PLURIPOTENTIAL STEM CELLS IN MOUSE EMBRYO LIVER

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In embryonic development the first appearance of lymphoid cells is in the thymus, and it is only later that these cells begin to appear in the lymph nodes and spleen (Ball and Auerbach, 1960). The origin of these first lymphocytes has long been a matter for discussion. Some, following Kölliker (1879), have held that they stem from the epithelial cells of the thymus rudiment ; while others, of whom Maximow (1909) was among the first exponents, argued that the lymphoid cells entered the thymus rudiment from outside. The evidence from more recent work is still conflicting, and will be discussed later. Concerning lymphopoiesis in post-natal life the situation is a little clearer, and recent work has favoured the view of Maximow in that it has detected cells entering the thymus (Miller, 1962 ; Metcalf and Wakonig-Vaartaja, 1964 ; Harris, Barnes, Ford and Evans, 1964) while failing to reveal any stable population of stem-cells within the thymus—such as might be provided by the epithelial framework (Harris and Ford, 1964). It is well known that the recovery of both lymphoid and haematogenous tissues after they have been depleted by irradiation is favoured by the injection of bone marrow cells, and that cells from the injected marrow actually repopulate these tissues (Law, 1952 ; Ford, Hamerton, Barnes and Loutit, 1956). The repopulation is likely to be a function of the least differentiated types of cell, which have been termed stem-cells (Bloom, 1938 ; Yoffey, 1960).

This property of repopulating marrow and thymus is shared only weakly, if at all, by lymph node or thymus cells (Ford and Micklem, 1963), thoracic duct cells (Gowans, 1959 ; Gesner and Gowans, 1962) or blood and peritoneal exudate cells (Goodman, 1963). The question arises whether there is a distinct type of stem cell for each line of differentiation, or whether a single pluripotential species serves them all.

The object of the following experiments was first to take embryo liver at a stage before lymphoid differentiation of the thymus becomes apparent (12 days) and examine its capacity to repopulate the thymus of irradiated recipients. If lymphoid stem cells were thus demonstrable at this stage it would suggest that they might be the source of the first-appearing lymphocytes in the embryonic thymus. The second object was to examine embryo liver, or irradiated bone marrow for dissociation between lymphoid and haematogenous potentialities such as might indicate separate populations of stem cells.

The principle of the experiments was to mix the cells under test in known proportion with normal bone marrow cells which served as a standard, and to inject the mixtures into irradiated recipients. The number of mitoses (distinguished by the T6 chromosome) of " test " relative to " standard " cell origin

found in the recipient were taken as a measure of the proliferative capacity (PC) of the " test " cells relative to normal bone marrow. As obtained from the recipient thymus this figure was taken to relate to cells of lymphoid potentiality, and as from the bone marrow to cells of mainly erythroid and myeloid potentialities.

MATERIALS AND METHODS

Mice of the co-isogenic CBA and CBA/T6T6 strains were used. The latter strain, developed at Harwell (see Harris and Ford, 1964), is homozygous for a distinct chromosome translocation, and has been much used in tracing transferred cells in radiation chimaeras. Recipient mice of both strains were irradiated with 900 r immediately before the cell transfers, which were all made intravenously. Mice were killed by cervical dislocation and bone marrow obtained by squirting Gey's solution through the femora and subsequent pipetting to dissociate the cells further. The age of embryos was timed from the day of observation of the vaginal plug. Embryo livers were dissected out and the cells dissociated by gentle pipetting. Injections were made on the basis of viable nucleated cells. These were defined by their ability to exclude 0·1 per cent eosin. It is possible that the foetal liver suspensions were slightly contaminated by intestinal cells. They were about 70 per cent viable. Chromosome preparations were made by a modification currently employed by Ford (personal communication, 1963) of the drying method of Rothfels and Siminovitch (1958).

EXPERIMENTAL AND RESULTS

In the first experiment recipient mice were injected with CBA embryo liver and CBA/T6T6 bone marrow in various proportions (Table I). Some control mice received bone marrow alone in order to detect the contribution of mitoses by the irradiated hosts. The recipient mice were killed 3–8 weeks later, and chromosome preparations made from thymus and bone marrow. The results (Table I, Fig. 1) show that the proliferative capacity of both 12-day and 15-day embryo liver was about equal to that of normal adult bone marrow. This was so whether it was assessed in the recipient thymus or bone marrow.

In one experiment (Table I, expt. IV) the embryo cells were taken from a homogenate of embryo tissue. This was prepared from the trunk region but taking care to exclude liver and thymus. The cells were dissociated without trypsinisation, and only 20 per cent were viable. They had the same proliferative capacity per viable cell as embryo liver. The proportion of mitoses contributed by cells of the irradiated hosts was found to be very small (Table I, expt. V). There was a tendency (Fig. 1) with increasing time after transfer for the proportions of mitoses to drift away from the mean ; yet in spite of this the proportions in thymus remained in most cases highly correlated with those in bone marrow. In one recipient, however, there was a significant difference between the two tissues in this respect.

The proliferative capacity of bone marrow can be lastingly impaired by X-irradiation (Cudkowitz, Upton, Shearer and Hughes, 1964). In the second experiment the proliferative capacity of such "depleted" bone marrow was compared with that of normal marrow by the method just described. If any dissociation in the capacity to repopulate marrow and thymus occurred as a result of irradiation it should be readily detected in this way. The depleted donors were CBA/T6T6 mice which had survived a dose of 900 r (without marrow therapy) 9 weeks before the cell transfer.

The results (Table II) show that the proliferative capacity of depleted bone marrow cells had been depressed to the same extent (about 0·7 per cent) whether it

TABLE I.—*Comparison of Proliferative Capacities of Embryo Cells with Adult Bone Marrow Cells in the Thymus and Marrow of Irradiated Host Mice*

Expt.	Age of embryos (days)	No. of cells transferred: CBA embryo liver	No. of cells transferred: CBA/T6T6 adult marrow	Time after transfer (weeks)	Host	Mitoses scored in: Thymus T6T6/T6⁻	Thymus PC (per cent)	Marrow T6T6/T6⁻	Marrow PC (per cent)	P†
I	15	10^7	10^6	3	CBA	2/15	70	4/28	70	—
					CBA	4/29	73	8/29	36	>0·2
II	15	10^6	10^6	3	CBA/T6T6	6/9	150	16/24	150	—
					CBA/T6T6	5/8	160	15/17	113	—
					CBA	4/3	75	19/16	84	—
III	12	10^6	10^5	5	CBA/T6T6	12/2	17	13/2	15	—
					CBA/T6T6	14/13	93	*(4) 18/34	189	>0·1
					CBA	*(1) 25/2	8	*(5) 12/15	125	<0·001
					CBA	*(2) 8/7	88	7/8	114	—
				7	CBA/T6T6	5/10	200	5/10	200	—
					CBA/T6T6	4/6	150	8/7	88	—
					CBA	3/12	400	4/11	280	—
				8	CBA/T6T6	14/1	7	14/1	7	—
					CBA	9/3	33	11/4	36	—
		CBA embryo homog.	CBA/T6T6 adult marrow							
IV	11	5×10^5	5×10^5	6	CBA/T6T6	*(3) 24/20	83	*(6) 13/28	215	>0·05
					CBA/T6T6	6/14	233	3/7	234	—
V	—	—	10^6	6	CBA	24/1	4	25/0	4	—
					CBA	15/2	13	20/1	5	—
					CBA	24/1	4	20/1	5	—

* Numbers referring to Table III. † Probability of difference in karyotype distribution between thymus and bone marrow (χ^2 test).

PC = Proliferative capacity of embryo cells (Expts. I–IV) or host cells (Expt. V) relative to adult marrow cells.

TABLE II.—*Comparison of Proliferative Capacities of Stem-cell-depleted and Normal Bone Marrow Cells in the Thymus and Marrow of Irradiated Hosts. Karyotype Analyses were Performed 3 Weeks After Cell Transfer*

No. of marrow cells transferred: CBA/T6T6 (depleted)	CBA (normal)	Host	Mitoses scored in: Thymus T6T6	Thymus Clone	Thymus T6⁻	†PC (per cent)	Marrow T6T6	Marrow Clone	Marrow T6⁻	†PC (per cent)	‡P
10^6	10^5	CBA	1	0	35	0·3	2	1	33	0·9	—
10^7	10^5	CBA	5 *(7)	33	53	0·7	27 *(8)	7	50	0·7	<0·001

* Nos. of chromosome preparations referring to Table III.

† Proliferative capacity of " depleted " relative to normal bone marrow.

‡ Probability of difference in karyotype distribution between thymus and bone marrow (χ^2 test).

was assessed in recipient marrow or thymus. Further examination revealed that a proportion of cells had a distinctive chromosome anomaly, additional to the T6 translocation (Fig. 2). These cells were present in both marrow and thymus of the recipients, but were significantly more frequent in thymus (Table II).

In view of reports of small foci of granulopoiesis in the thymus of animals recovering from irradiation (Grégoire, 1938; Urso and Congdon, 1957) the chromosome preparations were examined for granulocytes. The ring or horseshoe form of granulocyte nuclei could easily be distinguished from round nuclei. While these made up about 25 per cent of the bone marrow cells they were extremely scarce in most of the thymus preparations, and certainly too few to account for the mitoses of embryonic origin (Table III).

The third experiment was an attempt to detect pluripotential stem cells in the

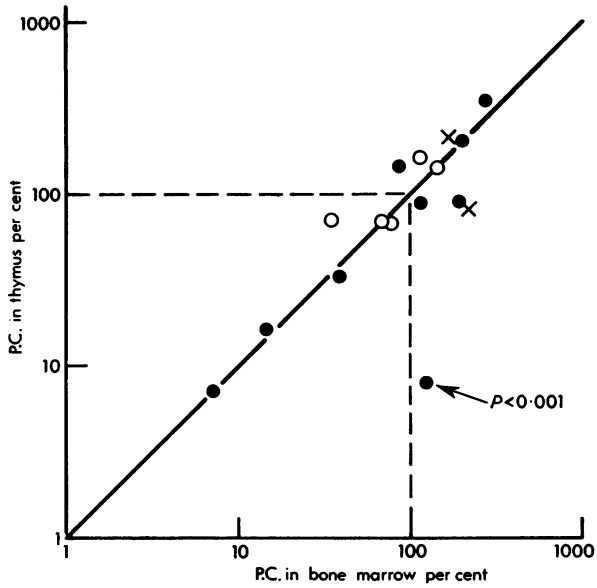


FIG. 1.—In this diagram the results from Table I are presented in graphical form. All values are of proliferative capacity (PC) of embryo cells relative to adult marrow cells. Each point represents one recipient mouse in which this quantity has been estimated in thymus (ordinate) and bone marrow (abscissa). The figure shows a strong correlation between the PC in thymus and bone marrow, although two points deviate significantly.

TABLE III.—*Classification of Nuclei in Chromosome Preparations from Tables I and II*

No. of preparation (* in Tables I and II)			Round nuclei	Polymorph nuclei	Mitoses	Polymorphs (per cent)
Table I	(1)	Thymus	1040	6	6	0·57
	(2)	Thymus	1016	8	12	0·77
	(3)	Thymus	329	7	6	2·00
	(4)	Marrow	246	50	50	17·0
	(5)	Marrow	154	44	2	22·0
	(6)	Marrow	281	131	9	31·0
Table II	(7)	Thymus	403	24	8	5·5
	(8)	Marrow	142	76	4	34·0

thymus by virtue of their ability to form macroscopic spleen colonies (Till and McCulloch, 1961). Irradiated (900 r) recipient mice were injected with various numbers of adult or neonatal thymus or bone marrow cells. Controls received no cells. To reduce mortality half of the mice in each group were given 2 intravenous injections of 0·5 ml. irradiated (900 r) heparinised mouse blood at 3 and 5 days after irradiation. The mice surviving to 10 days were killed and the spleens examined and weighed. Although cell colonies were plentiful in the spleens of mice receiving bone marrow, only one colony was found among mice receiving thymus cells (Table IV). This was not significant since there was also one colony

TABLE IV.—*Failure of Neonatal Thymus Cells to Form Spleen Colonies*

	Cells injected (millions)	Injection of blood	Relative spleen weight (mg./g. body wt.)	Spleen colonies
Bone marrow	0·25	+	2·6	Numerous
	0·25		2·4	,,
		−	4·1	,,
			4·4	,,
	0·05	+	1·9	~7 per spleen
			1·7	,,
		−	2·4	,,
			3·1	,,
Neonatal thymus	3·7	+	1·9	0
			1·8	0
		−	2·0	0
			1·6	1
	10·0	−	1·7	0
			1·6	0
	31·0	−	1·2	0
			1·0	0
Adult thymus	10·0	−	1·0	0
No transfer		+	1·2	0
			2·0	0
		−	1·3	1
			1·2	0
			1·2	0

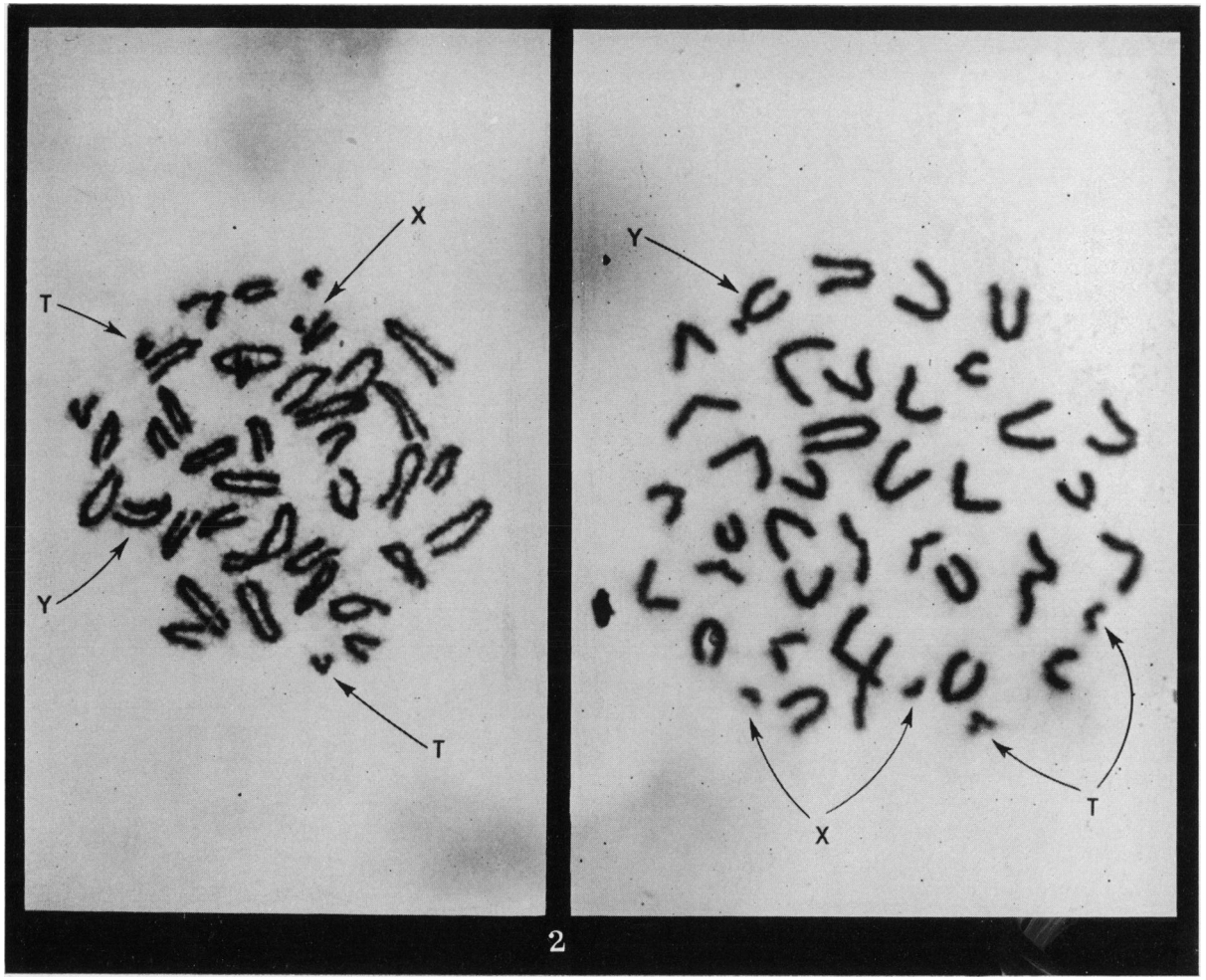
among control mice. The injection of blood, while not influencing mortality appreciably, had the effect of depressing the increase in spleen weight in the mice receiving bone marrow. The results of Siminovitch, McCulloch and Till (1963) suggest that some 17 per cent of the colony-forming units injected settle in the spleen. Therefore, if colony-forming units are present at all in the thymus they cannot make up more than 1 in 5×10^6 thymus cells, and there would be less than 2 such units in the neonatal mouse thymus.

DISCUSSION

Previous evidence concerning the origin in embryonic life of thymus lymphocytes has been mainly based either on histological observation (*e.g.* Maximow, 1909; Kölliker, 1879) or on the behaviour of explants of embryo thymus epithelium when denied of cellular exchange (*e.g.* Grégore, 1935; Auerbach, 1961).

EXPLANATION OF PLATE

FIG. 2.—Chromosomes of two cells belonging to the clone referred to in the text. The clone is characterised by 3 marker chromosomes in addition to the 2 T6 chromosomes (T). Two are very short (X) and one has a (displaced) subterminal centromere (Y).



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There are obvious uncertainties attached to histological observation of cellular transitions, while the second type of method suffers from the objections on the one hand that where lymphoid differentiation was not seen (Grégoire, 1935) this may have been due to the inadequacy of the nutritional medium; where, on the other hand, it was obtained (Auerbach, 1961) there is no proof that the lymphoid cells originated from the thymus epithelium itself and not from a few immigrant lymphoblasts which went undetected at the time the explant was removed from the embryo. In support of the immigration hypothesis, Grégoire (1935) found that the mitotic index among epithelial cells was far too low to account for the initial influx of lymphocytes—indeed even the overall mitotic index was too low. The most recent anatomical studies, by electron microscope, of adult mouse thymus, agree in failing to find cells transitional between epithelial and lymphoid morphology (Clark, 1963; Kohnen and Weiss, 1964) although such transitional cells have been reported in the bursa of Fabricius in the chick embryo (Ackerman, 1962) and thymus (Ackerman and Knouff, 1964).

The present experiments show that 12-day embryo liver contains cells capable of proliferation in the thymus of irradiated recipients. It seems very likely that the embryonic cells proliferating in the thymus were of lymphoid type, although this has not been proved. At least they were too many to be accounted for by the myeloid cells present in the thymus. The presence of extrathymic lymphoid stem cells in 9–12 day mouse embryos has been suggested by the experiments of Tyan and Cole (1963). Cells taken from the abdominal contents (including mainly liver cells) of parental strain (A) embryos were injected into irradiated F_1 hybrid (A $\times$ B) recipients. The subsequent activity of recipient spleen cells in causing graft-versus-host disease in a second F_1 hybrid (A $\times$ C) was interpreted as revealing the presence of immunologically competent cells of the parental type (A) derived from the original injection of embryo cells. In a later experiment Tyan (1964) found that thymectomy of the first host prevented this effect.

The entry of embryo liver or adult marrow cells into the thymus could be an artefact depending on the conditions of irradiation and cell transfer. But since an afferent stream of cells to the thymus still occurs between parabiosed pairs of mice which have suffered neither irradiation, nor thymus grafting, nor cell transfer (Harris *et al.*, 1964), it may be assumed that the present results reflect a real lymphoid potentiality present in 12-day embryo liver. However, this potentiality was evidently not confined to the liver and might at this stage be a general property of many embryonic tissues, including the thymus.

Dissociation of haemopoiesis from lymphopoiesis has been reported by Berman and Kaplan (1959) who found that treatment of bone marrow *in vitro* with urethane or irradiation impaired its ability to repopulate thymus more than its ability to repopulate bone marrow. However, this result is compatible with a unitary stem-cell hypothesis if one makes the assumption that in the irradiated recipient the demand for red cells is such that a limited number of stem-cells will tend to be diverted along an erythroid rather than a lymphoid pathway of differentiation. Such an interpretation is indeed suggested by the experiment of Urso and Congdon (1957) who injected marrow cells in a wide range of dosages, and found that the smaller doses induced disproportionately small regeneration on the part of the thymus as compared with bone marrow.

The findings here that irradiation depleted haemopoietic and lymphopoietic functions equally, and that (with one exception) embryo cells had these capacities

in exactly the same relative proportions as adult bone marrow cells, are also consistent with a unitary stem-cell hypothesis. More direct evidence for the existence of stem-cells combining lymphoid and haemopoietic potentialities came from Barnes, Ford, Gray and Loutit (1959) who examined mice after recovery from irradiation and identified karyotypically-distinct clones of cells. The cells of any clone were represented in marrow, thymus and lymph nodes, and sometimes these organs would consist almost exclusively of a single clone. A similar clone described here was represented in both marrow and thymus but much more frequently in the latter tissue. The mitoses in recipient thymus which originated from the CBA/T6T6 donor but did not belong to the clone were so few that they could have been accounted for by the proportion of granulocytes in the thymus. In this case one might postulate that the radiation damage which was responsible for the clone took place in a cell which had already undergone lymphoid determination. The clonal mitoses in the marrow would then represent a pool of specifically " lymphoid " stem-cells. Another possibility is that the thymus was reconstituted from a very few stem cells and that these had by chance been mostly members of this clone; or else it may be that irradiation damage could differentially affect the ability of cell clones to enter the thymus. The dissociation of proportions between marrow and thymus in one of the recipients of embryo cells (Fig. 1) evidently depended on an unusual circumstance in the recipients, since the majority of recipients, receiving the same cell suspension, showed no dissociation. It might be that during conditions of stress the afferent stream of cells to the thymus becomes interrupted and that this allows the proportions of the 2 cell types in the thymus to drift independently of their proportions in the marrow.

The failure to detect colony-forming cells in the neonatal thymus indicates either that the pluripotential stem-cells become determined on a lymphoid pathway before entering the thymus (as was suggested by the behaviour of the clone) or that if they do enter the thymus as such they must become determined soon afterwards.

SUMMARY

Cells under test were injected into lethally-irradiated recipients and allowed to proliferate competitively against a known proportion of normal adult bone marrow cells which served as a standard. Proliferating cells of different origin, distinguishable by means of the T6 marker chromosome, were scored in recipient bone marrow and thymus. The results indicate that stem-cells capable of lymphoid differentiation are present equally in adult bone marrow and in embryo liver at a stage (12 days) before lymphocytes become evident in the thymus rudiment. In bone marrow taken from mice 9 weeks after irradiation the proliferative capacity was found to have been depressed to the same extent (1 per cent) whether assessed in bone marrow or thymus. A karyotypically-distinct clone of cells which had arisen in the irradiated donors was present in both these tissues. This evidence is compatible with the hypotheses (a) that both haemopoetic and lymphopoietic capacities can reside in the same cell, and (b) that this cell is a precursor of the thymus lymphocytes both in adult and embryonic life.

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