoiesis in syngeneic spleen colonies. Whether irradiated thymocytes are also capable of promoting take in allogeneic conditions is not known. If so, it is probably feasible to employ irradiated human fetal thymocytes in attempts to promote the take of fetal liver cell grafts, because the human fetal thymocytes at the ages under consideration seem to have many of the characteristics of murine adult thymocytes (paragraph 4.2.5).

Finally, it should be stated that in a re-evaluation of the past clinical experiences with fetal liver cell transplantation, it was concluded that grafting of an insufficient number of cells might have been the principal cause for the disappointing results. In this respect, two factors were considered to be involved in the failures of these cells to take: a. the use of frozen cells; and b. the administration of the graft by the i.p. route (paragraph 1.3.6).

Our increased insight into the biology of fetal liver cell takeability provides a number of new possibilities for ensuring engraftment of fetal liver cells in human recipients. The following recommendations are made for future attempts at fetal liver cell transplantation.

7.4 Recommendations for fetal liver cell transplantation

Faced with the difficulty of engrafting fetal liver cells, the design of the transplantation protocol should place full emphasis on the take of the graft. In this respect, the strategy recommended for fetal liver cell grafting with its relatively low risks of acute GvH disease should differ from that for bone marrow grafting which is first of

all concerned with the prevention of GvH disease. With this in mind, a transplantation regimen is proposed which is deliberately centred on avoiding all possible factors which might in some way interfere with repopulation, such as freezing and gradient separation. The latter technical refinements may be added after sufficient experience with fetal liver cell takes has been gained, similar to the line of development seen in bone marrow transplantation.

I. Selection of patients

In view of the difficulties encountered in obtaining a take, it seems realistic to confine fetal liver cell grafting, for the present time, to CID patients. They represent the most readily accepting group of recipients, so that relatively low numbers of HSC can be expected to produce takes. This has indeed been borne out by bone marrow transplantation in CID patients. Excellent results have been obtained with bone marrow cells from genotypically HLA-identical donors and this treatment has resulted in either a surprisingly mild GvH disease or no GvH reaction at all [Van Bekkum, 1972]. However, such donors are not available in the majority of the cases. It is for this category of patients that transplantation of fetal liver cells must be considered.

Only when the problems of engraftment in CID patients can be regularly overcome and when it turns out that the GvH disease following transplantation of fetal liver cells, like in animals, is of a moderate type will it become useful to extend the fetal liver cell approach to the treatment of aplastic patients. Even then, the limited cell numbers obtained from one liver will preclude their therapeutic use in aplasia unless the present possibilities for *in vitro* multiplication are further developed.

II. Cell dose and age of fetus

In laboratory investigations concerned with transplantation of haemopoietic cells, no convenient model for CID recipients is available. For that reason, bone marrow transplantation research relies predominantly on experience in animals rendered aplastic by lethal irradiation. Such recipients need full repopulation, in contrast to CID recipients in which only the reconstitution of a single haemopoietic cell line (lymphocytic) is required. This explains why the CID patients require less cells for reconstitution than do aplastics. However, one may assume that the qualitative differences in repopulation properties between fetal liver and bone marrow cells, as observed in irradiated recipients, are essentially similar in CID hosts.

Taking into account the factors which have been demonstrated in murine experiments to have different effects on the repopulation by fetal and adult haemopoietic cells, and assuming that similar factors play a role in human recipients, the number of fetal liver cells needed to produce takes in histo-incompatible CID patients can be calculated from the number of bone marrow cells required. As immunocompetent lymphocytes syngeneic to the haemopoietic graft are known to markedly facilitate

the take in the allogeneic recipient [Van Bekkum and De Vries, 1967], the large numbers of lymphoid cells present in human bone marrow may significantly alter the analogy with the mouse. It seems more valid, therefore, to start the calculation from the number of cells of a purified human bone marrow graft needed for a take. This number is represented by the figure of 5×10^6 cells per kg of a HLA/MLC non-identical bone marrow stem cell concentrate [Van Bekkum and Dicke, 1972; Van Bekkum, 1972]. If we assume that the HSC population in the fraction is concentrated by a factor of 10 on the average [Dicke et al., 1968, 1970; Dicke, 1970] this figure is equivalent to 5×10^7 cells in terms of unfractionated bone marrow per kg. If we further assume (by analogy with the mouse) a 3-fold reduced stem cell concentration in fetal liver and a 2-fold allogeneic suppression of the efficiency of repopulation by embryonic HSC, the fetal liver cell number required for a take in an incompatible CID infant will be in the order of 3×10^8 cells per kg. This 6-fold larger cell dosage for fetal liver cell grafts is somewhat higher than the 4-fold difference between fetal liver and bone marrow cell numbers required to restore irradiated monkeys, which can be derived from the results published by Van Putten et al. [1968]. Therefore, we have adopted for our present recommendations a factor of 4–6 as the cell number difference between allogeneic fetal liver cell and bone marrow transplants. If we take the average body weight of a CID recipient to be 6 kg, a total cell number of $12–18 \times 10^8$ fetal liver cells would be required. According to data collected on human fetal liver cell yields (table 7.3), only livers of a gestational age of at least 16 weeks will serve to provide these numbers.

One should first await the results of the suggested animal experiments dealing with the question of the capacity of irradiated thymus cells to promote allogeneic fetal liver cell takes until deciding whether the additional i.v. injection of the thymocytes from the same embryo warrants application.

Table 7.3 *Cellularity and weight of developing human fetal liver*

maturation age (wks)	nr.	weight (gr)	cell yield	
			range	mean
8–12	3	1.5– 4.9	$0.4–1.0 \times 10^9$	0.6×10^9
16	2	4.3– 5.7	1.3×10^9	1.3×10^9
17–18	7	5.0–10.8	$1.3–3.6 \times 10^9$	2.5×10^9
20	1	19	4×10^9	4×10^9

III. Fresh versus frozen cells

Frozen cells offer three important advantages:

a. the possibility of using all suitable embryos received;
b. the opportunity to test the fetal liver cell graft on PHA activity before use; and
c. the availability of fetal liver cell grafts at all times.

In spite of the development of a storage method which allows the complete preservation of HSC without a decrease in the concentration of viable HSC [Schaefer et al., 1972a], one has to account for unavoidable losses of cells resulting from the extra steps included in the handling of the cell suspensions (e.g., repeated cell centrifugations). Because of the poor experience with frozen fetal liver cell transplants in the past [Soothill et al., 1971; Githens et al., 1973] and because of the necessity to eliminate all factors which might interfere with the results of fetal liver cell grafts in man, it is felt that preference should be given to the use of *fresh* fetal liver cells for the present time.

IV. Intravenous versus intraperitoneal administration

It is suggested that the cells be given by i.v. route, and not i.p., because of the marked increase in the chance of effective repopulation by i.v. administration [Schaefer et al., 1972b]. When the liver tissue is processed according to the procedure described in Materials and Methods (paragraphs 2.2.3 and 2.2.4), the intravascular administration does not carry a significant risk for embolism; in at least 3 human patients and in 10 monkeys treated so far, no complications due to the i.v. injection have been encountered.

V. Additional measures aimed at the prevention of GvH disease

According to the results in rodents reported here (chapter 6) and elsewhere [Uphoff, 1958, 1959; Urso et al., 1959; Lengerova, 1959; Crouch, 1959; Barnes et al., 1961], the amelioration of the GvH reaction by the use of fetal liver cells is not enough to assure complete survival. This may suggest the necessity for additional measures aimed at inhibition of delayed GvH disease in human fetal liver cell transplantation. In this respect it should be noted that one should avoid immunosuppressive treatment for mitigation of the GvH reaction, if possible, because it interferes with the objective of the treatment in CID.

The frequent occurrence of complicating bacterial infections during GvH disease in conjunction with only minor lesions directly attributable to the GvH reaction (paragraph 6.1) is a strong argument in support of the idea that the fetal liver GvH reaction, similar to the GvH events after bone marrow transplantation [Van Bekkum et al., 1974], does not directly cause death, but plays a permissive role in the occurrence of fatal infections throughout the prolonged period of immune incompetency. Consequently, it seems important to institute powerful prophylaxis to prevent infections. When applied throughout the major period of immune deficiency, the gnotobiotic state has proven to be an effective way of preventing endogenous as well as exogenous bacterial infections in rodents [Van der Waay et al., 1970; Van Bekkum et al., 1974]. Recent results of bone marrow research in the monkey indicate restricted benefit from this method in histo-incompatible bone marrow grafting in primates. However, it must be considered that, in the situation of the milder delayed GvH syndrome

following allogeneic fetal liver cell transplantation, the beneficial effect of germfree conditions may accordingly increase, as was seen in the comparatively mild bone marrow GvH disease encountered in rodents [Van Bekkum et al., 1974].

Because it is necessary to use donors older than 16 weeks for collecting sufficiently large fetal livers from a point of view of takeability (see above) and because 3 out of 10 grafts in our series of fetal livers beyond this age manifested a clearly positive PHA response (table 7.1), the possibility must be considered that – contrary to the rodent and monkey transplantation models – acute GvH disease may complicate the transplantation of fetal liver cells in man. Using the PHA reactivity as a specific indicator for acute GvH activity [Dicke et al., 1968, 1970; Dicke, 1970], a PHA assay could serve to test the cell suspensions prior to transplantation in order to discard potentially hazardous fetal liver cell grafts. This underlines the great importance of a rapid PHA test measuring the response within a few hours [Van den Berg, 1974]. A very recently reported rapid MLC test (1 h) may offer promise for the development of such an assay in the near future [Parsa and Kountz, 1974].

Another possibility is to subject the fetal liver cell grafts to cell separation [Dicke et al., 1968, 1970; Dicke, 1970] for removal of GvH reactive cells. However, both PHA testing and gradient separation require preservation of fetal liver cells, at present with the disadvantages mentioned above. Apart from the PHA monitoring, the gradient has the relative disadvantage in that certain losses of HSC will occur. It must be concluded, therefore, that, although purification of fetal liver stem cells is technically possible, this procedure should not be used until experience with whole fetal liver cell grafts has demonstrated the necessity for additional measures for preventing acute GvH complications.

The present investigation has disclosed some of the special factors which are operative in the take of fetal liver cell grafts. No allowance has been made for these factors in fetal liver cell transplantation history. It is concluded that fetal liver cell transplantation has occurred under suboptimal conditions in the past and it is likely that this has had an important bearing on previous failures. Because of this, the restorative potencies of these grafts in man are still obscure. Taking these findings into account, a strategy for fetal liver cell transplantation in the human has been suggested. Due to the rare incidence of CID, however, it will take a long time before insight into the potentialities of fetal liver cell grafts will be gained. From a practical standpoint, it is therefore of great importance to conduct international cooperative trials. This is the only way to evaluate the usefulness of fetal liver cell grafts for the human being within a reasonable space of time. If, indeed, takes of fetal liver cell grafts can be repeatedly obtained in CID patients with the procedure proposed, it will become apparent whether the dissimilarity in GvH inducing properties of fetal and adult HSC, which has been found in the mouse, also applies to the human species.

SUMMARY AND CONCLUSIONS

Fetal liver cell transplantation deserves intensified interest because, according to previous experimental evidence, it may represent a useful approach to reduce or avoid severe Graft-versus-Host (GvH) reactions following treatment with allogeneic haemopoietic cell grafts. The application of fetal liver cells in man has not been very successful so far. The present investigation in the mouse was concerned with the practical issues of elucidating the causes for the repeated failures of fetal liver cell grafting in patients and of establishing the applicability and limitations of these grafts. This study also had interesting physiological aspects. It allowed us to investigate the specific growth properties of the embryonic haemopoietic stem cells (HSC) and to add information to our incomplete knowledge of factors controlling the differentiation and proliferation of these cells.

The study was introduced by a chapter (1) reviewing the present status of allogeneic bone marrow transplantation. After a discussion of some basic principles of haemopoietic cell transplantation and the stage of development of methods for the mitigation of GvH disease, specific consideration was given to fetal liver cell transplantation. A description of the ontogenetic development and the characteristics of fetal liver haemopoiesis was provided and this was followed by a review of the experiences with fetal liver cell transplants in animals and humans as reported in the literature. From this it was particularly evident that failures of engraftment have hitherto represented the most serious single barrier in the successful application of fetal liver cell grafts.

The materials and methods employed in this study were described in chapter 2.

Chapter 3 was concerned with defining the cellular constitution of the fetal liver cell transplant, with major reference to the HSC population. Using assays for estimation of CFU-s numbers as well as the spleen colony forming efficiency of fetal liver stem cells made possible an enumeration of the HSC population in the fetal liver cell suspension. It was found that the HSC in the embryonic liver were present in approximately 3 × lower frequency than in the bone marrow tissue. The methodology of the assays was fully discussed. Discontinuous albumin density gradient centrifugation was employed to prepare fetal liver stem cell concentrates for transplantation purposes. Using this method it was confirmed that fetal liver HSC (1.057 g/cm^3) were of lighter density than bone marrow HSC (1.064 g/cm^3).

The problems encountered in engrafting fetal liver cells prompted a study of the mechanism of take (chapter 4). Stem cell kinetics after haemopoietic cell transplantation were determined in direct relation to take. Several factors which determine

engraftment and which differently influence fetal liver and bone marrow cell descendant repopulation were clarified:

a. The transfusion of the large number of more differentiated cells together with the HSC in the fetal liver cell transplant was shown not to have a beneficial influence on engraftment. This supported the concept that the HSC in the fetal liver cell transplant represented the major part of the cellular basis for haemopoietic reconstitution.

b. Under *syngeneic* transplantation conditions, fetal liver stem cells had the same repopulation proficiency as those from bone marrow. The 3 × higher nucleated cell numbers from fetal liver required for a successful take, as compared to bone marrow, correlated with the lower frequency of HSC in the embryonic liver tissue.

c. The equivalent repopulation efficiency of both kinds of HSC was in good accord with the proliferation kinetics of fetal liver and bone marrow HSC: doubling of fetal liver CFU in the haemopoietic tissue of the host was more rapid but the fraction (f) of the total number of administered fetal liver CFU which initiated repopulation was smaller; in addition, the generation of differentiated progeny per injected fetal liver CFU was shown to be lower.

d. From studies on fetal liver cell take in *allogeneic* recipients, it became apparent that the f-fraction of fetal-liver-derived CFU repopulation was repressed by a factor of 2 in allogeneic conditions which was in contrast to that of bone marrow; this accounted for a 2-fold difference in HSC numbers and a 6-fold difference in nucleated cell numbers needed for effective fetal liver cell versus bone marrow allotransplantation.

e. The 5–10 times larger cell numbers needed for allografting of both kinds of grafts (as compared to isografting) was partly due to a reduction in the f-fraction of CFU and partly to inhibited differentiation but could not be fully accounted for. It was shown that impaired repopulation in allogeneic hosts in the first week following transplantation was mostly caused by so-called environmental resistance factors of the recipient to the donor type cells. Arguments were provided in support of the fact that at later stages immunological processes (cell mediated allograft rejection, GvH reaction) also interfered with reconstitution.

f. The addition of thymocytes to the haemopoietic cell graft markedly enhanced haemopoietic repopulation in the host (as measured by spleen colony formation) which indicated the presence of allogeneic resistance and even syngeneic resistance (ontogenetic) to grafted fetal liver HSC.

g. Attempts to promote take by employment of thymus cells in allotransplantation proved not convincing enough to warrant practical application (narrow effective dose range, risks for GvH disease, etc.). Some interesting possibilities for further exploration and development of this line of research were discussed, in particular, the employment of irradiated thymus cells.

Another possible approach to enhance fetal liver cell take was outlined in chapter 5. It was attempted to increase the number of stem cells in the transplant by multiplication of the number of HSC prior to grafting. For this purpose, a liquid culture system in which *in vitro* proliferation of HSC could be measured was devised; proliferation of HSC was determined by estimating the maintenance of CFU numbers and the cycling status of CFU at regular intervals. Various factors were tested for their effect on these two parameters of CFU in culture: serum from lethally irradiated mice, haemolysate, irradiated fibroblasts (i.e. the stimulating source of the so-called thin agar layer system (TALS) for myeloid colony growth *in vitro*). Evidence was obtained that self-replication of HSC in cultures containing fibroblasts or fibroblast-conditioned medium occurred and that it was dependent on the action of a stem cell activating factor (SAF). This factor was shown to be distinct from the colony stimulating factor (CSF) and from another product (haemolysate) which was able to sustain increased survival of HSC in culture. The experiments may offer promising possibilities for producing HSC *in vitro* for transplantation purposes. As yet, the method has not reached a sufficient stage of development to allow net production from limited numbers of HSC.

As reported in the literature, fetal liver cell grafts compared favourably with bone marrow cells in that they elicited less severe delayed GvH reactions in our experiments. In specially designed experiments based on stem cell purification and quantitation of HSC numbers in the graft, the basis for the distinctly weak delayed GvH disease following fetal liver cell engraftment was traced back to intrinsic properties of the fetal liver HSC (chapter 6). According to the findings in this chapter, a somewhat modified classification of GvH disease was proposed which distinguished, apart from the acute GvH reaction which is directly caused by grafted immunocompetent cells, two other types: a severe delayed GvH reaction mediated by the lymphocytic progeny of adult HSC in the graft and a less severe delayed GvH disease mediated by lymphocytic descendant cells from transplanted fetal HSC.

In chapter 7 the results of these investigations were put into the perspective of fetal liver cell transplantation in man; in this context, a strategy for attempts at fetal liver cell transplantation in patients suffering from severe combined immunodeficiency disease (CID) was proposed.

SAMENVATTING/SUMMARY IN DUTCH

De late Graft-versus-Host (GvH) reactie vormt hedentendage waarschijnlijk de belangrijkste hinderpaal voor uitgebreide toepassing van allogene beenmergtransplantatie. Afgaande op dier-experimentele waarnemingen uit het verleden, mag de foetale leverceltransplantatie worden beschouwd als een alternatieve vorm van transplantatie van bloedvormende cellen die gepaard gaat met minder risico's van late GvH reacties. Hieraan ontleent de foetale leverceltransplantatie zijn betekenis. Opmerkelijk genoeg hebben pogingen van foetale leverceltransplantatie bij de mens echter tot op heden weinig effect gesorteerd; hierbij stonden moeilijkheden inzake het aanslaan van foetale levercellen centraal. De oorzaken hiervan waren grotendeels onbekend. Inzicht in de achtergrond van deze problemen is van rechtstreeks belang voor de ontwikkeling van foetale leverceltransplantatie tot een praktische methode voor transplantatie van haemopoietische cellen met minder risico van GvH ziekte. De huidige studie, uitgevoerd in de muis, was gericht op een opheldering van de oorzaken van de mislukkingen bij de mens en op het afbakenen van de mogelijkheden en beperkingen van foetale leverceltransplantatie. Daarbij waren de groeikarakteristieken van embryonale bloedvormende cellen en de factoren die de proliferatie en differentiatie van cellen van het haemopoietische systeem na transplantatie reguleren, belangrijke aspecten van onderzoek. Dit was met name mogelijk dank zij een aantal recente ontwikkelingen in inzichten en methodologie betreffende de haemopoiese en de beenmergtransplantatie waardoor de rol van de pluripotente stamcel in het transplantatieproces (aanslaan, GvH reactie) in het onderzoek kon worden betrokken.

In hoofdstuk 1 van deze studie werd een overzicht gegeven van de betekenis en de ontwikkeling van de beenmergtransplantatie. Nadat enkele basisprincipes van beenmergtransplantatie waren beschreven, werd specifieke aandacht geschonken aan de foetale leverceltransplantatie zelf. Achtereenvolgens kwamen aan de orde: de ontogenetische ontwikkeling en aard van de foetale lever haemopoiese en een evaluatie van de foetale leverceltransplantaties in proefdier en mens uit de literatuur.

In hoofdstuk 2 werden de materialen en methoden beschreven, die in het onderzoek van dit proefschrift zijn gebruikt.

In hoofdstuk 3 werd aan de hand van microscopisch onderzoek en functionele testen op een aantal specifieke celtypes, de cellulaire samenstelling van het foetale leverceltransplantaat gedefinieerd. De resultaten wezen op het opvallend erythropoietische karakter van de bloedcelvorming in de embryonale lever en het gebrek aan lymphopoietische en myelopoietische activiteit. Wat betreft de afwezigheid van deze laatste cellijn kon worden aangetoond dat ook de myeloide voorloper cellen (CFU-c)

numeriek in geringere mate aanwezig zijn. Ruime aandacht werd geschonken aan de populatie van de pluripotente haemopoietische stamcel (HSC) in foetale lever. Met behulp van de miltknobbelproef en een methode ter bepaling van de efficiëntie waarmee deze test stamcellen detecteert in een intraveneus toegediende celsuspensie, was het mogelijk om het aantal stamcellen in de foetale levercelsuspensie bij benadering vast te stellen. Het bleek dat de HSC in de embryonale lever in een gemiddeld 3 × lagere concentratie voorkomt dan in het beenmerg. De methodologie van deze bepaling werd uitvoerig ter discussie gesteld.

Voor het bereiden van stamcel-concentraten uit foetale lever werd de discontinue albumine dichtheid-gradient techniek toegepast. Daarmee konden gezuiverde stamcel suspensies worden verkregen die ontdaan waren van 80–90% van de rijpere cellen die naast de HSC in het transplantaat aanwezig zijn. Vergelijking van deze foetale levercelfracties met ongefractioneerde celsuspensies opende de mogelijkheid voor het maken van een onderscheid tussen de rol van HSC en de meer gedifferentieerde cellen in de repopulatie en het ontstaan van GvH ziekte na transplantatie (zie onder). Impliciet kon worden bevestigd dat de dichtheid van de foetale HSC (1,057 g/cm^3) geringer is dan die van zijn volwassen tegenhanger (1,064 g/cm^3).

Het moeilijke aanslaan in dier-experimentele en humane foetale leverceltransplantaties vormde de aanleiding voor het onderzoek naar het mechanisme en de kinetiek van de repopulatie na transplantatie (hoofdstuk 4). In titratie-studies, waarin groepen letaal bestraalde muizen toenemende hoeveelheden stamcellen ontvingen, werd nagegaan wat de minimum benodigde hoeveelheid cellen was om 80% overleving op dag 30 te bereiken (als maat voor protectie tegen sterfte aan het beenmerg-stralingssyndroom). Voorts werd de stamcelproliferatie-activiteit van foetale lever en beenmerg rechtstreeks gemeten door bepaling van de aantallen HSC in beenmerg en milt met regelmatige tijdsintervallen na de transplantatie. Deze proeven, die werden uitgevoerd zowel in syngenetische als in allogenetische ontvangers, brachten de volgende gegevens aan het licht:

a. de HSC in het transplantaat is voornamelijk, zo niet geheel, verantwoordelijk voor repopulatie; de eliminatie van de rijpere cellen uit het transplantaat had geen effect op de overleving;

b. in *syngenetische* transplantatie condities zijn foetale en volwassen stamcellen evengoed in staat om letaal bestraalde muizen te repopuleren; dit kon in verband worden gebracht met de repopulatie-kinetiek van beide soorten stamcellen: de proliferatiesnelheid van de foetale HSC was hoger t.o.v. die van de beenmerg HSC, maar de fractie (f) van de ingespoten portie stamcellen die de repopulatie initieerde was lager; voorts werden aanwijzingen verkregen dat de differentiatie van foetale levercellen geringer was op een per-ingespoten-stamcel basis.

c. hoewel voor het aanslaan van het foetale leverceltransplantaat evenveel stamcellen dienden te worden toegediend, lagen de totale celaantallen benodigd voor

foetale leverceltransplantatie ongeveer 3 × hoger dan voor beenmerg, in verband met de lagere concentratie van HSC in dit weefsel.

d. in *allogene* transplantatie waren zelfs 6 × zoveel foetale levercellen nodig voor aanslaan, vergeleken met beenmergtransplantatie; dit extra-verschil kwam overeen met een behoefte aan 2 × meer stamcellen voor foetale lever en kon worden toegeschreven aan een 2 × zo sterke suppressie van de proliferatieve fractie, f, van de ingespoten stamcellen in de allogene gastheer.

e. aanwijzingen werden verkregen dat in de beginperiode na transplantatie vooral de zgn. microenvironment-factoren van de gastheer (allogeen) verantwoordelijk zijn voor de groeiremming; het was aannemelijk dat in een latere fase ook immunologische reactiemechanismen de repopulatie verstoren.

f. pogingen om de haemopoiese van foetale levercellen te stimuleren door toevoegen van een hoge dosis thymuscellen aan het transplantaat hadden een positief effect zowel in syngenetische als allogenetische condities. Deze bevindingen konden worden geinterpreteerd in termen van het bestaan van een syngenetische (evt. ontogenetische), respectievelijk allogenetische barriere tegen getransplanteerde foetale lever haemopoiese die door de thymuscellen werd doorbroken.

g. intussen stuitten pogingen om deze werking van thymuscellen toe te passen in een allogeen transplantatiesysteem ter bevordering van de kans op aanslaan vooralsnog op moeilijkheden, met name de inductie van acute GvH reacties. Foetale thymuscellen i.p.v. volwassen thymocyten gaven geen GvH reactie, maar hadden het bezwaar in hun activiteit gebonden te zijn aan een smal dosisgebied van foetale levercellen. Tenslotte werden wegen voor verdere ontwikkeling van deze methodiek ten behoeve van praktische toepassing aangegeven, waarbij in het bijzonder het gebruik van bestraalde thymuscellen, die niet meer GvH reactief zijn, interessant leek.

Het probleem van het aanslaan van foetale levercellen was relatief in die zin dat het kon worden overwonnen door de toediening van hogere aantallen stamcellen. Een benaderingswijze die op dit uitgangspunt gebaseerd was, werd in hoofdstuk 5 geexploreerd. Deze hield een poging in om een beperkte hoeveelheid beschikbare HSC te vermeerderen. Daartoe werd een *in vitro* kweeksysteem opgezet, dat was afgeleid van een eerder in dit laboratorium ontwikkelde techniek voor kolonievorming van haemopoietische cellen in agar. Doordat ons systeem een vloeibare basis had, kon de kinetiek van HSC in de aanwezigheid van een aantal stimuli technisch gemakkelijk worden onderzocht. Dit geschiedde aan de hand van twee parameters: de aantallen HSC *in vitro* en hun cyclusstatus. Uit het feit dat HSC in bepaalde condities in staat van actieve celdeling werden gebracht en in hogere aantallen uit de kweken werden verkregen, kon worden geconcludeerd, dat *in vitro* proliferatie van HSC optrad. Replicatie van zowel beenmerg als foetale lever HSC kon worden opgewekt. De HSC delingsactiviteit bleek afhankelijk van de werking van een mediator die door fibro-

blasten wordt afgegeven. Met grote waarschijnlijkheid kon worden aangetoond dat de stamcel activerende factor (SAF) een andere is dan de kolonie stimulerende factor (CSF), die myeloide precursor cellen activeert. Helaas is de methode vooralsnog niet bruikbaar als preparatieve techniek ten behoeve van een netto produktie van stamcellen voor transplantatiedoeleinden.

In hoofdstuk 6 werd bevestigd dat na allogenetische foetale leverceltransplantatie een relatief milde GvH reactie van het vertraagde type optreedt die met minder sterfte gepaard gaat. In experimenten die gebaseerd waren op stamcelzuivering en kwantificering van HSC in het transplantaat, kon worden aangetoond dat het mildere type GvH reactie berust op specifieke eigenschappen van de embryonale stamcel. Dit leidde tot een gemodificeerde formulering van de gebruikelijke indeling van GvH reacties: a. een acute GvH reactie die rechtstreeks afkomstig is van lymphocyten in het transplantaat, b. een ernstige vertraagde GvH reactie die wordt veroorzaakt door de lymphocyten-nakomelingschap van de beenmerg HSC in het transplantaat, en c. de minder ernstige late GvH reactie die berust op de activiteit van lymphocyten afstammend van de embryonale lever HSC.

In hoofdstuk 7 werden de gegevens die in het onderhavige onderzoek werden verkregen en de implicaties daarvan getoetst aan de foetale leverceltransplantatie bij de mens. Via extrapolatie van deze bevindingen konden de veelvuldige mislukkingen van het aanslaan van deze transplantaten bij de mens worden verklaard, nl. uit het niet voldoen aan de kwantitatieve voorwaarden van celaantallen die nodig zijn voor aanslaan. Deze nieuwe gegevens leidden onder meer tot het formuleren van aanbevelingen voor toekomstige toepassing van foetale levercel-therapie in de kliniek, voorlopig voor het behandelen van kinderen met ernstige gecombineerde immuundeficienties (CID).

ABBREVIATIONS

cAMP	adenosine cyclic monophosphate
BSA	bovine serum albumin
CFC	colony forming cells – represents the whole class of cells which share the capacity of giving rise to colonies *in vivo* (a proportion of the same population in an oculum produce spleen colonies)
CFU-c	colony forming units in culture
CFU-s	colony forming units spleen – refers to the single cells in a haemopoietic suspension which actually produce spleen colonies
CID	(severe) combined immunodeficiency disease
Con A	concanavalin A
CSF	colony stimulating factor
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dpm	desintegrations per minute
f-factor	fraction of injected CFU that form macroscopic colonies on the surface of the spleen following injection into lethally irradiated mice (– spleen colony forming efficiency – CFU/CFC ratio)
FCM	fibroblast conditioned medium
GvH	graft versus host
HBSS	Hanks' balanced salt solution
HLA	human leucocyte antigens (major histocompatibility complex in man)
HSC	pluripotent haemopoietic stem cell(s)
^{3}HTdR	tritiated thymidine
i.m.	intramuscular
i.v.	intravenous
i.p.	intraperitoneal
LPS	*E.coli* lipopolysaccharide
MEM	minimum essential medium
MLC	mixed lymphocyte culture
n.d.	not done
PHA	phytohaemagglutinin
SAF	stem cell activating factor
s.c.	subcutaneous
S.D.	standard deviation
S.E.	standard error
TALS	thin agar layer system
T_D	(mean) population doubling time
TdR	unlabelled thymidine

ACKNOWLEDGEMENTS

These investigations were conducted at the Department of Radiobiology of the Erasmus University (Rotterdam) and the Radiobiological Institute TNO (Rijswijk) (director: Prof. Dr. D. W. van Bekkum).

The author wishes to thank Prof. Dr. D. W. van Bekkum and Dr. K. A. Dicke for introducing him into the field of bone marrow transplantation and for the opportunity to benefit from the specific expertise in this area which has been acquired at Rijswijk over many years. The fact that this study "could not outgrow its embryonic state" was inherent to the name of the subject and was in spite of the inspiring help and interest of both of them throughout these investigations.

Furthermore, he wishes to express his thanks to:
Miss H. M. C. de Groot and Mrs. M. G. C. Hogeweg-Platenburg for their excellent technical assistance;
Miss N. Riou for her enthusiastic cooperation in some of the experiments;
Miss H. T. M. Nieuwerkerk and Mrs. B. M. Kooistra-Wierda for performing the tests for lymphocyte stimulation;
Mrs. J. A. T. Nooteboom-Den Herder for dedicated typing of the manuscript;
Dr. A. C. Ford for correcting the English text;
Mr. J. Ph. de Kler, Mr. H. J. van Westbroek and Mr. E. J. van der Reijden for realisation of the photographs and designing the front cover.

The author is also very grateful to Prof. Dr. O. Vos, Prof. Dr. J. Abels and Dr. L. J. Dooren for critical reading of the manuscript, and to Dr. S. D. Lawler (Fetal Tissue Bank, Royal Marsden Hospital, London), Prof. Dr. E. V. van Hall (Department of Gynaecology, University Hospital, Leiden) and Dr. P. E. R. Rhemrev (Department of Gynaecology, Elisabeth Gasthuis, Haarlem) for providing him with specimens of human embryonic tissues.

Finally, he is very much indebted to the members of the REPGO-TNO Institutes for their considerable, pluripotential support: Prof. Dr. L. M. van Putten, Drs. G. J. van den Engh, Dr. H. B. Kal, Dr. C. Zurcher, Miss P. H. M. Scheffers, Mrs. N. van Westbroek-Van der Zwan, Mr. H. T. Madhuizen, and many others.

REFERENCES

ABERLE, S. B. DE – A study of the hereditary anaemia of mice. American J. Anat. **40**, p. 219–249, 1927.

ANDERSSON, J., SJÖBERG, O. and MÖLLER, G. – Induction of immunoglobin and antibody synthesis "in vitro" by lipopolysaccharides. Eur. J. Immunol. **2**, p. 349–353, 1971.

ANDERSSON, J., SJÖBERG, O. and MÖLLER, G. – Mitogens as probes for immunocyte activation and cellular cooperation. Transplant. Rev. **11**, p. 131–177, 1972.

ATTFIELD, M. J. – Inherited macrocytic anaemias in the house mouse. III. Red blood cell diameters. J. Genetics, **50**, p. 250–263, 1951.

BALNER, H. – Identification of peritoneal macrophages in mouse radiation chimeras. Transplantation **1**, p. 217–223, 1963.

BARCHILON, J. and GERSHON, R. K. – Synergism between thymocytes and bone marrow derived cells in graft-versus-host disease. Fed. Proc. **29**, p. 430, 1970.

BARNES, D. W. H. and LOUTIT, J. F. – Immunological and histological response following spleen treatment in irradiated mice. In: Progress in Radiobiology; ed. by J. S. Mitchell, B. E. Holmes and C. L. Smith. London, Oliver & Boyd, p. 291–295, 1956.

BARNES, D. W. H., LOUTIT, J. F. and MICKLEM, H. S. – "Secondary disease" in lethally irradiated mice restored with syngeneic or allogeneic foetal liver cells. In: Mechanism of Immunological Tolerance; ed. by M. Hasek, A. Lengerova, and M. Vojtiskova. Prague, House of the Czechoslovak Academy of Sciences, p. 371–377, 1961.

BARNES, D. W. H., FORD, C. E. and LOUTIT, J. F. – Haemopoietic stem cells. The Lancet **I**, p. 1395–1396, 1964.

BATCHELOR, J. R. and HOWARD, J. G. – Synergic and antagonistic effects of isoantibody upon graft versus host disease. Transplantation **3**, p. 161–169, 1965.

BECKER, A. J., MCCULLOCH, E. A. and TILL, J. E. – Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature **197**, p. 452–454, 1963.

BECKER, A. J., MCCULLOCH, E. A., SIMINOVITCH, L. and TILL, J. E. – The effect of differing demands for blood cell production on DNA synthesis by haemopoietic colony-forming cells of mice. Blood, **26**, p. 296–308, 1965.

BEKKUM, D. W. VAN, VOS, O. and WEYZEN, W. W. H. – The pathogenesis of the secondary disease after foreign bone marrow transplantation in X-irradiated mice. J. Nat. Cancer Inst. **23**, p. 75–89, 1959.

BEKKUM, D. W. VAN – The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. I. Effect of storage at 4°C. Transplantation **2**, p. 393–404, 1964.

BEKKUM, D. W. VAN – Immunological reactivity and specific tolerance of the graft in radiation chimeras and the influence of the thymus. In: La Greffe des Cellules hématopoiétiques allogéniques, Paris 7–9 Sept. 1964. Editions du Centre National de la Recherche Scientifique, p. 381–389, 1965.

BEKKUM, D. W. VAN, and VRIES, M. J. DE – Radiation chimaeras. London, Logos/Acad. Press, 1967.

BEKKUM, D. W. VAN – Bone marrow transplantation and partial body shielding for estimating cell survival and repopulation. In: Comparative Cellular and Species Radiosensitivity; ed. by V. P. Bond and T. Sugahara. Tokyo, Igaku Shoin Ltd., p. 175–192, 1969.

BEKKUM, D. W. VAN – Prevention and control of secondary disease following allogeneic bone marrow transplantation. In: Bone Marrow Conservation, Culture and Transplantation. International Atomic Energy Agency, Vienna, p. 35–45, 1969a.

BEKKUM, D. W. VAN, NOORD, M. J. VAN, MAAT, B. and DICKE, K. A. – Attempts at identification of hemopoietic stem cell in mouse. Blood, **38**, p. 547–558, 1971.

BEKKUM, D. W. VAN – Use and abuse of haemopoietic cell grafts in immune deficiency diseases. Transplant. Rev. **9**, p. 3–53, 1972.

BEKKUM, D. W. VAN and DICKE, K. A. – Treatment of immune deficiency disease with bone marrow stem cell concentrates. In: Ontogeny of Acquired Immunity. A CIBA Foundation Symposium (1971). Amsterdam, Elsevier, p. 223–247, 1972.

BEKKUM, D. W. VAN, ROODENBURG, J., HEIDT, P. J. and WAAY, D. VAN DER – Mitigation of secondary disease of allogeneic mouse radiation chimeras by modification of the intestinal microflora. J. Nat. Cancer Inst. **52,** p. 401–404, 1974.

BERG, K. J. VAN DEN – The role of amino acids in the mitogenic activation of lymphocytes. Thesis Leiden, 1974.

BLEIBERG, I., LIRON, M. and FELDMAN, M. – Reversion by erythropoietin of the suppression of erythroid clones caused by transfusion induced polycythemia. Transplantation **3,** p. 706–710, 1965.

BLOMGREN, H. – Synergism between thymocytes and lymph node cells in the graft-versus-host response. Effect of cortisone treatment of the thymus cell donor. J. Immunol. **110,** p. 144–147, 1973.

BODLEY SCOTT, R., MATTHIAS, J. Q., CONSTANDOULAKIS, M., KAY, H. E. M., LUCAS, P. F. and WHITESIDE, J. D. – Hypoplastic anemia treated by transfusion of foetal haemopoietic cells. Brit. Med. Journ. **II,** p. 1385–1388, 1961.

BORANIC, M., TONKOVIC, I. and BLAZI, M. – Quantitative aspects of destruction of haemopoietic tissue in mouse radiation chimaeras. Biomedicine **19,** p. 104–107, 1973.

BORGHESE, E. – The present state of research on WW mice. Acta Anat. **36,** p. 185–220, 1959.

BORTIN, M. M. and SALTZSTEIN, E. C. – Immunologic incompetence of mouse perinatal liver hematopoietic cells against transplantation antigens. J. Immunol. **100,** p. 1215–1218, 1968.

BORTIN, M. M. and SALTZSTEIN, E. C. – Graft versus host inhibition: fetal liver and thymus cells to minimize secondary disease. Science **164,** p. 316–318, 1969.

BORTIN, M. M. – A compendium of reported human bone marrow transplants. Transplantation **9,** p. 571–587, 1970.

BORTIN, M. M. and RIMM, A. A. – Graft-versus-host inhibition. III. Fetal thymic factor and fetal liver cells to minimize secondary disease. Transplantation **9,** p. 310–321, 1970.

BORTIN, M. M., RIMM, A. A. and SALTZSTEIN, E. C. – Survival and immune competence of murine radiation chimeras. Fed. Proc. **30,** p. 652, 1971.

BORTIN, M. M., RIMM, A. A. and SALTZSTEIN, E. C. – Potentiating effect of fetal thymus on fetal liver cells for the promotion of recovery from the radiation injury in murine allogeneic radiation chimeras or a little bit of thymus goes a long, long way. In: Second Int. Workshop on Immunodeficiency Disease in Man; ed. by R. A. Good and D. Bergsma. Nat. Found. Press, 1973. In press.

BRADLEY, T. R. and METCALF, D. – The growth of mouse bone marrow cells in vitro. Austr. J. Exp. Biol. Med. Sci. **44,** p. 287–300, 1966.

BRADLEY, T. R., STANLEY, E. R. and SUMNER, M. A. – Factors from mouse tissues stimulating colony growth of mouse bone marrow cells in vitro. Austr. J. Exp. Biol. Med. Sci. **49,** p. 595–603, 1971.

BRADLEY, T. R. – Technical manual data. In: In Vitro Culture of Hemopoietic Cells. Proc. of a Workshop/Symposium on In Vitro Culture of Hemopoietic Cells; ed. by D. W. van Bekkum and K. A. DICKE (1971). Rijswijk, Radiobiological Institute TNO, p. 447–448, 1972.

BRADLEY, T. R., FRY, P., SUMNER, M. A. and MCINERNY, E. – Factors determining colony forming efficiency in agar suspension cultures. Austr. J. Exp. Biol. Med. Sci. **50,** p. 813–818, 1972.

BUCKLEY, R. H. – Reconstitution: grafting of bone marrow and thymus. Progr. Immunol. **1,** p. 1061–1080, 1971.

BUCKLEY, R. H., AMOS, B., KREMER, W. B. and STICKEL, D. L. – Incompatible bone marrow transplantation in lymphopenic immunologic deficiency. Circumvention of fatal graft versus host disease by immunologic enhancement. New Engl. J. Med. **285,** p. 1035–1042, 1971.

BUURMAN, W. A., VOS, O. and BRUGGEN, I. VAN – Genetic resistance in F_1 recipient mice after different treatments. Abstract of the Int. Soc. for Exp. Haematology, Houston, April 1974. To be published in Exp. Haemat.

BYRD, W. J., BOEHMER, H. VON and ROUSE, B. T. – The role of the thymus in maturational development of phytohemagglutinin and pokeweed mitogen responsiveness. Cell. Immunol. **6,** p. 12–24, 1973.

BYRON, J. W. – Evidence for a β-adrenergic receptor initiating DNA synthesis in haemopoietic stem cells. Exp. Cell Res. **71**, p. 228–232, 1971.

CANTOR, H. – The effects of anti-theta antiserum upon graft-versus-host activity in spleen and lymph node cells. Cell. Immunol. **3**, p. 461–469, 1972.

CANTOR, H. and ASOFSKY, R. – Synergy among lymphoid cells mediating the graft versus host response. III. Evidence for interaction between two types of thymus-derived cells. J. Exp. Med. **135**, p. 764–779, 1972.

CHUI, D. H. K., DJALDETTI, M., MARKS, P. A. and RIFKIND, R. A. – Erythropoietin effects on fetal mouse erythroid cells. I. Cell population and hemoglobin synthesis. J. Cell. Biol. **51**, p. 585–595, 1971.

COLE, L. J., GARVER, R. M. and O'KUNEWICK, J. P. – Lethal graft-versus-host reaction induced in X-irradiated F_1 hybrids by parental strain leukocytes. Transplant. Bull. **6**, p. 429–432, 1959.

COLE, R. J. and PAUL, I. – The effects of erythropoietin on haemosynthesis in mouse yolk sac and cultured foetal liver cells. J. Embryol. Exp. Morph. **15**, p. 245–260, 1966.

CONGDON, C. C. and URSO, I. S. – Homologous bone marrow in the treatment of radiation injury in mice. Amer. J. Path. **33**, p. 749–767, 1957.

COOPER, M., KEIGHTLEY, R. G., WU, L. Y. P. and LAWTON, A. R. – Developmental defects of T and B cell lines in humans. Transplant. Rev. **16**, p. 51–84, 1973.

COPENHAGEN STUDY GROUP OF IMMUNODEFICIENCIES – Bone-marrow transplantation from an HL-A non-identical but mixed-lymphocyte-culture identical donor. The Lancet, **I**, p. 1146–1149, 1973.

CRAIG, M. L. and RUSSEL, E. S. – A developmental change in haemoglobins correlated with an embryonic red cell population in the mouse. Develop. Biol. **10**, p. 191–201, 1964.

CROUCH, B. G. – Transplantation of fetal hemopoietic tissues into irradiated mice and rats. In: Proc. Seventh Congress of the Eur. Soc. for Haematology, London, p. 973–978, 1959.

CUDKOWICZ, G. and STIMPFLING, J. H. – Deficient growth of C57BL marrow cells transplanted in F_1 hybrid mice. Association with the histocompatibility-2 locus. Immunology **7**, p. 291–306, 1964.

CUDKOWICZ, G. and BENNETT, M. – Peculiar immunobiology of bone marrow allografts. I. Graft rejection by irradiated responded mice. J. Exp. Med., **134**, p. 83–102, 1971.

DICKE, K. A., HOOFT, J. I. M. VAN and BEKKUM, D. W. VAN – The selective elimination of immunologically competent cells from bone marrow and lymphatic cell mixtures. II. Mouse spleen cell fractionation on a discontinuous albumin gradient. Transplantation **6**, p. 562–570, 1968.

DICKE, K. A., TRIDENTE, G. and BEKKUM, D. W. VAN – The selective elimination of immunologically competent cells from bone marrow and lymphocyte cell mixtures. III. In vitro test for detection of immunocompetent cells in fractionated mouse spleen cell suspensions and primate bone marrow suspensions. Transplantation **8**, p. 422–434, 1969.

DICKE, K. A. – Bone marrow transplantation after separation by discontinuous albumin density gradient centrifugation. Thesis, Leiden, 1970.

DICKE, K. A., LINA, P. H. C. and BEKKUM, D. W. VAN – Adaptation of albumin density gradient centrifugation to human bone marrow fractionation. Rev. Europ. d'études Clin. Biol. **15**, p. 305–309, 1970.

DICKE, K. A., PLATENBURG, M. G. C. and BEKKUM, D. W. VAN – Colony formation in agar: in vitro assay for haemopoietic stem cells. Cell Tissue Kinet. **4**, p. 463–477, 1971.

DICKE, K. A. and BEKKUM, D. W. VAN – Preparation and use of stem cell concentrates for restoration of immune deficiency diseases and bone marrow aplasia. Rev. Europ. d'études Clin. Biol. **17**, p. 645–649, 1972a.

DICKE, K. A. and BEKKUM, D. W. VAN – Transplantation of haemopoietic stem cell (HSC) concentrates for treatment of immune deficiency disease. In: Microenvironmental aspects of immunity; ed. by B. D. Jankovic and K. Isakovic. New York, Plenum Press, p. 337–342, 1973.

DICKE, K. A., NOORD, M. J. VAN, MAAT, B., SCHAEFER, U. W. and BEKKUM, D. W. VAN – Attempts at morphological identification of the haemopoietic stem cell in primates and rodents. In: Haemopoietic Stem Cells. CIBA Foundation Symposium 13 (New Series). Amsterdam-Elsevier, p. 47–69, 1973b.

DICKE, K. A., NOORD, M. J. VAN, MAAT, B., SCHAEFER, U. W. and BEKKUM, D. W. VAN – Identifica-

tion of cells in primate bone marrow resembling the hemopoietic stem cell in the mouse. Blood **42**, p. 195–208, 1973c.

DICKE, K. A., ENGH, G. J. VAN DEN. LÖWENBERG, B. and BEKKUM, D. W. VAN – The behaviour of the hemopoietic stem cell in various culture systems. In: Second Workshop on Hemopoiesis in Culture; held in Airlie House Virginia, May 23–26, 1973; ed. by W. A. Robinson, p. 31–43, 1974.

DÖHMANN, U. – Personal communication 1974.

DORIA, G., GOODMAN, J. W., GENGOZIAN, N. and CONGDON, C. C. – Immunologic study of antibody-forming cells in mouse radiation chimaeras. J. Immunol, **88**, p. 20–30, 1962.

DUPLAN, J. F. – Etude comparative des cellules souches hématopoiétiques de la moelle osseuse et du foie foetal. Nouvelle Revue Française d'Hématologie, **8**, p. 445–456, 1968.

DUPLAN, J. F. – Radiosensitivity of the hematopoietic colony-forming cells of the mouse. In: Haemopoietic Cellular Proliferation; ed. by F. Stohlman Jr. New York, Grune and Stratton, p. 189–196, 1970.

DUPLAN, J. F. and LEGRAND, E. – Therapeutic efficiency of CFU belonging to different hemopoietic organs. In: Abstracts of the 8th Annual Meeting of the Eur. Soc. for Radiation Biology, Basto Polje, Yugoslavia, p. 37–38, 1971.

DUPONT, B., ANDERSEN, V., ERNST, P., FABER, V., GOOD, R. A., HANSEN, G. S., KOCH, C., MÜLLER-BERAT, N., PARK, B. H., SVEJGAARD, A., THOMSON, M. and WILK, A. – Immunologic reconstitution in severe combined immunodeficiency with HL-A-incompatible bone marrow graft; donor selection by mixed lymphocyte culture. Transplant. Proc. **5**, p. 905–908, 1973.

ENGH, G. J. VAN DEN – Quantitative in vitro studies on stimulation of murine haemopoietic cells by colony stimulating factor. Cell Tissue Kinet. **7**, p. 537–548, 1974.

FANTONI, A., BANK, A. and MARKS, P. A. – Globin composition and synthesis of hemoglobins in developing fetal mice erythroid cells. Science, **157**, p. 1327–1328, 1967.

FEFER, A., BUCKNER, C. D., CLIFT, R. A., FASS, L., LERNER, K. G., MICKELSON, E. M., NEIMAN, P., RUDOLPH, R., STORB, R. and THOMAS, E. D. – Marrow grafting in identical twins with hematologic malignancies. Transplant. Proc. **5**, p. 927–931, 1973.

FLOERSHEIM, G. L. and STORB, R. – Knochenmarktransplantation bei aplastischen Anämien und akuten Leukämien. Einsichten und Aussichten. I. Schweiz. Med. Wschr. **104**, p. 3–10, 1974.

FOWLER, J. H., WU, A. M., TILL, J. E., MCCULLOCH, E. A. and SIMINOVITCH, L. – The cellular composition of haemopoietic spleen colonies. J. Cell. Comp. Physiol. **69**, p. 65–72, 1967.

GATTI, R. A., MEUWISSEN, H. J., ALLEN, H. D., HONG, R. and GOOD, R. A. – Immunological reconstitution of sex-linked lymphopenic immunological deficiency. The Lancet, **II**, p. 1366–1369, 1969.

GEIGY – Scientific tables 1970. Statistical Methods, par. 18; CIBA-Geigy Ltd., Basle, Switzerland, p. 174–179, 1971.

GELFAND, E. W., ET AL. – In: Second International Workshop on the Primary Immuno-deficiency Diseases in Man, Florida, U.S.A. (1973). In press.

GENGOZIAN, N., RABETTE, B. and CONGDON, C. C. – Abnormal immune mechanism in allogeneic radiation chimeras. Science, **149**, p. 645–647, 1965.

GENGOZIAN, N. and CONGDON, C. C. – Immunological memory in radiation chimeras. Transplantation **16**, 32–40, 1973.

GERY, I., KRUGEL, J. and SPIESEL, S. Z. – Stimulation of B lymphocytes by endotoxin. Reactions of thymus deprived and karyotypic analysis of dividing cells in mice bearing T_6T_6 thymus grafts. J. Immunol. **108**, p. 1088–1091, 1972.

GILLER, R. H., BORTIN, M. M., RIMM, A. A., GLASSPIEGEL, J. S. and SALTZSTEIN, E. C. – Combined fetal liver and thymus for hematopoietic reconstitution of allogeneic radiation chimeras. Proc. Soc. Exp. Biol. Med. **139**, p. 1022–1027, 1972.

GITHENS, J. H., FULGINITI, V. A., SUVATTE, V., SCHROTER, G., HATHAWAY, W. E., PEARLMAN, D. S., KAY, H. E. M., TERASAKI, P. I., HILL, G. J., KEMPE, C. H. and COX, S. T. – Grafting of fetal thymus and hematopoietic tissue in infants with immune deficiency syndromes. Transplantation **15**, p. 427–434, 1973.

GOOD, R. A., MARTINEZ, C. and GABRIELSEN, A. E. – Progress toward transplantation of tissues in man. Advan. Paediat. **13**, p. 93–127, 1964.

GOODMAN, J. W. and CONGDON, C. C. – Blood-marrow mixtures in irradiated mice effect of blood injection on bone marrow transplantation in lethally irradiated mice. Arch. Pathol. **72**, p. 18–26, 1961.

GOODMAN, J. W. and SHINPOCK, S. G. – Influence of thymus cells on erythropoiesis of parental marrow in irradiated hybrid mice. Proc. Soc. Exp. Biol. Med. **129**, p. 417–422, 1968.

GOODMAN, J. W. and WHEELER, H. B. – Factors that modify poor growth of parental marrow cells in F_1 hybrid mice. In: Advance in Transplantation; ed. by J. Dausset, J. Hamburger and G. Mathé. Copenhagen, Munksgaard, p. 427–430, 1968.

GOODMAN, J. W. and GRUBBS, C. G. – The relationship of the thymus to erythropoiesis. In: Hemopoietic Cellular Proliferation; ed. by F. Stohlman Jr. New York, Grune & Stratton, p. 26–35, 1970.

GOODMAN, J. W., BURCH, K. T. and BASFORD, N. L. – Graft-vs-host activity of thymocytes: relationship to the role of thymocytes in haemopoiesis. Blood **39**, p. 850–861, 1972.

GOSS, C. M. – Experimental removal of the blood island of Amblystoma punctatum embryos. J. Exp. Zool. **52**, p. 45–63, 1928.

GRAW JR., R. G., LEVENTHAL, B. G., YANKEE, R. A., ROGENTINE, G. N., WHANG-PENG, J., GINN, M. H., HERZIG, G. P., HALTERMAN, R. H. and HENDERSON, E. S. – HLA- and mixed lymphocyte culture matched allogeneic bone marrow transplantation in patients with acute leukaemia. Transpl. Proc. **3**, p. 405–408, 1971.

GREGORY, C. J., MCCULLOCH, E. A. and TILL, J. E. – Erythropoietic progenitors capable of colony formation in culture: state of differentiation. J. Cell. Physiol. **81**, p. 411–420, 1973.

HASKILL, J. S. and MOORE, M. A. S. – Two dimensional cell separation: comparison of embryonic and adult haemopoietic stem cells. Nature **226**, p. 853–854, 1970.

HELLSTRÖM, K. A. and HELLSTRÖM, I. – Syngeneic preference and allogeneic inhibition. In: Isoantigens and Cell Interactions; ed. by J. Palm. Philadelphia, The Wistar Institute Press, p. 79, 1965.

HENDRIKS, W. D. H. – Personal communication 1974.

HENDRY, J.H. – The f number of primary transplanted splenic colony-forming cells. Cell Tissue Kinet. **4**, p. 217–223, 1971.

HITZIG, W. H., LANDOLT, R., MÜLLER, G. and BODMER, P. – Heterogeneity of phenotypic expression in a family with Swiss type agammaglobulinaemia: observations on the acquisition of agammaglobulinaemia. J. Pediat. **78**, p. 968–980, 1971.

ISCOVE, N. N., TILL, J. E. and MCCULLOCH, E. A. – The proliferative status of mouse granulopoietic progenitor cells. Proc. Soc. Exp. Biol. Med. **134**, p. 33–36, 1970.

JACOBSON, L. O., MARKS, E. K. and GASTON, E. O. – Studies on erythropoiesis. XII. The effect of transfusion-induced polycythemia in the mother on the fetus. Blood **14**, p. 644–653, 1959.

JANOSSY, G. and GREAVES, M. F. – Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. Clin. Exp. Immunol. **9**, p. 483–498, 1971.

KEIGHTLEY, R. G., LAWTON, A. R., WU, L. Y. P. and COOPER, M. D. – Differentiation of fetal liver stem cells along lymphocyte lines in an infant with combined immunodeficiency disease (CID). Exp. Haemat. **1**, p. 253–254, 1973.

KONING, J. DE, DOOREN, L. J., BEKKUM, D. W. VAN, ROOD, J. J. VAN, DICKE, K. A. and RADL, J. – Transplantation of bone marrow cells and fetal thymus in an infant with lymphopenic immunological deficiency. The Lancet **1**, p. 1223–1227, 1969.

KUBANEK, B., RENCRICCA, N., PORCELLINI, A., HOWARD, D. and STOHLMAN JR., F. – The pattern of recovery of erythropoiesis in heavily irradiated mice receiving transplants of fetal liver. Proc. Soc. Exp. Biol. Med. **131**, p. 831–834, 1969.

LAHIRI, S. K. and PUTTEN, L. M. VAN – Distribution and multiplication of colony forming units from bone marrow and spleen after injection in irradiated mice. Cell Tissue Kinet. **2**, p. 21–28, 1969.

LAHIRI, S. K., KEIZER, H. J. and PUTTEN, L. M. VAN – The efficiency of the assay for haemopoietic colony forming cells. Cell Tissue Kinet. **3**, p. 355–362, 1970.

LAJTHA, L. G. – Response of bone marrow stem cells to ionizing radiations. In: Current Topics in Radiation Research; ed. by M. Ebert and A. Howard. Amsterdam, North-Holland Publ. Comp., Vol. I, p. 139–162, 1965.

LAJTHA, L. G., POZZI, L. V., SCHOFIELD, R. and FOX, M. – Kinetic properties of haemopoietic stem cells. Cell Tissue Kinet. **2**, p. 39–49, 1969.

LENGEROVA, A. – Comparison of the therapeutic efficiency of homologous and heterologous embryonic haematopoietic cells administered to lethally irradiated mice. Folia Biologica **5**, p. 18–29, 1959.

LEVEY, R. H., KLEMPERER, M. R., GELFAND, E. W., SANDERSON, A. R., BATCHELOR, J. R., BERKER, A. I. and ROSEN, F. S. – Bone marrow transplantation in severe combined immunodeficiency syndrome. The Lancet **II**, p. 571–575, 1971.

LÖWENBERG, B., DICKE, K. A. and BEKKUM, D. W. VAN – Isolation of haemopoietic stem cell in foetal liver. A preliminary step to the evaluation of fetal liver transplantation in the treatment of bone marrow aplasia after lethal irradiation. Int. J. Radiat. Biol. **23**, p. 191, 1973.

LÖWENBERG, B., DICKE, K. A. and BEKKUM, D. W. VAN – Different behaviour of fetal liver and bone marrow colony forming cells following grafting. Exp. Hemat. **1**, p. 244, 1973.

LÖWENBERG, B., PLATENBURG, M. G. C. and DICKE, K. A. – Proliferation of haemopoietic stem cells in culture. Submitted for publication.

LÖWENBERG, B. and DICKE, K. A. – Studies on the *in vitro* proliferation of pluripotent haemopoietic stem cells. Proc. Int. Symp. on Leukemia and Aplastic Anemia, Naples, Sept. 1974. In press.

LÖWENBERG, B., DICKE, K. A. and BEKKUM, D. W. VAN – Quantitative studies on the take of fetal liver haemopoietic transplants. Proc. 5th Int. Congr. Transplant. Soc., Jerusalem, August 1974. In press.

LÖWENBERG, B. and VRIESENDORP, H. M. – Unpublished observations.

LORD, B. I. – The relationship between spleen colony production and spleen cellularity. Cell Tissue Kinet. **4**, p. 211–216, 1971.

LORD, B. I. and HENDRY, J. H. – Observations on the settling and recoverability of transplanted hemopoietic colony-forming units in the mouse spleen. Blood **41**, p. 409–415, 1973.

LOTZOVA, E., CUDKOWICZ, C., GALLAGHER, M. T., RAUCHWERGER, J. M. and TRENTIN, J. J. – Suppression of resistance of irradiated mice to foreign marrow grafts by antimacrophage agents; prevention of suppression by a macrophage stabilizer. Abstract of the Int. Soc. for Exp. Haematology, Houston, April 1974. To be published in Exp. Haematology.

LOUTIT, J. F. – Recovery from the lethal effects of radiation. In: Lectures on the Scientific Basis of Medicine, V; 1955–1956. London, Athlone Press. p. 439–450, 1957.

MATHÉ, G., AMIEL, J. L., BREZIN, C. and CHOQUET, C. – Comparaison de la réactivité contre l'hôte de thymocytes et de cellules ganglionnaires semi-allogéniques. Rev. franc. Etudes clin. Biol. **9**, p. 988–990, 1964.

MATIOLI, G., VOGEL, H. and NIEWISCH, H. – The dilution factor of intravenously injected haemopoietic stem cells. J. Cell. Physiol. **72**, p. 229–234, 1968.

MAXIMOW, A. and BLOOM, W. – Textbook of Histology. Philadelphia, Saunders, 1934.

MCCULLOCH, E. A. and TILL, J. E. – Repression of colony-forming ability of C57BL hematopoietic cells transplanted into non-isologous hosts. J. Cell. Comp. Physiol. **61**, p. 301–308, 1963.

MCCULLOCH, E. A., GREGORY, C. J. and TILL, J. E. – Cellular communication early in haemopoietic differentiation. In: Haemopoietic Stem Cells. CIBA Foundation Symposium 13 (New Series). Amsterdam, Elsevier, p. 183–199, 1973.

METCALF, D. and MOORE, M. A. S. – Haemopoietic Cells: their Origin, Migration and Differentiation (Frontiers of Biology, vol. 24). Amsterdam, North-Holland Publ. Comp., 1971a.

METCALF, D. and MOORE, M. A. S. – In: Haemopoietic Cells: their Origin, Migration and Differentiation (Frontiers of Biology, vol. 24). Amsterdam, North-Holland Publ. Comp., p. 184, 1971b.

METCALF, D. and MOORE, M. A. S. – In: Haemopoietic Cells: their Origin, Migration and Differentiation (Frontiers of Biology, vol. 24). Amsterdam, North-Holland Publ. Comp., p. 189–195, 1971c.

METCALF, D. and MOORE, M. A. S. – In: Haemopoietic Cells: their Origin, Migration and Differentia-

tion (Frontiers of Biology, vol. 24). Amsterdam, North-Holland Publ. Comp., p. 205–207, 1971d.

METCALF, D. and MOORE, M. A. S. – In: Haemopoietic Cells: their Origin, Migration and Differentiation (Frontiers of Biology, vol. 24). Amsterdam, North-Holland Publ. Comp., p. 208, 1971e.

METCALF, D. and MOORE, M. A. S. – In: Haemopoietic Cells: their Origin, Migration and Differentiation (Frontiers of Biology, vol. 24). Amsterdam, North-Holland Publ. Comp., p. 204, 1971f.

MICKLEM, H. S. – In: MICKLEM, H. S. and LOUTIT, J. F. – Tissue Grafting and Radiation. New York/London, Academic Press, p. 84, 1966.

MICKLEM, H. S. and FORD, C. E. – Competitive in vivo proliferation of haemopoietic cells of fetal and adult origin. Abstract for the 1st Int. Congr. of Transplant. Soc. Paris, p. 262, 1966.

MICKLEM, H. S. and LOUTIT, J. F. – Tissue Grafting and Radiation. New York/London, Academic Press, 1966.

MICKLEM, H. S. – Cell proliferation in hematopoietic spleen colonies of mice: difference between colonies derived from injected adult bone marrow and foetal liver cells. Cell Tissue Kinet. **5,** p. 159–164, 1972.

MILLER, J. F. A. P. – Immunological function of the thymus. The Lancet **II,** p. 748–749, 1961.

MILLER, J. F. A. P. – Immunological significance of the thymus of the adult mouse. Nature **195,** p. 1318–1319, 1962.

MOORE, M. A. S. and OWEN, J. J. T. – Stem cell migration in the developing myeloid and lymphoid systems. The Lancet **I,** p. 658–659, 1967.

MOORE, M. A. S., MCNEILL, T. A. and HASKILL, J. S. – Density distribution analysis of in vivo and in vitro colony forming cells in developing fetal liver. J. Cell. Physiol. **75,** p. 181–192, 1970.

NIEWISCH, H., HAJDIK, I., SULTANIAN, I., VOGEL, H. and MATIOLI, G. – Hemopoietic stem cell distribution in tissues of fetal and newborn mice. J. Cell. Physiol. **76,** p. 107–116, 1970.

NOWELL, P. C., HIRSCH, B. E., FOX, D. H. and WILSON, D. B. – Evidence for the existence of multipotential lympho-hematopoietic stem cells in the adult rat. J. Cell. Physiol. **75,** p. 151–158, 1970.

PARSA, I. and KOUNTZ, S. L. – Rapid mixed lymphocyte culture test based on relative increase of protein synthesis. Nature **250,** p. 675–678, 1974.

PEGG, D. E. – A quantitative study of bone marrow grafting: implications for human bone marrow infusion. Brit. J. Cancer **16,** p. 400–416, 1962.

PLUZNIK, D. H. and SACHS, L. – The cloning of normal "mast cells" in tissue culture. J. Cell. Comp. Physiol. **66,** p. 319–324, 1965.

PLUZNIK, D. H. and SACHS, L. – Induction of clones of normal mast cells by a substance from conditioned medium. Exp. Cell. Res. **43,** p. 553–563, 1966.

PUTTEN, L. M. VAN – Thymectomy: effect on secondary disease in radiation chimeras. Science **145,** p. 935–936, 1964.

PUTTEN, L. M. VAN, BEKKUM, D. W. VAN and VRIES, M. J. DE – Transplantation of foetal haemopoietic cells in irradiated rhesus monkeys. In: Radiation and the Control of the Immune Response. Proc. of a panel held in Paris, June 22–24, 1967. International Atomic Energy Agency, Vienna, p. 41–49, 1968.

RIFKIND, R. A., CHUI, D. and DJALDETTI, M. – Erythroid differentiation in fetal mouse liver. Fed. Proc. **27,** p. 724, 1968.

RIFKIND, R. A., CHUI, D. and EPLER, H. – An ultrastructural study of early morphogenetic events during the establishment of fetal hepatic erythropoiesis. J. Cell. Biol. **40,** p. 343–365, 1969.

RUBINSTEIN, A., SPECK, B. and JEANNET, M. – Successful bone marrow transplantation in a lymphopenic immunologic deficiency syndrome. New Engl. J. Med. **285,** p. 1399–1402, 1971.

RUGH, R. – X-irradiation effects on the human fetus. J. Pediat. **52,** p. 531–538, 1958.

SAFFORD, JR., J. W. and TOKUDA, S. – Suppression of the graft-versus-host reaction by passive immunisation of donor against recipient antigens. Proc. Soc. Exp. Biol. Med. **133,** p. 651–654, 1970.

SAYK, J. – Cytologie der cerebro-spinal Flüssigkeit. Ergebnisse vergleichender Untersuchungen. Jena, Gustav Fischer Verlag, 1960.

SCHAEFER, U. W., DICKE, K. A. and BEKKUM, D. W. VAN – Recovery of haemopoiesis in lethally

irradiated monkeys by frozen allogeneic bone marrow grafts. Rev. Europ. d'études Clin. Biol. **17**, p. 483–488, 1972a.

SCHAEFER, U. W., SNEL, D. P. B. M. and BOORMAN, G. A. – Intraperitoneal administration of allogeneic bone marrow cells in lethally irradiated rhesus monkeys. Exp. Haematology **22**, p. 98–99, 1972b.

SCHOFIELD, R. – A comparative study of the repopulating potential of grafts from various haemopoietic sources: CFU repopulation. Cell Tissue Kinet. **3**, p. 119–130, 1970.

SHADDUCK, R. K., RICHARD, K. A., HOWARD, D. E. and STOHLMAN JR., F. – The effect of pre-irradiation of recipient mice on the proliferation of transplanted hemopoietic stem cells. Blood **37**, p. 330–339, 1971.

SHORTMAN, K., BYRD, W. J., CEROTTINI, J. C. and BRUNNER, K. T. – Characterisation and separation of mouse lymphocyte subpopulations responding to phytohaemagglutinin and Pokeweed mitogen. Cellular Immunol. **6**, p. 25–40, 1973.

SILINI, G., POZZI, L. V. and PONS, S. – Studies on the haemopoietic stem cells of the mouse fetal liver. J. Embryol. Exp. Morphol. **17**, p. 303–318, 1967.

SILINI, G., PONS, S. and POZZI, L. V. – Quantitative histology of spleen colonies in irradiated mice. Brit. J. Haemat. **14**, p. 489–500, 1968.

SIMINOVITCH, L., MCCULLOCH, E. A. and TILL, J. E. – The distribution of colony forming cells among the spleen colonies. J. Cell. Comp. Physiol. **62**, p. 327–336, 1963.

SIMONSEN, M. – Graft-versus-host reactions. Their natural history and applicability as tools of research. Progr. Allergy **6**, p. 349, 1962.

SIMONSEN, M. and JENSEN, E. – The graft versus host assay in transplantation chimaeras. In: Biological Problems of Grafting; ed. by F. Albert and G. Lejeune-Ledant. Oxford, Blackwell, p. 214–236, 1959.

SMITH, L. H. and VOS, O. – Radiation sensitivity of mouse lymph node cells relative to their proliferative capacity *in vivo*. Radiat. Res. **19**, p. 485–491, 1963.

SOOTHILL, J. F., KAY, H. E. M. and BATCHELOR, J. R. – Graft restoration of primary immunodeficiency. In: Cell Interaction and Receptor Antibodies in Immune Responses; ed. by O. Makelo, A. Cross and T. E. Kosmen. New York, Academic Press, p. 41–52, 1971.

SPECK, B., DOOREN, L. J., KONING, J. DE, BEKKUM, D. W. VAN, EERNISSE, J. G., ELKERBOUT, F., VOSSEN, J. M. and ROOD, J. J. VAN – Clinical experience with bone marrow transplantation. Failures and success. Transpl. Proc. **3**, p. 409–413, 1971.

STEINER, R. S. and VOGEL, H. – On the kinetics of erythroid cell differentiation in fetal mice. I. Microspectrophotometric determination of the hemoglobin content in erythroid cells during gestation. J. Cell. Physiol. **81**, p. 323–338, 1973.

STEPHENSON, J. R., AXELRAD, A. A., MCLEOD, D. L. and SHREEVE, M. M. – Induction of colonies of hemoglobin synthesizing cells by erythropoietin in vitro. Proc. Nat. Acad. Sci. **68**, p. 1542–1546, 1971.

STIEHM, E. R., LAWLER, JR., G. J., KAPLAN, M. S., GREENWALD, H. L., NEERHOUT, R. C., SENGAR, D. P. S. and TERASAKI, P. I. – Immunologic reconstitution in severe combined immunodeficiency without bone marrow chromosomal chimerism. New Engl. J. Med. **286**, p. 797–803, 1972.

STOBO, J. D. – Phytohaemagglutinin and concanavalin A; probes for murine T cell activation and differentiation. Transplant. Rev. **11**, p. 60–86, 1972.

STOBO, J. D. and PAUL, W. E. – Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of T cells to phytohemagglutinin and concanavalin A as a probe for T cell subsets. J. Immunol. **110**, p. 362–375, 1973.

STORB, R., EPSTEIN, R. B., BRYANT, J., RAGDE, H. and THOMAS, E. D. – Marrow grafts by combined marrow and leukocyte infusions in unrelated dogs selected by histocompatibility typing. Transplantation **6**, p. 587–593, 1968.

STORB, R., THOMAS, E. D., BUCKNER, C. D., CLIFT, R. A., JOHNSON, F. L., FEFER, A., GLUCKSBERG, H., GIBLETT, E. R., LERNER, K. G. and NEIMAN, P. – Allogeneic marrow grafting for treatment of aplastic anemia. Blood **43**, p. 157–180, 1974.

STUTMAN, O. and GOOD, R. A. – Immunocompetence of embryonic haemopoietic cells after traffic to thymus. Transpl. Proc. **3**, p. 923–925, 1971.

SUMNER, M. A., BRADLEY, T. R., HODGSON, G. S., CLINE, M. J., FRY, P. A. and SUTHERLAND, L. – The growth of bone marrow cells in liquid culture. Brit. J. Haematol. **23**, p. 221–234, 1972.

TAYLOR, R. B. – Pluripotential stem cells in mouse embryo liver. Brit. J. Exp. Path. **46**, p. 376–383, 1964.

TESTA, N. G. and LAJTHA, L. G. – Comparison of the kinetics of colony forming units in spleen (CFU-s) and culture (CFU-c). Brit. J. Haematol. **24**, p. 367–376, 1973.

TESTA, N. G., LORD, B. I. and SHORE, N. A. – The in vivo seeding of hemopoietic colony-forming cells in irradiated mice. Blood **40**, p. 654–661, 1972.

THOMAS, E. D., COLLINS, J. A., KASAKURA, S. and FERREBEE, J. W. – Lethally irradiated dogs given infusions of fetal and adult hematopoietic tissue. Transplantation **1**, p. 514–520, 1963.

THOMAS, E. D., STORB, R., FEFER, A., SLICHTER, S. J., BRYANT, J. I., BUCKNER, C. D., NEIMAN, P. E., CLIFT, R. A., FUNK, D. D. and LERNER, K. G. – Aplastic anemia treated by marrow transplantation. The Lancet **I**, p. 1062–1080, 1972.

TIGELAAR, R. E. and ASOFSKY, R. – Kinetics of graft-versus-host splenomegaly in neonatal F_1 recipients of parental strain mouse lymph node cells and or thymocytes. Transplantation **14**, p. 805–807, 1972.

TIGELAAR, R. E. and ASOFSKY, R. – Graft-vs-host reactivity of mouse thymocytes: Effect of cortisone pretreatment of donors. J. Immunol. **110**, p. 567–574, 1973.

TILL, J. E. and MCCULLOCH, E. A. – A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. **14**, p. 213–222, 1961.

TILL, J. E. and MCCULLOCH, E. A. – The f-factor of the spleen colony assay for haemopoietic stem cells. Ser. Haematol. **5**, p. 15–21, 1972.

TRENTIN, J. J. – Mortality and skin transplantability in X-irradiated mice receiving isologous, homologous and heterologous bone marrow. Proc. Soc. Exp. Biol. Med. **92**, p. 688–693, 1956.

TRENTIN, J. J. – Induced tolerance and homologous disease in X-irradiated mice protected with homologous bone marrow. Proc. Soc. Exp. Biol. Med. **96**, p. 139–147, 1957.

TRENTIN, J. J. and FAHLBERG, W. J. – An experimental model for studies of immunologic competence in irradiated mice repopulated with "clones" of spleen cells. In: Conceptual Advances in Immunology and Oncology. p. 66–74, 1963.

TRENTIN, J. J. – Determination of bone marrow stem cell differentiation by stromal haemopoietic inductive microenvironments (HIM). Amer. J. Pathol. **65**, p. 621–628, 1971.

TYAN, M. L. and COLE, L. J. – Mouse fetal liver and thymus: potential sources of immunologically active cells. Transplantation **1**, p. 347–350, 1963.

TYAN, M. L. – Thymus: role in maturation of fetal lymphoid precursors. Science **145**, p. 934–935, 1964.

TYAN, M. L. and COLE, L. J. – Sources of potential immunologically reactive cells in certain fetal and adult tissues. Transplantation **2**, p. 241–245, 1964.

TYAN, M. L., COLE, L. J. and HERZENBERG, L. A. – Fetal liver: a source of immunoglobulin producing cells in the mouse. Proc. Soc. Exp. Biol. Med. **124**, p. 1161–1163, 1967.

TYAN, M. L. and HERZENBERG, L. A. – Immunoglobulin production by embryonic tissues: thymus independent. Proc. Soc. Exp. Biol. Med. **128**, p. 952–954, 1968a.

TYAN, M. L. and HERZENBERG, L. A. – Studies on the ontogeny of the mouse immune system. II. Immunoglobulin-producing cells. J. Immunol. **101**, p. 446–450, 1968b.

TYAN, M. L., NESS, D. B. and GIBBS, P. R. – Fetal lymphoid tissues, antibody production in vitro. J. Immunol. **110**, p. 1170–1173, 1973.

UMIEL, T. A., GLOBERSON, A. and AUERBACH, R. – Role of the thymus in the development of immunocompetence of embryonic liver cells in vitro. Proc. Soc. Exp. Biol. Med. **129**, p. 598–600, 1968.

UPHOFF, D. E. – Genetic factors influencing irradiation protection by bone marrow. I. The F_1 hybrid effect. J. Nat. Cancer Inst. **19**, p. 123–130, 1957.

UPHOFF, D. E. – Preclusion of secondary phase of irradiation syndrome by inoculation of fetal hematopoietic tissue following lethal total-body X-irradiation. J. Nat. Cancer Inst. **20**, p. 625–632, 1958.

UPHOFF, D. E. and LAW, L. W. – Genetic factors influencing irradiation protection by bone marrow. II. The histocompatibility – 2(H-2) locus. J. Nat. Cancer Inst. **20**, p. 617–624, 1958.

UPHOFF, D. E. – Alteration of survival pattern by homologous fetal hematopoietic tissue in lethally irradiated mice. Abstract. Radiat. Res. **11**, p. 474, 1959.

URSO, I. S., CONGDON, C. C. and OWEN, R. D. – Effect of foreign fetal and newborn blood-forming tissues on survival of lethally irradiated mice. Proc. Soc. Exp. Biol. Med. **100**, p. 395–399, 1959.

VOGEL, H., NIEWISCH, H. and MATIOLI, G. – The self-renewal probability of haemopoietic stem cells. J. Cell. Physiol. **72**, p. 221–228, 1968.

VOGEL, H., NIEWISCH, H. and MATIOLI, G. – Stochastic development of stem cells. J. Theor. Biol. **22**, p. 249–270, 1969.

VOGEL, H., HAJDIK, I., NIEWISCH, H., SULTANIAN, I. and MATIOLI, G. – Growth kinetics of hemopoietic fetal stem cells. J. Cell. Physiol. **76**, p. 117–126, 1970.

VOISIN, G. A. and KINSKY, R. – Protection against runting by specific treatment of newborn mice, followed by increased tolerance. In: Transplantation; a CIBA Foundation Symposium; ed. by G. E. W. Wolstenholme and M. P. Cameron. Boston, Little, Brown and Comp., p. 286–327, 1962.

VOISIN, G. A., KINSKY, R. and MAILLARD, J. – Protection against homologous disease in hybrid mice by passive and active immunological enhancement – facilitation. Transplantation **6**, p. 187–202, 1968.

VOS, O., VRIES, M. J. DE, COLLENTEUR, J. C. and BEKKUM, D. W. VAN – Transplantation of homologous and heterologous lymphoid cells in X-irradiated and non-irradiated mice. J. Nat. Cancer Inst. **23**, p. 53–72, 1959.

VOS, O., GOODMAN, J. W. and CONGDON, C. C. – Donor-type lymphatic tissue cells in lethally irradiated mice treated with homologous fetal liver. Transplant. Bull. **7**, p. 408–411, 1960.

VRIES, M. J. DE, DOOREN, L. J. and CLETON, F. J. – Graft versus host or autoimmune lesions in the Swiss type of agammaglobulinemia. Birth Defects **4**, p. 173–191, 1968.

VRIESENDORP, H. M. – Personal communication.

WAAY, D. VAN DER, VRIES, J. M. DE and LEKKERKERK, J. E. C. – Eliminating bacteria from monkeys with antibiotics. In: Infections and Immunosuppression in Subhuman Primates; ed. by H. Balner and W. I. B. Beveridge. Copenhagen, Munksgaard, p. 21–33, 1970.

WU, A. M., TILL, J. E., SIMINOVITCH, L. and MCCULLOCH, E. A. – A cytological study of the capacity for differentiation of normal hemopoietic colony forming cells. J. Cell. Physiol. **69**, p. 177–184, 1967.

WU, A. M., TILL, J. E., SIMINOVITCH, L. and MCCULLOCH, E. A. – Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cells of the lymphoid system. J. Exp. Med. **127**, p. 455–464, 1968.

YAMAMURA, M., NEWTON, R. C. F., JAMES, D. C. O., HUMBLE, J. G., BUTLER, L. J. and HOBBS, J. R. – Uncomplicated HL-A matched sibling bone marrow graft for combined immune deficiency. Brit. Med. J. **2**, p. 265–269, 1972.

ZALESKI, M. and MILGROM, F. – Immunosuppressive effect of graft-versus-host reaction. Cell. Immunol. **7**, p. 268–274, 1973.

CURRICULUM VITAE

Bob Löwenberg was born in Groningen (The Netherlands) in 1946. He commenced a medical course at the State University in Groningen in 1964, after graduation from the Praedinius Gymnasium (department β). His interest in biological-medical research was stimulated during participation in an experimental immunological program at the Department of Histology (Prof. Dr. F. J. Keuning). Part of his clinical training was taken at the St. Elisabeth Gasthuis in Curacao. In 1970, he was certified by the American Examination Council for Foreign Medical Graduates. He finished his study and was registered M.D. At the end of 1971, he joined the "haematology group" of the Radiobiological Institute TNO in Rijswijk (director: Prof. Dr. D. W. van Bekkum) as a member of the Department of Radiobiology of the Erasmus University (Rotterdam). This thesis comprises the investigations carried out at that Institute.