

IN VITRO STUDIES ON LYMPHOCYTE DIFFERENTIATION

I. Long Term In Vitro Culture of Cells Giving Rise  
to Functional Lymphocytes in Irradiated Mice\*

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The primary lymphoid organs of adult animals continue to generate large numbers of lymphocytes throughout life (1, 2). Knowledge of the regulation and cellular basis of lymphocyte generation is thus an integral part of an understanding of the working of the mature immune system. It is well established that adult animals contain pluripotential stem cells that can give rise not only to hemopoietic cells but also to lymphocytes (3). Little is known however about the steps between pluripotential stem cells and lymphocytes. Unanswered questions include those concerning the self-renewal capacity of cells at various steps of lymphocyte differentiation, the rate at which clones of lymphocytes with newly determined receptor specificities are being generated, possible regulating influences, and the manner in which self-reactive clones are controlled. A method for generating lymphocytes from pluripotential cells in vitro would greatly facilitate the study of these and other questions. Recently Dexter et al. (4) have developed a tissue culture system permitting the long-term proliferation of stem cells that form colonies of erythroid and myeloid cells in the spleens of irradiated mice. We have investigated the likelihood that this culture-system also supports the growth of cells able to give rise to lymphocytes. In this report we document the long-term in vitro culture of cells able to repopulate irradiated hosts with both T and B lymphocytes.

**Materials and Methods**

Mice (C57BL/6, CBA, BALB/c, DBA/2, and their F<sub>1</sub> hybrids) were bred under specific pathogen-free conditions at the Hall Institute. Alloantisera between these strains were prepared by the repeated intraperitoneal injection of  $50 \times 10^6$  spleen cells.

Tetramethylrhodamine (TMR)- and fluorescein (FLU)-labeled sheep antibodies to mouse Ig were donated by Ms. J. Layton, Walter and Eliza Hall Institute. FLU-labeled goat antibodies to mouse- $\gamma_2$ -globulin were obtained from Meloy Laboratories Inc. (Springfield, Va.).

Polymerized flagellin (POL) conjugated with the haptens 2-4 dinitrophenol (DNP-POL), 4 hydroxy-3 iodo-5-nitro-phenacetyl (NIP-POL), and FLU-POL were the gift of Ms. B. Pike.

Long-term cultures of bone marrow cells were set up as described by Dexter et al (4) with the following modifications. The medium used was Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 25% horse serum (batch 632.1 from Commonwealth Serum Laboratories, Parkville, Australia) and cultures were maintained at 33°C in 10% CO<sub>2</sub> and air. Plastic flasks (Corning 25100, Corning Glass Works, Science Products Div., Corning, N. Y. or Falcon 3013, Falcon Plastics, Div. of BioQuest,

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Oxnard, Calif.) were first coated with a monolayer of cells by culturing in each the cells flushed from one femur shaft in 10 ml of medium for 1 wk. The medium and nonadherent cells were then removed and long-term cultures were initiated by adding  $10^7$  viable nucleated bone marrow cells in 10 ml of fresh medium. Three times weekly, one-half the medium and about one-half of the nonadherent cells (which comprised the majority of the total cell population) were harvested and the cultures fed with 5 ml of fresh medium.

Lethal irradiation, preparation of antigens, and plaque assays for antibody-forming cells (AFC) were performed as described elsewhere (5).

The *in vitro* generation and assay of cytotoxic T cells, and the complement-mediated cytotoxicity by alloantisera were performed as before (6).

### Results and Discussion

After the 1st wk of culture, the numbers of readily suspended nonadherent cells stabilized for 6–7 wk at  $0.5\text{--}4 \times 10^6$  cells per culture. Thus, over this long period, the cultures appeared capable of continuously generating harvestable cells at a significant plateau rate. The majority of these cells ( $\cong 80\%$ ) were granulocytes or macrophages, the remaining cells being less differentiated blast cells. Cells resembling small lymphocytes morphologically were rare ( $<0.1\%$ ). The incidence of cells forming colonies of myeloid cells in soft agar in the presence of the appropriate colony-stimulating factors was between 0.1 and 0.5%, and that of cells forming macroscopic colonies in the spleens of irradiated mice,  $\cong 10$ -fold less. Surface immunoglobulin positive cells declined to undetectable levels during the 1st wk. There was no proliferative response of the cultured cells to the mitogens concanavalin A or lipopolysaccharide. Thus, the established cultures did not appear to produce mature T or B lymphocytes.

To investigate whether these cultures contained cells that were able to give rise to lymphocytes under different circumstances, small numbers of cultured cells ( $5\text{--}20 \times 10^4$ ) were injected *i.v.* into lethally irradiated mice. Both the spleen and thymus of these mice enlarged during the first 2 wk (Fig. 1). B cells, staining strongly with fluorescent anti-immunoglobulin reagents were detected in the spleen during the 2nd wk and increased in frequency in the 3rd and 4th wk (Fig. 1). Results were comparable when the donor cells were taken from cultures of 2- to 7-wk duration. Thus, the cultures appeared to contain cells capable of giving rise to both B cells and thymocytes in irradiated hosts.

These results raised three immediate questions. The first was whether the lymphocytes found in the irradiated hosts were indeed derived from the cultured cells, as it was conceivable that the injected erythroid and myeloid stem cells allowed the proliferation of residual host lymphoid precursor cells, either through direct supportive action or merely by preventing the early death of the host. The second question was whether the lymphocytes in the repopulated animals were fully functional and the third, whether these lymphocyte populations represented a severely restricted repertoire of antibody specificities (clonotypes). The experiments here reported established the donor origin of the lymphocytes and suggested that the lymphocytes had a normal range of functions and were not grossly restricted in their receptor-repertoire.

To investigate the origin of the lymphocytes in repopulated animals, advantage was taken of the fact that cultured bone marrow cells could reconstitute semiallogeneic hosts. Chimeras, formed by injecting parental cultured cells into lethally irradiated  $F_1$  hybrid recipients, were vigorous and healthy after 1 mo and showed no signs of graft-versus-host disease. Immunofluorescence techniques were then used to determine whether the surface alloantigens on the lymphocytes in these animals matched those

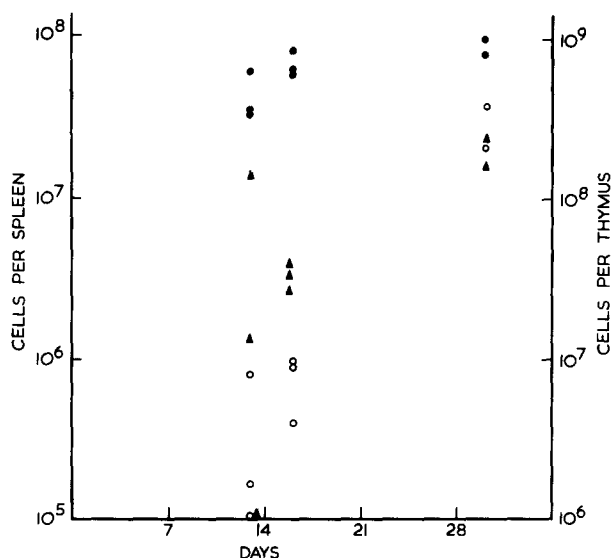


FIG. 1. Repopulation of spleen and thymus in irradiated hosts by cultured bone marrow cells. Lethally irradiated C57BL/6 mice were injected i.v. with  $1.4 \times 10^6$  cells from 14 day cultures of C57BL/6 bone marrow. Mice were killed at the time points indicated and the nucleated cells per spleen (●) and per thymus (▲) counted. Aliquots of  $10^6$  cells were incubated with  $10 \mu\text{l}$  of FLU-sheep anti-mouse Ig reagent in  $100 \mu\text{l}$  of medium for 20 min at room temperature, washed, and the percentage of strongly staining cells (B cells) determined by fluorescence microscopy. The number of B cells per spleen (○) was then calculated.

TABLE I  
Donor Origin of Immunoglobulin-Positive Cells in Irradiated Mice Reconstituted with Cultured Bone Marrow Cells

Chimera	Donor	Recipient	Immunoglobulin-positive cells per spleen*	Staining with alloantisera		
				Anti-C57BL/6	Anti-BALB	Anti-CBA
1	BALB/c¶	(BALB/c × C57BL/6)F <sub>1</sub>	$6 \times 10^6$ (20%)	—	+‡	ND
2	C57BL/6§	(BALB/c × C57BL/6)F <sub>1</sub>	$15 \times 10^6$ (38%)	+	—	ND
3	C57BL/6	(CBA × C57BL/6)F <sub>1</sub>	$21 \times 10^6$ (32%)	+	ND	—

Donor inocula were  $3.5 \times 10^6$  cells from 52 day cultures,¶ or  $9 \times 10^6$ § and  $4 \times 10^6$ || cells from 31 day cultures. Chimeras were killed on day 21 (1), or day 27 (2, 3). Immunoglobulin-positive cells per spleen\* were stained with TMR-sheep anti-mouse Ig antibodies under capping conditions. The cells were then exposed for 20 min at 4°C to  $10 \mu\text{l}$  of the indicated alloantisera in  $100 \mu\text{l}$  of phosphate-buffered saline (PBS) with  $\text{NaN}_3$  (20 mM) to prevent further capping. They were then washed and exposed to  $10 \mu\text{l}$  of FLU-goat anti-mouse- $\gamma_2$ -globulin antibodies for 20 min at 4°C in  $100 \mu\text{l}$  of PBS and  $\text{NaN}_3$  (20 mM), washed, and viewed by fluorescence microscopy. In positive reactions with the alloantisera‡ all immunoglobulin-positive cells (with bright red caps) also had bright green patches outside the caps. ND, not done.

of the parental donor or the F<sub>1</sub> hybrid. These experiments demonstrate that both the immunoglobulin-positive B cells (Table I), and the thymocytes (Table II), in such chimeras were derived from the cultured cells.

The reconstituted animals responded to immunization with a variety of antigens, and antibody-forming cells were demonstrable in the spleen. The antibody-forming cells in chimeric mice, made by injecting parental cultured cells into irradiated F<sub>1</sub> hybrids, were shown to be derived from the cultured cells (Table II). Table III shows the response of reconstituted mice to simultaneous immunization with three haptens. The reconstituted mice responded to all three haptens. Furthermore, although the responses were lower than those of controls, the ratios of the responses to the different haptens to each other were comparable in the reconstituted and the control mice,

TABLE II  
*Donor Origin of Antibody-Forming Cells and Thymus Cells in an Irradiated Host*

Cell source	Anti-NIP AFC in spleen after treatment with complement and*		Cells in thymus stained with‡	
	CBA Anti-C57BL/6 serum	C57BL/6 Anti-CBA serum	CBA Anti-C57BL/6 serum	C57BL/6 Anti-CBA serum
			%	
Chimera§	0.5	125	100	17¶
Chimera	0	90	100	9¶
C57BL/6	2.5	120	ND	ND
CBA	117	0	ND	ND
(CBA × C57BL/6)F <sub>1</sub>	14	1.5	100	100

Chimera§ were formed by injecting irradiated (CBA × C57BL/6)F<sub>1</sub> hybrid mice with  $1.4 \times 10^8$  cells from 14 day cultures of C57BL/6 bone marrow cells. 1 mo later these mice together with normal CBA, C57BL/6, and (CBA × C57BL/6)F<sub>1</sub> hybrid mice, were injected i.p. with 10 µg FLU-POL, 10 µg NIP-POL, and 5 µg DNP-POL. After 3 days the mice were killed and cell suspensions prepared from the spleens and thymuses. The spleen cells were assayed for antibody-forming cells and 0.5 ml aliquots adjusted so that 50 µl contained  $\approx 100$  anti-NIP. AFC were treated with alloantisera directed to either parental strain and then complement. The cells were resuspended in 0.5 ml and duplicate 50-µl aliquots were assayed for anti-NIP AFC\*. Similar results were obtained with anti-FLU or anti-DNP AFC.  $2 \times 10^8$  Thymus cells were treated with 10 µl of the indicated alloantisera‡ at 4°C for 30 min, washed, and then treated with sheep anti-mouse Ig antibodies (10 µl in 100 µl of medium) for 40 min at room temperature and washed. In some samples || all cells were brightly stained,  $\approx 10$ –15% of the cell being capped, and the remainder being patched. In other samples¶ only capped cells were present; these were probably B cells, as comparable numbers of capped stained cells (11%) were seen in samples of thymocytes from the chimeras after treatment with the FLU-sheep anti-mouse IgM alone.

TABLE III  
*Irradiated Mice Reconstituted With Cultured Bone-Marrow Cells Respond to a Variety of Antigens With Antibody Production*

	AFC per spleen			
	NIP	DNP	FLU	SRC
* Reconstituted	50,700	4,200	29,300	—
* Reconstituted Normal C57BL/6	13,300	1,020	8,700	—
† Reconstituted	—	—	—	4,200
‡ Reconstituted	—	—	—	9,300
‡ Reconstituted Normal (CBA × C57BL/6)F <sub>1</sub>	—	—	—	50,000

\* Two lethally irradiated C57BL/6 mice were injected with  $1.5 \times 10^8$  cells from 14 day cultures of C57BL/6 bone marrow. 1 mo later the mice, together with three normal C57BL/6 mice as controls, were injected i.p. with 10 µg FLU-POL, 10 µg NIP-POL, and 5 µg DNP-POL. 3 days later, spleen cell suspensions were assayed for cells secreting antibodies against each of the three haptens using appropriately coated sheep erythrocytes (SRC).

‡ In the second experiment shown, irradiated (C57BL/6 × CBA)F<sub>1</sub> mice were reconstituted with  $1.2 \times 10^8$  cells from 36 day cultures of (C57BL/6 × CBA)F<sub>1</sub> bone marrow and 30 days later, together with three normal (C57BL/6 × CBA)F<sub>1</sub> mice as controls, were injected i.p. with  $10^8$  sheep erythrocytes. 5 days later anti-sheep erythrocyte AFC were assayed.

suggesting no major defect in the clonotype repertoire of the reconstituted animals.

Reconstituted mice also responded to a thymus-dependent antigen, the sheep erythrocyte (Table III), indicating that functional helper T cells were present. Furthermore spleen cells from reconstituted mice-generated cytotoxic T cells when cultured with allogeneic-stimulating cells (Table IV).

These experiments establish that it is possible to maintain in liquid cultures for periods of at least 7 wk, cells that can repopulate irradiated hosts with functional T and B lymphocytes. Although mature lymphocytes were not generated in these cultures, we do not know how much of the differentiation sequence from pluripotential stem cell to lymphocyte is occurring in vitro. The present results suggest that the cells responsible for repopulating the irradiated animals have a large proliferative potential and are at a stage of differentiation before or during generation of the receptor-

TABLE IV  
*Irradiated Mice Reconstituted with Cultured Bone Marrow Cells Generate Cytotoxic T Cells*

Cell source	Lysis of target cells by dilutions of cultures			
	1	1/2	1/4	1/8
Reconstituted mouse*	81	90	77	64
"    "	94	79	58	33
Normal mouse	92	86	94	84
"    "	94	94	100	89

Spleen cells were prepared from normal C57BL/6 mice or irradiated C57BL/6 mice reconstituted\* 1 mo previously with  $1.4 \times 10^6$  C57BL/6 cells from 14 day bone marrow cultures. Spleen cells from each mouse ( $5 \times 10^6$ ) were cultured in quadruplicate for 5 days with mitomycin-C-treated DBA/2 spleen cells ( $3 \times 10^6$ ). Quadruplicate cultures were pooled, resuspended in 0.6 ml of medium, and 100  $\mu$ l aliquots of the indicated dilutions were incubated in duplicate with  $2.5 \times 10^4$   $^{51}\text{Cr}$ -labeled target cells of DBA/2 origin (the mastocytoma P815) for 4 h. Aliquots of the supernates were then counted and the cytotoxic lysis calculated (6).

repertoire. These cells could thus include pluripotential stem cells (including those giving rise to spleen colonies) and various stem cells committed to lymphocyte production. It is likely that a variety of techniques will be required to identify all the cell types that might be present in the culture. For example, cells that are positive for the enzyme terminal deoxynucleotidyltransferase, some of which are likely to be thymocyte progenitors, are generated in the present cultures.<sup>1</sup>

The demonstration that cells able to give rise to a functional lymphoid system can be easily maintained for long periods in vitro, opens new approaches to questions of the source and regulation of lymphocyte production, the mechanism of immunological tolerance, and the generation of diversity. In vitro culture of dissociated cells has in the past led to rapid accumulation of knowledge on cellular and humoral regulation of mature lymphocytes and has defined conditions that are now allowing the in vitro cloning of these cells. The present results also have possible clinical implications, in that the cultured bone marrow cells reconstituted lethally irradiated semiallogeneic hosts with both hemopoietic tissue and functional lymphocytes, without evidence of graft-versus-host disease. Preliminary in vitro culture of human marrow with similar conditions may thus be a useful technique for facilitating the transplantation of bone marrow from incompletely matched donors.

### Summary

In vitro cultures of mouse bone marrow cells, maintained for periods up to 7 wk, were shown to contain cells able to repopulate irradiated hosts with T and B lymphocytes. The lymphocytes were fully functional and there did not appear to be any gross restriction of their receptor repertoire. The cultured cells reconstituted irradiated semiallogeneic hosts without evidence of graft-versus-host disease, suggesting that culture of donor marrow might be a useful preliminary to transplantation when tissue matching is incomplete.

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<sup>1</sup> J. W. Schrader and I. Goldschneider. In vitro studies on lymphocyte differentiation. II. The in vitro generation of deoxynucleotidyltransferase positive cells. Manuscript in preparation.

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