

PROLONGED SURVIVAL IN VIVO OF UNPRIMED B CELLS  
RESPONSIVE TO A T-INDEPENDENT ANTIGEN

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The lifespan of B cells continues to be a controversial issue. From studies on the rate at which B cells incorporate tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) in vivo, several groups have concluded that the turnover of B cells in rodents is very rapid in the bone marrow (BM) (1) and in a compartment of the spleen, but slow in lymph nodes (LN) and thoracic duct lymph (TDL) (2-4). These findings imply that although most marrow and some spleen B cells have a rapid turnover, the majority of spleen cells and nearly all LN and TDL B cells remain in interphase for many weeks or months.

This notion has recently been challenged by Freitas and coworkers (5, 6). These workers assert that the vast majority of B cells, including LN and TDL B cells, have a lifespan of only 1-2 d. This conclusion stems largely from the finding that injecting mice with hydroxyurea, a compound toxic for dividing cells, causes a marked transient reduction of B cells in spleen, LN, and TDL as well as in the marrow (6). Outputs of B cells in TDL were reduced by up to 70% within 1 d of hydroxyurea injection and returned to normal by day 4. From these studies the authors concluded that the bulk of mature B cells in the secondary lymphoid tissues represent recent emigrants from the marrow.

In light of these data (5, 6), B cell turnover clearly needs to be reexamined. The evidence that most B cells in the peripheral lymphoid tissues are long-lived derives largely from a single experimental approach, namely examining the rate at which cells incorporate  $[^3\text{H}]\text{TdR}$ . In this paper we use an alternative approach to study B cell longevity. Making use of the fact that mice with X-linked immunodeficiency (*xid*) are unresponsive to the T-independent antigen, trinitrophenyl (TNP)-Ficoll, we studied TNP-Ficoll responses in *xid* mice reconstituted with normal LN B cells, a population of putatively long-lived B cells devoid of stem cells. The results show that the responses of the recipients to TNP-Ficoll fail to decline within 3 mo of B cell transfer. These findings indicate that LN B cells responsive to TNP-Ficoll are not short-lived.

#### Materials and Methods

*Mice.* (CBA/N  $\times$  BALB/c) $F_1$  and (CBA/N  $\times$  DBA/2) $F_1$  mice were provided by the animal facility at Scripps Clinic and Research Foundation. CBA/J mice were obtained from The Jackson Laboratory, Bar Harbour, ME.

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*Purification of Lymphoid Cells.* B cell-enriched populations from adult mice were prepared by treating pooled LN cells (cervical, inguinal, axillary, and mesenteric nodes) or TDL lymphocytes with a monoclonal anti-Thy-1.2 (J1j) (7) antibody plus complement (C'). T cell-enriched populations were prepared by treating pooled LN cells with the monoclonal antibody J11d (7) plus C'; this antibody lyses >98% Ig<sup>+</sup> cells in LN but does not lyse LN T cells. BM cells were treated with a cocktail of anti-Thy-1.2 and J11d monoclonal antibodies plus C'; J11d antibody lyses 70–90% of BM cells but spares pluripotential stem cells (7).

*PFC Assay.* As described elsewhere (8), TNP-specific direct PFC were enumerated 6 d after immunization of mice with 20  $\mu$ g TNP-Ficoll. The H-2 phenotype of PFC was determined by treating aliquots of spleen cells with a monoclonal antibody (28-11-5S), specific for H-2D<sup>b,d</sup> (9), plus C' before enumerating TNP-specific PFC (8).

*Irradiation.* Mice aged 10–14 wk were exposed to 500 rad  $\gamma$  irradiation (8) at least 3 h before cell transfer.

## Results

Our general approach was to transfer stem cell-deficient lymphoid cells (LN or TDL) from unprimed normal mice, e.g., (CBA/N  $\times$  BALB/c)F<sub>1</sub> females, into *xid* mice, e.g., (CBA/N  $\times$  BALB/c)F<sub>1</sub> males, then challenge groups of the recipients with TNP-Ficoll at various times after injection and measure anti-TNP PFC in the spleen 6 d later. To avoid the possibility of alloaggression directed either to donor (H-X) or host (H-Y) antigens, the donor cells were pretreated with anti-Thy-1 antibody plus C' and the recipients were exposed to 500 rad. Cell suspensions were injected intravenously.

A preliminary experiment in which groups of *xid* mice were injected with 10<sup>7</sup> LN B (Thy-1<sup>-</sup>) cells from normal mice is shown in Table I. Responses to TNP-Ficoll were invariably undetectable or negligible in *xid* mice given 500 rad but no B cells, as well as in unirradiated *xid* mice (data not shown). By contrast, *xid* mice injected with normal B cells responded well to TNP-Ficoll, whether the antigen was given immediately after cell transfer (week 0) or up to 9 wk after injection. These responses were generally two- to threefold less than the responses observed in unmanipulated normal control mice [(CBA/N  $\times$  BALB/c)F<sub>1</sub> females] (see Table I, footnote).

The results of two experiments in which the dose of B cells injected was varied

TABLE I  
*TNP-Ficoll PFC Response in Spleens of (CBA/N  $\times$  BALB/c)F<sub>1</sub> Male *xid* Mice Given 10<sup>7</sup> Normal F<sub>1</sub> Female LN B Cells 0–9 Wk Previously*

Mice tested	No. of mice per group	Total number of direct PFC per spleen			
		Week 0	Week 1	Week 3	Week 9
500 rad (CBA/N $\times$ BALB/c)F <sub>1</sub> $\delta$ given 10 <sup>7</sup> LN B cells from (CBA/N $\times$ BALB/c)F <sub>1</sub> $\phi$	4	5,890 (1.17)	5,760 (1.06)	17,190 (1.17)	26,010 (1.23)
Normal (CBA/N $\times$ BALB/c)F <sub>1</sub> $\phi$	3	18,180 (1.12)	16,050 (1.38)	23,770 (1.16)	50,320 (1.08)
500 rad (CBA/N $\times$ BALB/c)F <sub>1</sub> $\delta$	2	<200	<200	<200	520 (1.02)

(CBA/N  $\times$  BALB/c)F<sub>1</sub> male mice were exposed to 500 rad and injected within 1 d with 10<sup>7</sup> viable anti-Thy-1.2 + C'-treated LN cells taken from (CBA/N  $\times$  BALB/c)F<sub>1</sub> female mice. All mice were immunized with 20  $\mu$ g TNP-Ficoll given intraperitoneally at different times after reconstitution. Direct (IgM) PFC responses were measured on day 6. Results are expressed as geometric means ( $\times/\pm$  SE). The higher response to TNP-Ficoll seen in the normal F<sub>1</sub> female mice used as controls at week 9 is probably fortuitous and was not seen in other experiments.

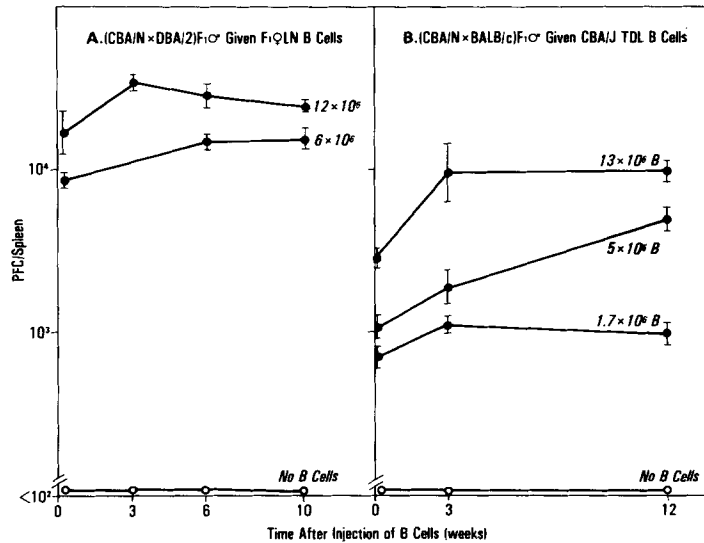


FIGURE 1. TNP-Ficoll response of 500 rad-irradiated *xid* mice injected 0–12 wk previously with varying numbers of purified B cells obtained from LN (A) or TDL (B) of normal mice (●). Control *xid* mice received no B cells (○). The data show mean number of anti-TNP PFC per spleen measured 6 d after intraperitoneal injection of 20  $\mu$ g TNP-Ficoll; three to four mice per group.

are shown in Fig. 1; the B cells were prepared from LN in Fig. 1A and from TDL in Fig. 1B. Two points are evident. First, the responses observed were roughly proportional to the number of B cells injected. Second, the responses failed to decline, even at 10–12 wk after transfer; the responses were higher at week 3 than at week 0 but then tended to reach a plateau.

Table II lists a number of control experiments that were carried out in parallel with the experiments mentioned above. As expected, *xid* mice given purified normal T cells gave no response to TNP-Ficoll (Table II, group 1). Transfer of normal marrow cells to *xid* mice gave negligible responses at week 0, low responses at week 3, and very high responses at week 12 (group 2). The kinetics of these responses make it most unlikely that the responses observed with transfer of normal B cells reflected contamination with pluripotential stem cells. If LN or TDL B cells did contain stem cells, the progeny of these stem cells would be expected to seed the marrow of the recipients. If this marrow were then used to reconstitute additional *xid* mice, these latter mice should eventually develop responsiveness to TNP-Ficoll. Testing this prediction (group 4) showed no response to TNP-Ficoll even at 11 wk after marrow transfer. The experiment shown in group 5 was designed to rule out the possibility that transfer of B cells to *xid* mice somehow induced responsiveness of host-type B cells. To examine this possibility, (CBA/N  $\times$  BALB/c) $F_1$  male (*xid*) mice were injected with normal CBA/J TDL B cells ( $H-2^k \rightarrow H-2^k \times H-2^d$ ) and then immunized with TNP-Ficoll 13 wk later. It can be seen that splenic anti-TNP PFC from the recipients were highly resistant to pretreatment with monoclonal anti- $H-2^d$  (anti-host) antibody plus C'. Since this treatment reduced the response of normal (CBA/N  $\times$  BALB/c) $F_1$  female mice by 99%, these data imply that virtually all PFC in the group 5

TABLE II  
*TNP-Ficoll Response in xid Mice Injected With Normal B Cells vs. T Cells or Marrow Cells:  
 Origin of PFC*

Group	Mice tested*		Time between lymphoid cell transfer and immunization with TNP-Ficoll	No. of mice per group	Pretreatment of spleen cells assayed for anti-TNP PFC	Total No. of direct (IgM) anti-TNP PFC per spleen
	Host strain	Cells transferred to host strain				
			<i>wk</i>			
1	(CBA/N × DBA/2)F <sub>1</sub> ♂ (500 rad)	5 × 10 <sup>6</sup> F <sub>1</sub> ♀ LN T cells	0	4	—	<200
			6	4	—	<200
			10	4	—	<200
2	(CBA/N × BALB/c)F <sub>1</sub> ♂ (500 rad)	2 × 10 <sup>6</sup> F <sub>1</sub> ♀ BM cells	0	4	—	300 (1.15)
			3	4	—	3,210 (1.30)
			12	4	—	93,900 (1.03)
3	(CBA/N × BALB/c)F <sub>1</sub> ♂ (500 rad)	10 <sup>7</sup> F <sub>1</sub> ♀ LN B cells	9	4	—	26,010 (1.23)
4	(CBA/N × BALB/c)F <sub>1</sub> ♂ (500 rad)	2 × 10 <sup>6</sup> BM from group	1	4	—	<200
			6	4	—	<200
			11	4	—	<200
5	(CBA/N × BALB/c)F <sub>1</sub> ♂ (500 rad)	13 × 10 <sup>6</sup> CBA/J ♀ TDL B cells	13	3	C' only α-H-2 <sup>d</sup> + C'	10,820 (1.16) 9,950 (1.08)
			—	1	C' only α-H-2 <sup>d</sup> + C'	71,800 64,000
6	Normal CBA/J ♂ (unirradiated)	—	—	1	C' only α-H-2 <sup>d</sup> + C'	53,400 600
7	(CBA/N × BALB/c)F <sub>1</sub> ♀ (unirradiated)	—	—	1	C' only α-H-2 <sup>d</sup> + C'	13,660 (1.08) 13,890 (1.16)
8	(CBA/N × BALB/c)F <sub>1</sub> ♂ (unirradiated)	10 <sup>7</sup> F <sub>1</sub> ♀ LN B cells	1	3	—	13,660 (1.08)
			4	4	—	13,890 (1.16)
9	(CBA/N × BALB/c)F <sub>1</sub> ♂ (unirradiated)	—	—	4	—	<200

The table shows controls for the experiments shown in Table I and Fig. 1. Group 1: Control for the experiment shown in Fig. 1A. 5 × 10<sup>6</sup> J11d + C'-treated F<sub>1</sub> female LN T cells were injected intravenously into *xid* F<sub>1</sub> males. Group 2: Control for the experiment shown in Fig. 1B. 2 × 10<sup>6</sup> J11d + anti-Thy-1 + C'-treated CBA/J BM cells were injected intravenously into *xid* F<sub>1</sub> males. Group 3: Data from Table I. Group 4: Control for the experiment shown in Table I. *xid* F<sub>1</sub> male mice were injected with 2 × 10<sup>6</sup> J11d + anti-Thy-1 + C'-treated BM cells from *xid* F<sub>1</sub> male mice given 10<sup>7</sup> normal F<sub>1</sub> female LN B cells 9 wk before. Group 5: Identity of PFC in the spleens of *xid* F<sub>1</sub> male mice reconstituted with 13 × 10<sup>6</sup> CBA/J TDL B cells. Effect of pretreating aliquots of spleen cells with monoclonal anti-H-2<sup>d</sup> + C' or C' alone. Groups 6 and 7: Negative and positive controls for anti-H-2<sup>d</sup> antibody. Groups 8 and 9: Effect of using unirradiated mice as recipients of B cells.

\* Mice were immunized with 20 μg TNP-Ficoll 6 d before spleens were assayed. See Table I.

mice were of donor origin. Finally, the experiment shown for group 8 demonstrates that unirradiated *xid* recipients of normal LN B cells generated high responses to TNP-Ficoll, both at 1 and 4 wk after transfer. This finding would seem to overcome the objection that the prolonged survival of B cells in the above experiments was somehow related to preconditioning the hosts with irradiation.

### Discussion

The main finding in this paper is that transfer of normal LN or TDL B cells to *xid* mice enabled the recipients to respond to TNP-Ficoll for up to 3 mo after injection. Trivial explanations for the data, e.g., B cell differentiation from contaminating stem cells or de novo responsiveness of host-type B cells, seem very unlikely (see Table II). The surprising finding was that the responsiveness of the B cell-injected recipients to TNP-Ficoll showed no tendency to decrease with time. As a population, the transferred B cells can thus be viewed as very long-lived. Whether the B cells remained in interphase or underwent division on transfer, however, cannot be deduced from the data. In this respect the results

do not constitute evidence against the claim of Freitas et al. (6) that most B cells, including LN and TDL B cells, have a rapid turnover. Nevertheless, the data do argue against the broader conclusion (6) that LN and TDL B cells are short-lived cells of recent marrow origin. If the transferred B cells in the present study did undergo division on transfer, B cell death and replacement from Ig<sup>+</sup> precursors clearly would have to be equally balanced.<sup>1</sup> Thus, although TNP-Ficoll responses tended to rise within the first 3 wk after transfer, the responses thereafter remained relatively constant. It should be noted that the early rise in the response to TNP-Ficoll was not seen when unirradiated *xid* mice were used as B cell recipients (Table II, group 8). Hence, the initial rise in irradiated recipients of B cells probably did not denote expansion of the B cells but simply suboptimal responses resulting from transient disorganization of the lymphoid tissues caused by the dose of 500 rad.

In view of the evidence that LN and TDL B cells incorporate [<sup>3</sup>H]TdR at only a slow rate in vivo (2-4), the simplest interpretation of the present data is that most of the injected B cells remained in interphase from the time of injection or divided at only rare intervals. However, whether the transferred B cells divided intermittently or remained in interphase, the key finding is that the responding B cells did not decline in number even after long periods. This is in line with the report (10) that B cells clonable in agar can be recovered from *xid* mice (which lack clonable B cells) for several weeks after transfer of normal LN cells. Likewise, we recently observed (unpublished data) that, when parental strain LN B cells were transferred to H-2-semiallogeneic F<sub>1</sub> mice, Ig<sup>+</sup> cells of donor origin remained in the recipients in approximately constant numbers for at least 2 mo after transfer. These data imply that prolonged survival of B cells on transfer applies not only to TNP-Ficoll-responsive cells but to LN B cells in general. The corollary follows that, once the level of B cells in LN and TDL reaches adult levels, no further input of these long-lived B cells from the marrow is required. It does not necessarily follow, however, that B cell production in adult marrow is redundant. It has been suggested elsewhere (11) that the pool of recirculating B cells may be memory cells primed as the result of prior contact with environment or self antigens. According to this idea, B cells specific for antigens that do not crossreact with self or environmental antigens would not be represented in the recirculating pool. These B cells would be short-lived cells and their existence would require constant replenishment by the marrow. Although there are isolated reports (11, 12) that B cells specific for certain antigens do have a rapid turnover and are not found in the recirculating lymphocyte pool, there is a clear need for quantitative information on the putative holes in the repertoire of long-lived recirculating B cells.

### Summary

Despite earlier evidence to the contrary, it has recently been claimed that most B lymphocytes, including lymph node (LN) and thoracic duct B cells, are short-

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<sup>1</sup> It might be argued that most of the injected B cells died soon after injection and that clonal expansion of residual donor B cells accounted for the response measured. If this were the case, however, the recipients should have manifested an initial trough of unresponsiveness to TNP-Ficoll. This was not found.

lived cells of recent marrow origin. To seek direct information on this question, we transferred unprimed LN or thoracic duct B cells from normal mice to *xid* mice, i.e., mice unresponsive to the T-independent antigen, trinitrophenyl (TNP)-Ficoll. At varying periods after B cell transfer the recipients were challenged with TNP-Ficoll; anti-TNP plaque-forming cells were assayed in the spleen 6 d later. The results showed that the B cell recipients retained responsiveness to TNP-Ficoll for at least 3 mo after transfer. Responsiveness increased within the first 3 wk but then remained relatively constant. These findings imply that, at least for TNP-Ficoll-reactive cells, B cells residing in LN and thoracic duct lymph are not short-lived cells of recent marrow. Indeed, the data suggest that once the pool of recirculating B cells is fully formed in adult mice, further input of newly formed cells from the marrow into the recirculating pool is very limited.

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