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Immunology

Long-term cultured B lymphocytes and their precursors reconstitute the B-lymphocyte lineage *in vivo*

(hematopoietic reconstitution/culture *in vitro*/humoral immunity/immune deficiency)

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ABSTRACT **Long-term *in vitro* cultured B lymphocytes and their precursors were tested for their ability to reconstitute a functional B-cell lineage *in vivo*. Continuous *in vitro* production of cells representing early stages of the B-lymphocyte lineage originates from a bone marrow cell type that can be distinguished from a multipotential hematopoietic stem cell. Progenitors of the long-term cultured B-lymphoid cells are recoverable *in vitro* after *in vivo* passage into lethally irradiated mice. Long-term cultured B-lymphoid cells and their progenitors reconstituted the B-lymphocyte lineage and restored normal humoral immunity in genetically defective mice. Cultured B-lymphoid cells did not cause lymphoid tumors *in vivo* in irradiated syngeneic mice. These findings indicate that multipotential hematopoietic stem cells are not directly required for the continued production of functionally competent B lymphocytes *in vitro*, and they suggest the existence of a B lymphoid progenitor cell with *in vitro*, and possibly *in vivo*, stem cell-like qualities.**

Early in ontogeny multipotential stem cells become developmentally restricted to each of the hematopoietic lineages (1–4). The earliest identifiable B-lymphoid cells are distinguished by the presence of cytoplasmic immunoglobulin μ heavy chain (5, 6). These pre-B cells are thought to be the direct progenitors of B lymphocytes bearing membrane-bound immunoglobulin (6, 7), which subsequently develop into antibody-secreting B cells or memory B lymphocytes (8). Cells that are not yet able to synthesize immunoglobulin μ heavy chain but have undergone a rearrangement in the gene encoding this protein, might be envisioned to be the progenitors of pre-B cells. Such phenotypes have been identified among lymphoma cell lines (9, 10).

A major problem in studying early B-lymphocyte development is the low frequency of B-lymphoid progenitor cells and their separation from coexisting multipotential stem cells (3, 11, 12). By modifying the long-term bone marrow cultures of Dexter *et al.* (13, 14), we established cultures that were selective for the growth of B-lymphoid cells and their precursors (15). The B-lymphoid populations consist of pre-B cells—a subpopulation of null lymphoid cells lacking both cytoplasmic immunoglobulin μ heavy chain and the B220 antigen associated with pre-B cells and B lymphocytes (16, 17)—and a minor population of polyclonal B lymphocytes demonstrating multiple species of immunoglobulin μ heavy and light chains (15).

We report the reconstitution of the B-lymphocyte lineage and the restoration of normal humoral immunity in mice adoptively transferred with long-term *in vitro* cultured B-lymphoid cells and their precursors. Development of the B-lymphocyte lineage *in vitro* and *in vivo* is shown to be maintained in the absence of detectable multipotential hematopoietic stem cells. These studies suggest that a lymphoid cell type capable of extended growth and differentiation *in vitro* can repopulate *in vivo* or, alternatively, produce differentiated progeny that can function *in vivo*.

MATERIALS AND METHODS

Mice. BALB/c (Cumberland View Farms, Clinton, TN) and BAB-14 (gift of I. Weissman, Stanford University) mice were bred and maintained in our facility. Homozygous CBA/N and (CBA/N × BALB/c)F_1 ♂ mice were obtained from Dominion Laboratories (Dublin, VA).

Establishment of Long-Term B-Lymphoid Cultures. Long-term bone marrow cultures were established from the femoral contents of 3- to 4-week-old BALB/c mice (Cumberland View Farms, Clinton, TN) as previously described (15, 18). Adherent bone marrow feeder layers from BAB-14 mice were prepared under the identical culture conditions, except that the initial bone marrow cell concentration was 1/10th (18).

Assay of Colony-Forming Units—Spleen (CFU-S). CFU-S, which define populations of multipotential and myeloid-erythroid restricted hematopoietic stem cells (4), were assayed by the method of Till and McCulloch (19).

***In Vivo* Adoptive Transfer of Cultured B-Lymphoid Cells.** B-lymphoid cells harvested from different-age cultures were mixed with a standard (2×10^6) number of bone marrow filler cells from 3- to 4-week-old homozygous CBA/N mice and intravenously injected into sublethally irradiated (400 roentgens; 1 roentgen = 2.6×10^{-4} coulomb per kilogram) 8- to 10-week-old (CBA/N × BALB/c)F_1 ♂ mice. Fresh bone marrow from 3- to 4-week-old BALB/c and CBA/N mice were used as positive and negative controls, respectively. Recipients were intraperitoneally immunized with 75 μg of 2,4,6-trinitrophenyl coupled to Ficoll (Pharmacia) (TNP-AECM-Ficoll) (20), and sacrificed 5 days later.

Clonable B-Lymphocyte Assay. Colony-forming units—B lymphocyte (CFU-B) were assessed in soft agar cultures containing Iscove's modified Dulbecco's medium with 0.3% Bacto-agar (Difco), 50 μM 2-mercaptoethanol, 10 μg of endotoxin lipopolysaccaride (*Salmonella typhosa*, W0901, Difco), and 1% packed, washed sheep erythrocytes (SRBC, Flow Laboratories) as previously described (21, 22).

Assay of Cells Forming Plaques in Response to TNP (TNP-PFC). TNP-PFC were assessed in a direct hemolytic plaque assay (23–25) using 1–2% (vol/vol) packed TNP-conjugated SRBC (TNP-SRBC) (20) and SRBC-absorbed guinea pig complement (GIBCO, 22N5723).

Assay for Anti-TNP Hemagglutinating Antibody. Serum anti-TNP antibody was assayed by the hemagglutination of TNP-SRBC (23) in round-bottom 96-well plates containing serial serum dilutions and a 1–2% (vol/vol) suspension of indicator SRBC.

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Abbreviations: CFU-S, colony-forming unit—spleen; CFU-B, colony-forming unit—B lymphocyte; TNP, 2,4,6-trinitrophenyl; SRBC, sheep erythrocytes; PFC, plaque-forming cell; kb, kilobase(s).

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Southern Blot Analysis. Samples (10 μg) of each DNA were digested with *Eco*RI, fractionated on 0.8% agarose gels, and transferred to nitrocellulose (26). The filter-bound DNA was hybridized (27) with a CDNA clone containing the 3′ portion of the secreted constant μ region mRNA (28).

RESULTS

Long-Term B-Lymphoid Cell Cultures Lack Detectable CFU-S. Growth of B lymphocytes and their precursors in long-term mouse bone marrow cultures is initiated and sustained by a population of hematopoietic progenitor cells that survive *in vitro* adaptation. These cells proliferate after the disappearance of other hematopoietic cells, giving rise to discrete foci of B-lineage cells that expand into mass culture (15). There are a few surface IgM-positive lymphocytes (5–10%), a major population of cytoplasmic μ-positive, surface IgM-negative pre-B cells (50–60%), and a null lymphoid cell population (20–35%) lacking cytoplasmic μ and other B- or T-cell surface antigens such as B220 or Thy1 (refs. 15, 16, and 18; unpublished data). Further suggestion of early lymphoid cell types in these cultured populations has been obtained by Southern blot analysis of the μ heavy chain loci. All long-term B lineage cultures we have examined (between 4 and 30 weeks of age) have retained a significant fraction (10–30%) of their μ loci in the germ line configuration (45). These data were considered suggestive evidence for the existence of a cell population that possessed progenitor-like qualities, since it could generate heterogeneous B-lymphoid progeny. The progenitor cells of such cultures might be self-renewing multipotential hematopoietic stem cells that under our selective culture conditions failed to differentiate into the other hematopoietic lineages and gave rise only to B-lymphoid cells.

We investigated whether multipotential hematopoietic stem cells existed in the cultures, as assessed by the *in vivo* spleen colony (CFU-S) assay (2, 19). As shown in Table 1, injection of fresh BALB/c bone marrow gave rise to normal numbers of CFU-S, but none were detected in the spleens of mice that received cultured cells. Multipotential hematopoietic stem cells do not appear to contribute to the maintenance phase of *in vitro* B-lymphoid cell growth.

CFU-S are heterogeneous (30) and are primarily restricted to the myeloid and erythroid cell lineages (4, 31). To test the possibility that the B-lymphoid cultures contained a multipotential hematopoietic stem cell population that was not detected by the CFU-S assay, we examined the bone marrow contents of the lethally irradiated recipients for evidence of general hematopoietic reconstitution. The bone marrow of these animals was markedly hypocellular compared to animals that had received fresh bone marrow cells and there was no evidence of granulopoiesis or erythropoiesis.

Table 1. Absence of CFU-S in long-term B-lymphoid cultures

Recipient	Donor cells	Cell inoculum $\times 10^{-6}$	CFU-S per spleen*
BALB/c	BALB/c bone marrow	0.1	19 ± 2
	6-week cultured	0.5	0
	6-week cultured	5	0
BALB/c	BALB/c bone marrow	0.05	11 ± 2
	BALB/c bone marrow	0.1	22 ± 3
	10-week cultured	0.1	0
	10-week cultured	0.5	0
(CBA/N	BALB/c bone marrow	0.1	30 ± 4
× BALB/c)F_1	14-week cultured	0.1	0
	14-week cultured	0.5	0

*Mean number of CFU-S ± SD detected in the spleens of two or three lethally irradiated (1025 roentgens) 8- to 10-week-old BALB/c and (CBA/N × BALB/c)F_1 ♂ mice 10–14 days after injection of BALB/c cultured cells or fresh BALB/c bone marrow. The use of different recipients and positive control fresh bone marrow is used to show that the absence of CFU-S in the long-term cultures was not due to hybrid resistance, in which some parental hematopoietic grafts can be rejected by F_1 recipients (29).

Progenitors of the Long-Term Cultured B-Lineage Cells Can Be Recovered After Passage *in Vivo*. The bone marrow contents of several lethally irradiated recipients of cultured cells were pooled and placed back *in vitro* under the original culture conditions in dishes containing adherent bone marrow stromal cell layers from BAB-14 mice. These adherent feeder layers were used to provide stromal cells and growth factors required for *in vitro* adaptation of the *in vivo* passaged cells (15).

The femoral contents of the lethally irradiated recipients of the cultured cells contained very few nucleated cells, but after 3–4 weeks small foci of nonadherent cells appeared above the adherent cell layer which were expanded for further study. All of these cells were lymphoid in appearance and showed the same spectrum of lymphoid phenotypes as in the primary long-term cultures.

To demonstrate that the B-lymphoid cells arising in the secondary cultures were derived from the original cultured cells passaged *in vivo*, we used restriction fragment length polymorphisms as genetic markers. Cells from BALB/c mice can be distinguished from BAB-14 and CBA/N cells. BAB-14 mice are isogeneic with BALB/c but contain the immunoglobulin μ heavy chain gene from C57BL/6 mice (32). DNAs extracted from nonadherent cells of the secondary B-lymphoid cell cultures were digested with *Eco*RI and hybridized to a cDNA probe containing sequences from the constant region of the μ gene (28). DNA blot analysis revealed only the 12.7-kilobase (kb) fragment of the BALB/c genotype, confirming that these cells were derived from the original cultured cells and not from host cells of the (CBA/N × BALB/c)F_1 ♂ recipients or cells emerging from the BAB-14 adherent bone marrow layer (Fig. 1).

***In Vivo* Reconstitution of the B-Lymphocyte Lineage by Long-Term Cultured B-Lymphoid Cells.** Long-term cultured BALB/c B-lymphoid cells were passaged into immunodeficient (CBA/N × BALB/c)F_1 ♂ mice. CBA/N mice possess a defective X-chromosome-linked gene that leads to a series of B-lymphocyte abnormalities (33, 34), including absence of

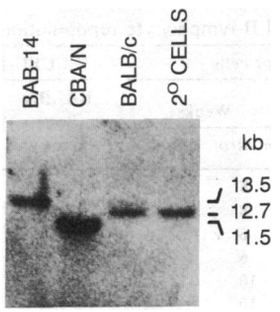

FIG. 1. Restriction fragment length polymorphism of cells arising in secondary long-term cultures. Lethally irradiated recipients of the cultured cells were analyzed for the presence in their bone marrow of cells capable of establishing secondary long-term B-lymphoid cultures. Cells appearing in secondary cultures seeded onto pre-established BAB-14 adherent marrow feeder layers were analyzed for a restriction fragment length polymorphism in the immunoglobulin μ heavy chain gene to determine whether they originated from the BALB/c primary long term cultures. Liver cell DNA from BAB-14, CBA/N, BALB/c, and the nonadherent lymphoid cells from the secondary cultures (2° cells) was analyzed by Southern blot (26). An *Eco*RI restriction polymorphism in the μ heavy chain gene gives rise to different size restriction fragments distinguishing BAB-14 (13.5 kb), CBA/N (11.5 kb), and BALB/c (12.7 kb). Secondary cultured B-lymphoid cells contained the 12.7-kb BALB/c DNA fragment.

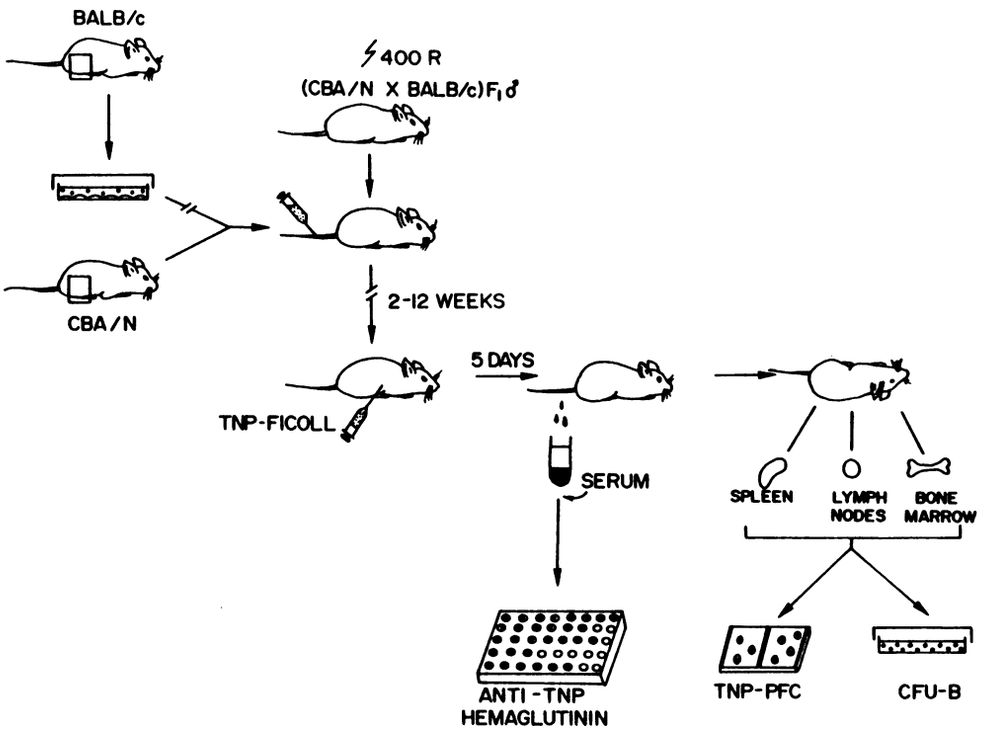

FIG. 2. Schematic representation of adoptive transfer studies assessing the capability of long-term cultured cells to repopulate the B-lymphocyte lineage of genetically immune defective mice. Sublethally irradiated (400 roentgens) immune defective (CBA/N × BALB/c)F_1 ♂ mice were intravenously injected with various numbers of cells from different age BALB/c long-term bone marrow cultures in conjunction with low numbers of CBA/N bone marrow filler cells. At various times after adoptive transfer, the recipients were immunized with TNP-Ficoll and examined 5 days later for clonable B lymphocytes (CFU-B), TNP-PFC, and anti-TNP antibody.

a population of B lymphocytes that clone in soft agar (CFU-B) (35) and unresponsiveness to TNP-Ficoll and other type II thymus-independent antigens (24, 34, 36). These defects allow the B-lymphocyte compartment of CBA/N mice to be selectively repopulated by hematopoietic cells without the need for lethal irradiation (11, 35). This repopulation is potentiated by low-dose radiation (37) and the co-transfer of low numbers of CBA/N bone marrow filler cells (12, 38). This adoptive transfer system permitted the survival of recipients for long periods of time, which was necessary for B-lymphocyte reconstitution. The experimental method is shown schematically in Fig. 2.

Table 2 (and graphic summary in Fig. 3) shows the results of 14 groups of adoptive transfers in which various transplant inocula prepared from different age long-term cultures were tested.

Low numbers of CFU-B were detectable at 2 weeks after adoptive transfer of the cultured cells, and by 6–8 weeks represented approximately 50% of the numbers of clonable B cells found in the spleens of mice receiving equivalent numbers of fresh bone marrow cells. By 12 weeks after adoptive transfer the total number of CFU-B was collectively greater than 65% of the positive control recipients.

Attempts at detecting TNP-PFC in the spleens of (CBA/N × BALB/c)F_1 ♂ mice at 2 weeks after adoptive transfer of the cultured cells were unsuccessful, despite the appearance of TNP-PFC in the spleens of cohort mice that received fresh bone marrow cells (Table 2). Low numbers of TNP-PFC did

Table 2. Functional B-lymphocyte repopulation of (CBA/N × BALB/c)F_1 ♂ mice by adoptive transfer of long-term cultured cells

	Donor cells			CFU-B × 10^{-3} per spleen			TNP-PFC × 10^{-3} per spleen			Reciprocal anti-TNP HA titer		
Exp.	Cell inoculum × 10^{-6}	Weeks *In vitro*	Weeks *In vivo*	BALB/c bone marrow	CBA/N bone marrow	Long-term cultured cells	BALB/c bone marrow	CBA/N bone marrow	Long-term cultured cells	BALB/c bone marrow	CBA/N bone marrow	Long-term cultured cells
A_1	10	8	2	38	<0.2	16	2	<0.1	<0.15	32	<2	<2
A_2	1	8	2	3	<0.2	2	0.3	<0.1	<0.15	16	<2	<2
B_1	10	6	6	118	<0.2	16	55	<0.1	0.6	128	2	8
B_2	1	6	6	24	<0.2	16	9	<0.1	0.5	256	<2	4
C_1	5	10	5	185	<0.2	68	24	<0.1	0.7	64	<2	<2
D_1	7	10	6	184	<0.2	124	52	<0.1	8	128	<2	4–8
E_1	7	14	4	77	<0.2	38	5	<0.1	2	64–128	<2	2
E_2	7	14	8	272	<0.2	163	86	<0.1	30	128	<2	16–32
F_1	7	6	4	212	<0.2	29	19	<0.1	0.25	64	2	2
F_2	7	6	8	304	<0.2	129	44	<0.2	25	128–256	2	32–128
F_3	7	6	12	733	<0.2	484	36	<0.1	19	256–512	<2	32–64
G_1	7	20	4	212	<0.2	14	19	<0.1	<0.1	64	<2	2
G_2	7	20	8	304	<0.2	48	44	<0.2	19	128–256	2	32
G_3	7	20	12	733	<0.2	398	36	<0.1	20	256–512	<2	32

The results are shown for 14 groups of adoptive transfer experiments in which the recipients were concurrently examined for splenic CFU-B and TNP-PFC and serum anti-TNP hemagglutinin (HA). CFU-B and TNP-PFC are expressed as the geometric means of the total numbers of functional B lymphocytes detectable in the spleens of sublethally irradiated (CBA/N × BALB/c)F_1 ♂ mice. Anti-TNP hemagglutinin is expressed as the range in the inverse titers of anti-TNP hemagglutinin measured by serial dilution of the sera from each of these same recipients. Sources of donor cells included BALB/c long-term cultured cells, fresh BALB/c bone marrow, and fresh CBA/N bone marrow cells. The data are tabulated to illustrate the effects of different size transplant inocula obtained from separate sets of various age cultures and the period of time in which the cells were resident in the recipients.

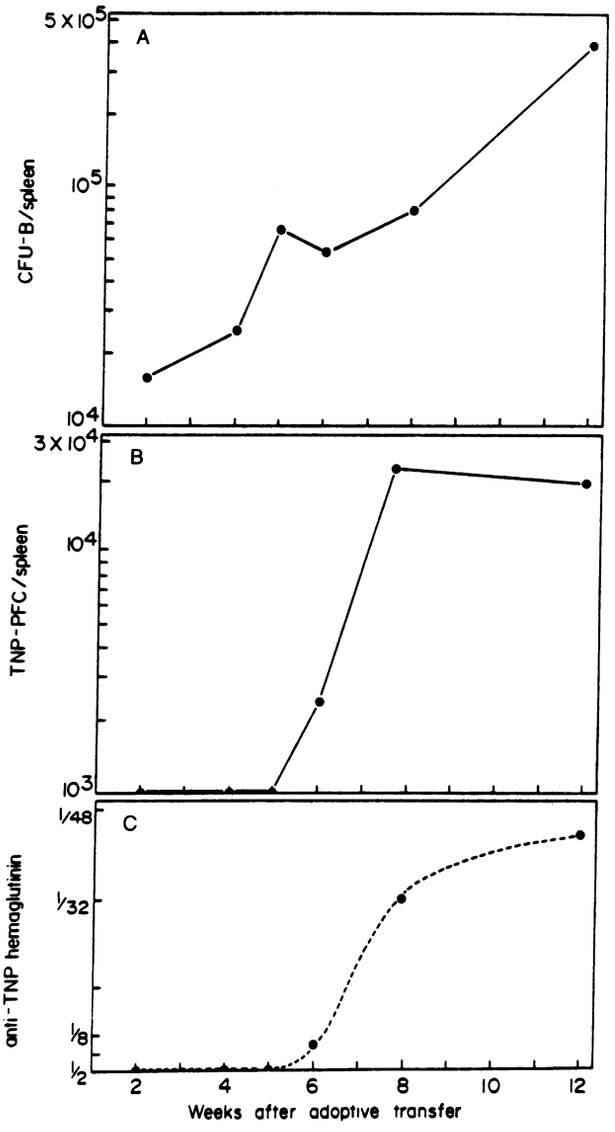

FIG. 3. Kinetics of appearance of functional B lymphocytes and hapten-specific antibody after *in vivo* adoptive transfer of long-term cultured B-lymphoid cells. Geometric means of the total numbers of splenic CFU-B (*A*) and TNP-PFC (*B*), and serum titers of anti-TNP hemagglutinin (*C*) detectable in sublethally irradiated (CBA/N × BALB/c)F_1 ♂ mice at various times after the adoptive transfer of long-term cultured cells. The results represent the combined data of all adoptive transfers performed with various sources of long-term cultured cells irrespective of the length of time the cells were maintained in culture, or the size of the transplant inocula. These include the data from Table 2, in addition to results obtained from adoptive transfers performed with cells pooled from different age BALB/c and CBA/J bone marrow cultures. All adoptive transfers were performed in combinations with a standard (2×10^6) number of CBA/N bone marrow filler cells as described in Fig. 2. (CBA/N × BALB/c)F_1 ♂ mice reconstituted with CBA/N bone marrow cells alone failed to demonstrate detectable CFU-B or significantly greater than background levels of TNP-PFC and anti-TNP hemagglutinin.

arise in the spleens of some of the recipients 4 weeks after adoptive transfer, and by 6 weeks all of the recipients of the cultured cells contained appreciable numbers of TNP-PFC. Between 8 and 12 weeks, the numbers of splenic TNP-PFC remained relatively constant. Both the lag period preceding the appearance of TNP-PFC and the plateau in total numbers of TNP-PFC occurred irrespective of the age of the long-term cultures from which the adoptively transferred cells were obtained (Table 2).

We have detected both TNP-PFC and clonable B cells in mesenteric lymph node and bone marrow, but the frequency of functional B lymphocytes in these sites is considerably lower than in spleen. At 8 weeks after the adoptive transfer of 14-week-old cultured cells, a similar pattern of distribution in both TNP-PFC and CFU-B was observed in spleen ($45/10^5$ cells for TNP-PFC, $243/10^5$ cells for CFU-B), mesenteric lymph nodes ($18/10^5$ cells for TNP-PFC, $96/10^5$ cells for CFU-B), and bone marrow ($5/10^5$ cells for TNP-PFC, $11/10^5$ cells for CFU-B).

Anti-TNP hemagglutinin titers in mice adoptively transferred with the cultured cells closely followed the emergence of splenic TNP-antigen responsive B lymphocytes (Table 2 and Fig. 3). Anti-TNP antibody was not detectable before 6 weeks, increased severalfold by 8 weeks, and remained constant through week 12. In contrast, both TNP-PFC and anti-TNP hemagglutinin was detected at 2–4 weeks after the adoptive transfer of fresh bone marrow (Table 2). This earlier response may be due to more mature antigen-responsive B cells that are present in adult bone marrow (39). At no time did TNP-PFC appear in the spleens of mice that did not also contain CFU-B, nor was there a significant titer of serum hemagglutinin in mice that did not contain splenic TNP-PFC.

Recipients of cultured cells were examined by autopsy for evidence of tumor formation. In no cases did tumors develop within lymphoid organs or as extranodal masses in any of the adoptively transferred animals.

DISCUSSION

This work provides evidence for the existence of a class of hematopoietic cells that can function *in vitro* as progenitors of early stages of the B-cell lineage. The mature progeny of such cells that develop both *in vitro* and during passage *in vivo* were functionally competent to participate in a normal humoral immune response. Multipotential stem cells do not contribute to steady-state B-lymphocyte production occurring in these *in vitro* cultures. Such *in vitro* culture conditions may artificially expand the proliferative potential of an otherwise rare progenitor cell type.

The precise nature of the reconstituting cell that gives rise to TNP-PFC and CFU-B *in vivo* is unknown. The possibility exists that mature B lymphocytes within the cultures, as well as pre-B cells destined to develop into functional B lymphocytes after transfer *in vivo*, contributed to TNP-PFC and CFU-B. Previous studies utilizing adoptive transfers of adult spleen cells and 12- to 14-day fetal liver cells have demonstrated that CFU-B are generated independently by B lymphocytes and pre-B cells, respectively (3, 11, 40).

An intriguing possibility is that the progenitor cells of the *in vitro* cultures themselves differentiated *in vivo* to the stage of mature functional B lymphocytes capable of colony formation and secretion of hapten-specific antibody. This is not proven but suggested by our accumulated evidence documenting (*i*) the diversity of immunoglobulin μ heavy and light chain species present among cultured B-lymphoid cells (15), (*ii*) the finding of a minor null lymphoid cell population lacking in cytoplasmic immunoglobulin and the B220 surface antigen (ref. 16; unpublished data), and (*iii*) the observation that there is a significant fraction of cultured lymphoid cells whose immunoglobulin μ heavy chain genes are in the germline configuration (unpublished data). The kinetics of functional B-lymphocyte reconstitution by the cultured cells is similar to that which occurs after the adoptive transfer of early gestational age fetal hematopoietic tissues. Day 12–13 fetal liver containing pre-B cells and their precursors, and 9- to 10-day yolk sac devoid of pre-B cells but known to contain hematopoietic stem cells (1), required 2 weeks and 8 weeks, respectively, to generate CFU-B *in vivo* (3, 11, 40). In contrast, transfers of adult spleen caused the emergence of

CFU-B by 18 hr that rose to near normal levels by 10 days (3, 11, 40).

The role of a restricted B-cell progenitor population in our reconstitution studies should be considered. Evidence for restricted progenitor cell types for erythroid and other lineages has been reported (41). The lag period for reconstitution (Fig. 3) of the (CBA/N × BALB/c)F_1 mice is suggestive of a progenitor cell type that must expand and differentiate *in vivo* prior to producing functional cells. Further experiments to enrich or isolate the active cell population will be necessary to resolve this point.

Recently, another model system of the SCID (severe combined immune deficiency) mouse has been reported (42). These mice can be reconstituted for their defective T- and B-cell lineages with cells grown in the long-term marrow system described by Dexter (13, 43), but only for B cell function with cells from cultures of B-lineage cells similar to those in our studies (44). Serial reconstitution with bone marrow to secondary recipients of B-lineage cultured cells was not observed. Repopulation from more mature B-cell elements was thus considered likely. Our ability to regrow *in vitro* B-lineage cultures from the bone marrow of mice inoculated with B-lineage populations 2 weeks previously (Fig. 1) suggests that some form of progenitor or stem-like cells was retained in the marrow.

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