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The differentiation of transplanted haematopoietic cells derived from bone marrow, spleen and fetal liver

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INTRODUCTION

Haematopoietic insufficiency has been prevented in mice during the 30 day period following exposure to a lethal dose of whole-body X-irradiation (675 r) by the intravenous injection of 10^5 syngeneic bone marrow cells within 3 h of exposure (van Bekkum & Vos, 1957). The substitution of syngeneic fetal liver cells for bone marrow cells necessitated the administration of $1{\cdot}6 \times 10^6$ cells in order to ensure the same result under exactly comparable conditions in the same laboratory (Crouch, 1960). This discrepancy was attributed to some relative deficiency of the fetal liver stem cell population, for it had already been demonstrated that the prevention of haematopoietic insufficiency in lethally irradiated mice after the injection of haematopoietic cell suspensions is due to repopulation of the virtually acellular lympho-myeloid complex by cells of donor origin (Ford, Hamerton, Barnes & Loutit, 1956). It was supposed *either* that a cell suspension prepared from fetal liver contains a smaller proportion of stem cells than a bone marrow cell suspension, *or* that the stem cells derived from fetal liver do not proliferate as rapidly as stem cells derived from bone marrow in an irradiated host. The further possibility that the differentiation capacity of the stem cells derived from fetal liver is limited in comparison with that of the stem cells derived from bone marrow was not considered.

The pattern of haematopoietic activity in human fetal liver has been shown to be quite different from that in human fetal bone marrow (Thomas, Russell & Yoffey, 1960). This difference is consistent with the possibility that the haematopoietic differentiation capacity of the stem cells in fetal liver is restricted. The bone marrow contains erythroblasts, granulocytes and lymphocytes in similar proportions, each accounting for approximately one quarter of the haematopoietic cell population (Yoffey & Thomas, 1961). In contrast, the vast majority of the haematopoietic cells in the liver are erythroblasts, and even the few granulocytes and lymphocytes which are observed in smears of liver cell suspensions may be derived from the blood in the hepatic sinusoids (Thomas & Yoffey, 1962, 1964). Thus there is no convincing evidence of granulocyte or lymphocyte production in human fetal liver which can be used to establish the granulocytopoietic or lymphocytopoietic capacity of the hepatic stem cell population. The pattern of hepatic haematopoiesis in the mouse fetus on the fifteenth day of gestation is similar to that in the human fetus (Thomas, 1966).

Hypoplasia of the lymphoid tissues has been described in lethally irradiated mice treated with intravenous injections of syngeneic fetal liver cells (Barnes, Loutit & Micklem, 1962). This cannot be due to immunological interactions between the

antigenically congruous host and donor cells. The possibility that it may result from the absence of donor cells with a lymphocytopoietic potential has therefore been considered (Thomas, 1963). A similar explanation has been considered by Barnes and his colleagues at Harwell to account for their observation that lethally irradiated CBA-T6T6 mice given C57BL fetal liver cells together with (CBA-T6T6 × C57BL)F_1 lymph node cells differ from similar mice given syngeneic bone marrow and lymphoid cells in having a high proportion of metaphases derived from the lymphoid cell donor in their lymphoid tissues (Barnes *et al.* 1967). Adequate comparisons of the differentiation capacities of the stem cell populations in bone marrow and in fetal liver, or in other sites of extramedullary haematopoiesis, have not, however, been reported. The postulated differences have not therefore been conclusively demonstrated nor has identity been established.

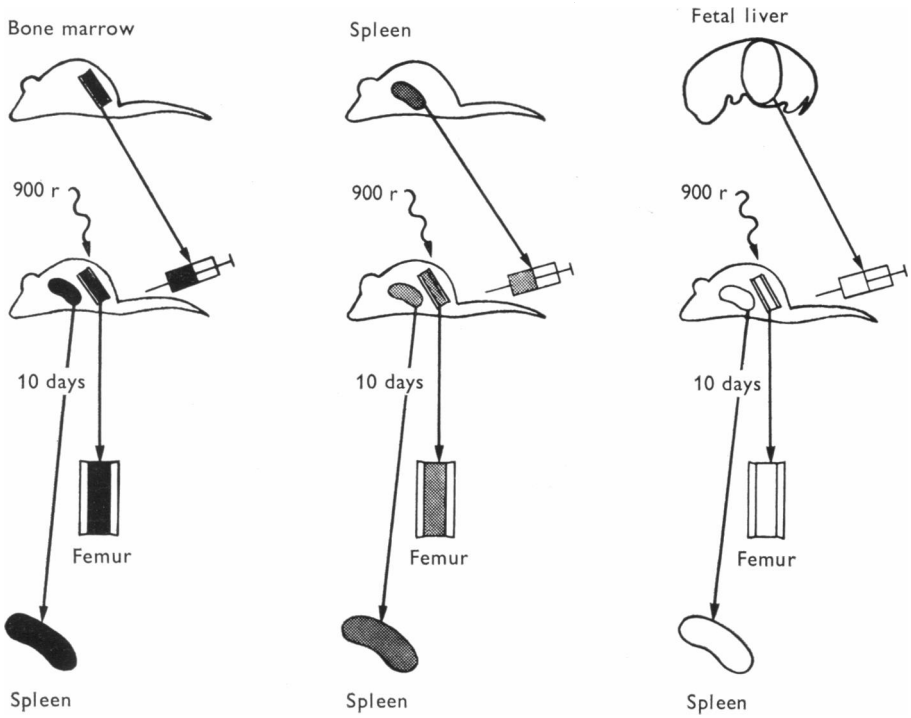

Fig. 1. Scheme of experimental procedure.

It has now been shown that the granulocytopoietic capacity of transplanted bone marrow cells is expressed to different degrees in the medullary cavity of the femur and in the spleen. This has been attributed to differences in the haematopoietic micro-environments provided by the medullary cavity and the extramedullary site (Thomas, 1970). Similar differences in the haematopoietic micro-environments provided by the medullary cavities and the liver of the fetus may account for some of the differences between the patterns of medullary and hepatic haematopoiesis. Fetal liver may thus contain cells which are capable of producing erythrocytes *in situ* and

granulocytes in an appropriate environment (Thomas, 1961) but which are incapable of producing lymphocytes. This is an intriguing possibility, as the existence of such stem cells has been postulated by Hayhoe and his collaborators in the light of their extensive study of the acute leukaemias (Hayhoe, Quaglino & Doll, 1964).

The present investigation has been devised to assess the dependence of differences between the patterns of hepatic and medullary haematopoiesis, which may co-exist in the same organism, upon differences in the differentiation capacities of the hepatic and medullary stem cell populations and upon differences in the micro-environments provided for haematopoiesis by the liver and the medullary cavities. The differentiation of cells derived from the three principal murine haematopoietic tissues – bone marrow, spleen and fetal liver – following transplantation to lethally irradiated mice has been analysed (Fig. 1). Each of these tissues has previously been shown to contain cells capable of proliferating in the virtually acellular haematopoietic and lymphoid tissues of a lethally irradiated recipient and thus establishing cell populations which may prevent haematopoietic insufficiency and death (Ford *et al.* 1956; Micklem, Ford, Evans & Gray, 1966; Barnes *et al.* 1967).

MATERIALS AND METHODS

(i) *Animals.* Specific pathogen-free albino mice of the CSI strain, supplied by the Scientific Products Farm, Ash, Kent, have been used throughout this investigation. Haematopoietic cell recipients, bone marrow cell donors and spleen cell donors were females weighing between 20 and 25 g. Liver cells were obtained from fetuses delivered by abdominal hysterotomy on the seventeenth day of gestation, determined by counting the date of appearance of a vaginal plug as the first day of gestation.

(ii) *Irradiation conditions.* Animals were accommodated in the radially disposed compartments of a ventilated circular lucite cage during exposure to a lethal dose (900 r) of whole-body X-irradiation. A Watson ST150 X-ray set was operated at 140 kVp and 7 mA, with 1 mm copper + 0·2 mm aluminium added filtration to give a dose rate of 60 r per minute at a point 26 cm from the anode.

(iii) *Preparation of donor cell suspensions.* Suspensions of donor cells in chilled Eagle's tissue culture medium (Wellcome Reagents Ltd.) were prepared by gentle homogenization of bone marrow fragments flushed from the femoral diaphysis, or small blocks of spleen or fetal liver, using dissecting needles. Any debris remaining after a suspension had been aspirated several times through a fine needle was discarded. The nucleated cell content of suspensions was estimated using routine haemocytometric techniques in an improved Neubauer counting chamber (Dacie & Lewis, 1970). Suspensions were then diluted with chilled Eagle's medium to provide the cell doses desired. Throughout the short interval between collection and injection the cells in suspension were gently agitated on a Matburn wheel in a refrigerator at 4 °C. Sterile instruments and containers were used. Fatal embolism was avoided by adding 0·1 mg (12 i.u.) of heparin sodium dissolved in isotonic saline to each millilitre of suspensions containing 2×10^7 or more cells per ml.

(iv) *Injection of donor cell suspensions.* Within 2 h of X-irradiation an intravenous injection of bone marrow cells, spleen cells or fetal liver cells suspended in 0·5 ml of Eagle's medium was administered to each recipient. Dilatation of the tail veins

was ensured by warming the recipients for half an hour or so under a 60 W electric light bulb before transferring them to the tubular holder in which they were immobilized while the injection was administered. A Gillette sterile needle (25 G × $\frac{5}{8}$, no. 20) was used for this purpose.

(v) *Animal accommodation and management.* During the 10 days following irradiation, and the subsequent injection of haematopoietic cell suspensions, groups of not more than five mice were housed in freshly sterilized cages. Water containing sulphadimidine (1·5 g/l) and saccharin elixir (8 drops per litre), added to conceal the bitter taste of the drug, was provided in freshly sterilized bottles. The cages and water bottles were changed every two days. Animals which were not progressing satisfactorily were isolated immediately and the remaining animals were transferred to a fresh cage.

(vi) *Biopsy procedure and the preparation of smears.* After 10 days the animals were killed by exposure to ether vapour. Smears prepared from suspensions of femoral bone marrow cells and of spleen cells, in serum from syngeneic donors, were stained using the following variation of the Jenner–Giemsa technique.

Air dried smears were fixed in methanol for 10 min or so and then immersed in a freshly prepared mixture of Jenner stain (113 ml) and water buffered to pH 6·4 (67 ml) for 5 min. After removal of excess stain by rapid washing in water buffered to pH 6·4 the smears were immersed in a freshly prepared mixture of improved Giemsa 'R 66' (30 ml) and water buffered to pH 6·4 (150 ml) for 10 min, washed thoroughly and differentiated in water buffered to pH 6·4, blotted and mounted in neutral mounting medium when dry. The Jenner stain, improved Giemsa 'R66', buffer tablets and neutral mounting medium were all supplied by George T. Gurr, Ltd. This variation of the Jenner–Giemsa technique has proved to be most satisfactory for murine haematopoietic cells which, as Dunn (1954) has emphasized, are exceedingly difficult to distinguish from one another in routinely stained preparations.

(vii) *Differential counts.* At least 500 consecutive cells were examined and classified in smears prepared from the femoral bone marrow and the spleen of each chimaera and in smears prepared in a similar fashion from the three donor tissues – femoral bone marrow, spleen and fetal liver. Damaged cells and cells which defied classification were scored as others.

RESULTS

The bone marrow is rendered virtually acellular and the lymphoid tissues severely atrophic in animals which do not receive injections of haematopoietic cells after exposure to the dose of whole-body X-irradiation used throughout this series of experiments (900 r). None survives longer than 13 days. In contrast a high proportion of animals which receive injections of haematopoietic cell suspensions survive for 7 months or more. Although these chimaeras are slightly smaller than untreated controls and most of them develop bilateral cataracts within 6 months of irradiation, they are in other respects unremarkable (unpublished observations).

(1) *Bone marrow*

Fifty-six per cent of the cells in femoral bone marrow are granulocytes at various stages of maturation; they outnumber erythroblasts eight to one. Lymphocytes

Table 1. *The cellular composition of the three principal murine haematopoietic tissues and of the cell populations derived from each in a medullary cavity and in an extramedullary site*

Site of differentiation ...	In donor tissue			In medullary cavity			In spleen		
Source of cells	Marrow	Spleen	Fetal liver	Marrow	Spleen	Fetal liver	Marrow	Spleen	Fetal liver
Number of cells injected	—	—	—	5×10^6	20×10^6	5×10^6	5×10^6	20×10^6	5×10^6
Dose of X-irradiation (r)	—	—	—	900	900	900	900	900	900
Granulocytes (%)*	56(±3)	5(±2)	3(±1)	50(±4)	34(±6)	46(±5)	26(±8)	12(±2)	24(±8)
Erythroblasts (%)*	7(±2)	5(±2)	90(±3)	12(±2)	11(±6)	23(±6)	39(±10)	40(±16)	55(±15)
Lymphocytes (%)*	30(±2)	81(±1)	1(±1)	18(±2)	43(±2)	17(±4)	24(±7)	39(±14)	10(±4)

* Damaged cells and cells which defied classification account for the residual portion of the haematopoietic cell population. Standard errors are quoted throughout.

account for nearly one-third of this population. One-fifth of the lymphocytes are intermediate in form between generalized blast cells and small lymphocytes.

The composition of the cell population which is established in the medullary cavity of the femur following the transplantation of bone marrow cells to lethally irradiated recipients is very similar to that of the donor tissue. However, the cell population which is established in the spleen differs both from that which usually occupies this site and from that of the donor tissue. Myeloid metaplasia in the spleen of the radiation chimaera results in the establishment of a cell population in which erythroblasts outnumber granulocytes and lymphocytes. For a given number of erythroblasts, the cell population established in the bone marrow contains six times as many granulocytes as that established in the spleen by cells derived from the same source – the injected bone marrow cell suspension.

Table 2. *The granulocyte: erythroblast ratios in cell populations established in a medullary cavity (M.C.) and an extramedullary site (E.M.S.)*

Donor tissue	Granulocytes per 100 erythroblasts after transplantation: M.C. (femur)	E.M.S. (spleen)	Granulocytes per 100 erythroblasts M.C. / Granulocytes per 100 erythroblasts E.M.S.
CBA bone marrow*	320	45	7·1
CSI bone marrow	410	66	6·2
CSI spleen	320	29	11·0
CSI fetal liver	200	45	4·4
Average	313	46	7·2

* Previously reported data (Thomas, 1970).

(2) *Spleen*

In untreated controls most of the cells in the spleen are lymphocytes and very few granulocytes or erythroblasts are present.

Following the transplantation of spleen cells to lethally irradiated recipients, granulocytes account for 34 % of the cells in the medullary cavity of the femur, where they outnumber erythroblasts three to one, although lymphocytes still constitute the largest group of cells. In the chimaera spleen erythroblasts and lymphocytes occur in similar proportions, each accounting for 40 % or so of the haematopoietic cell population, while granulocytes account for only 12 %. Thus, for a given number of erythroblasts, the medullary cavity contains eleven times as many granulocytes as the spleen.

(3) *Fetal liver*

Ninety per cent of the haematopoietic cells in fetal liver are erythroblasts, the numbers of granulocytes and lymphocytes being insignificant by comparison.

The composition of the cell population which is established in the medullary cavity of the femur following the transplantation of cells derived from fetal liver to irradiated recipients does not, however, reflect the pronounced differences between the cellular composition of fetal liver and that of bone marrow. On the contrary, this cell

population is very similar to that established by cells of bone marrow origin in the same environment. In the spleen erythroblasts again predominate, and the medullary cavity contains more than four times as many granulocytes for a given number of erythroblasts as the extramedullary site.

DISCUSSION

Murine bone marrow contains granulocytes, erythroblasts and lymphocytes. If these lymphocytes are produced locally and replaced every few days, as they are in the guinea-pig (Osmond & Everett, 1964), the bone marrow stem cell population is able to maintain granulocytopoiesis, erythrocytopoiesis and lymphocytopoiesis in the medullary cavity. Each of these differentiation capacities is suggested by the cellular composition of bone marrow.

The cellular composition of fetal liver does not indicate a corresponding range of differentiation capacities for the hepatic stem cell population. In the absence of significant numbers of granulocytes and lymphocytes, only its ability to maintain erythrocytopoiesis is evident.

Although the spleen contains a few erythroblasts and granulocyte precursors, the vast majority of the cells in the spleen are lymphocytes. Many of these lymphocytes may be derived from extrasplenic sources and the rate of turnover of a high proportion of splenic lymphocytes is very slow (Everett, Caffrey & Reike, 1964). It would not, therefore, be justifiable to assume that the cellular composition of the spleen provides a useful indication of the differentiation capacity of its haematopoietic stem cell population. This appears to be very small in comparison with that of the bone marrow (McCulloch & Till, 1963).

Following transplantation to the femoral medullary cavity of an irradiated recipient, cells derived from bone marrow, spleen and fetal liver proliferate and establish similar populations which do not exhibit the contrasting patterns of cellular composition displayed by the donor tissues. Thus the stem cell populations of these three tissues behave similarly when exposed to similar environmental influences. In each case the composition of the cell population established in the medullary cavity of the femur approximates to that of the femoral bone marrow in untreated controls. The different patterns of haematopoietic activity in these tissues appear therefore to result from differences in the micro-environments which they provide for haematopoiesis, rather than from differences in the differentiation capacities of their stem cell populations. It would not of course be justifiable to infer that the differentiation capacities of the stem cell populations in the three haematopoietic tissues investigated are identical.

Stem cell populations derived from bone marrow, spleen and fetal liver also behave in similar fashions following transplantation to the spleen of an irradiated recipient. The cell populations established by transplanted cells from each of these sources are all essentially similar to one another but differ from that of the spleen in untreated controls. Myeloid metaplasia results in the establishment of significant populations of granulocytes and erythroblasts in the spleen. The relative proportions of granulocytes and erythroblasts are quite different in the spleen and in the medullary cavity of the femur (Table 2), although the cell populations which occupy both sites

are in each instance derived from the same source – the injected haematopoietic cell suspension (Ford *et al.* 1956) and are subject to the same regulatory mechanisms within the same organism.

Following the administration of bone marrow cells to lethally irradiated recipients, erythroid colonies outnumber granulocytic colonies three to one in the spleen (Curry, Trentin & Wolf, 1964 and 1967; Juraskova, Tkadlecek & Drasil, 1964; Lewis & Trobaugh, 1964; Lewis, O'Grady & Trobaugh, 1968), while in the bone marrow granulocytic colonies outnumber erythroid colonies two to one (Wolf & Trentin, 1968). For a given number of erythroid colonies the bone marrow thus contains approximately six times as many granulocytic colonies as the spleen. From this it may be inferred that, for a given input to the erythroblast compartment, the number of cells leaving the stem cell compartment to enter the medullary granulocyte compartment is six times the number of cells entering the splenic granulocyte compartment. The different degrees to which the granulocytopoietic and erythrocytopoietic capacities of transplanted stem cell populations are expressed in these two micro-environments are reflected in the composition of the cell populations to which they give rise. On average the granulocyte compartment of the bone marrow contains seven times as many cells as the granulocyte compartment of the spleen for a given number of erythroblasts (Table 2 and Thomas, 1970). The discrepancy could be explained by supposing that unipotent cells destined for the granulocyte compartment enter the medullary cavity in preference to the spleen, while cells destined for the erythroblast compartment enter the spleen rather than the medullary cavity. This explanation would not, however, account for the discrepancy between the number of granulocytes for a given number of erythroblasts in fetal liver and in the cell population established in the medullary cavity of the femur by transplanted cells derived from fetal liver (Table 1). The stem cell population of fetal liver is capable of initiating and maintaining granulocytopoiesis in a suitable micro-environment. Thus the insignificant numbers of granulocytes in fetal liver cannot be attributed to the absence or small size of a stem cell population capable of initiating and maintaining granulocytopoiesis, but rather to the failure of this stem cell population to express its granulocytopoietic potential in an inappropriate micro-environment.

Transplanted cells derived from the bone marrow, spleen or fetal liver establish significant lymphocyte populations in the femoral medullary cavity, although lymphocytes account for variable proportions of the haematopoietic cell populations derived from different sources. These variations in the relative size of the medullary lymphocyte compartment should be interpreted with extreme caution. It would not be justifiable to suppose that they reflect differences in the relative lymphocytopoietic capacities of the various stem cell populations which have been compared. Thus the cell population derived from transplanted spleen cells contains a higher proportion of lymphocytes than those derived from transplanted bone marrow or fetal liver cells. Lymphocytes occur in similar high proportions in animals treated with small doses of bone marrow or fetal liver cells (unpublished observations). The high proportion of lymphocytes in the medullary cavities of animals which received injections of spleen cells may similarly reflect the administration of small numbers of stem cells. Although the dose of spleen cells used in this investigation was four times the dose of bone marrow or fetal liver cells selected, a given number of bone marrow cells

may contain between 5 and 25 times as many colony-forming units as the same number of spleen cells (McCulloch & Till, 1963). The relative capacities of the stem cell populations of murine bone marrow, spleen and fetal liver to effect regeneration of the lymphoid tissues cannot be inferred from the results of the present investigation.

It is concluded that the haematopoietic differentiation capacities of the stem cell populations of murine bone marrow, spleen and fetal liver are very similar, though not necessarily identical. Each is capable of initiating and subsequently maintaining granulocytopoiesis, erythrocytopoiesis and lymphocytopoiesis in a suitable micro-environment. The micro-environment provided by the medullary cavities is more conducive to the transfer of cells from the stem cell compartment to the granulocyte compartment than the micro-environments provided in extramedullary sites such as the spleen or liver. The extramedullary sites provide micro-environments which are better suited to erythrocytopoiesis than they are to granulocytopoiesis.

These generalizations apply, of course, to cell populations and not to individual cells. This investigation does not attempt to establish whether the stem cell populations studied are mixtures of unipotent cells, each of which is capable of giving rise to a single variety of end product, or homogenous populations of cells, any one of which can give rise to progeny of any variety, depending upon the nature of the regulatory mechanisms to which it is exposed.

SUMMARY

The differentiation of transplanted haematopoietic cells derived from murine bone marrow, spleen and fetal liver has been compared. Intravenous injections of cell suspensions prepared from these tissues were administered to mice which had been exposed to a lethal dose of whole-body X-irradiation less than 2 h previously. Ten days later the compositions of the cell populations established in the femoral medullary cavity and in the spleen were analysed and found to be different. However, in each site the cell populations which were established were all of similar composition and did not reflect the different patterns of haematopoietic activity exhibited by the donor tissues.

As each of the stem cell populations studied differentiates in a similar fashion when exposed to similar environmental influences, it is inferred that the differentiation capacities of the stem cell populations of murine bone marrow, spleen and fetal liver are similar but not necessarily identical.

The different patterns of haematopoietic activity in the donor tissues are attributed to differences in the micro-environments which they provide for haematopoiesis. The micro-environment provided by the medullary cavities is more conducive to the transfer of cells from the stem cell compartment to the granulocyte compartment than to the erythroblast compartment, whereas in the micro-environments provided by extramedullary sites of haematopoiesis more cells enter the erythroblast compartment than the granulocyte compartment.

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