Brief Definitive Report

PHYSIOLOGY OF B CELLS IN MICE WITH X-LINKED IMMUNODEFICIENCY

II. Influence of the Thymus and Mature T Cells on B Cell Differentiation

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Mice of the CBA/N strain exhibit X-linked immunodeficiency (*xid*) affecting B lymphocytes (1). B cells in *xid* mice are substantially reduced in number and lack the mature Lyb-5$^+$ late-appearing subset of B cells found in normal mice (1). *Xid* B cells are viewed as immature B cells, although whether these cells are the exact counterpart of the early B cells found in normal mice is still unclear.

Recently, two groups (2, 3) reported that *xid nu nu* mice contain far fewer B cells than euthymic *xid* mice. This finding suggests that the thymus might play a role in the differentiation of *xid* B cells. Since the defect in the nude mouse is pleiomorphic, however, the above studies could not exclude the possibility that factors other than the absence of the thymus caused the reduction in B cell numbers. In this respect, one would like to know: (*a*) Does surgical thymectomy lead to the B cell defect seen in *xid nu nu* mice? (*b*) If so, does the defect reflect an influence of mature T cells rather than thymic humoral factors? This paper addresses these questions.

Materials and Methods

Mice. (C57B6/6 [B6] × CBA/J)F_1, (B6 × DBA/2)F_1, and CBA/Ca mice were obtained from The Jackson Laboratories, Bar Harbor, ME. CBA/N and (CBA/N × DBA/2)F_1 mice were kindly provided by Dr. D. Mosier, Institute for Cancer Research, Philadelphia, PA. Male mice were used in all experiments.

Thymectomy. Mice aged 6–8 wk were thymectomized by the method of Miller (4) and were left for 4–6 wk before irradiation and marrow reconstitution.

Preparation of Chimeras. Mice were irradiated and reconstituted with anti-Thy-1.2 and complement (C′)–treated marrow as described previously (5). Chimeras were maintained on neomycin and polymyxin B in the drinking water for 3–5 wk postreconstitution (5) and housed in a positive pressure isolation unit.

Irradiation. Mice were exposed to ^{137}Cs γ irradiation using a Gamma cell irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 92 rad/min.

Antibodies Used for Cytotoxicity Assays. The following antibodies were used: monoclonal anti-Thy-1.2 (JIj) antibody (6) (ascites fluid diluted to 1:20); affinity-purified rabbit anti-mouse IgG (RαMIg) antiserum obtained from Cappel Laboratories, Cochranville, PA; monoclonal antibody J11d (6) (culture supernatant diluted 1:5); monoclonal anti-I-A^k (11-5.2) antibody (ascites fluid diluted to 1:10) from hybridoma cells kindly provided by the Herzenberg group, Stanford University; and monoclonal anti-K^{b}D^b (28-8-6S) antibody

This work was supported by grants CA-33958 and AI-21687 from the U. S. Public Health Service.

(culture supernatant diluted to 1:5), cells obtained through the courtesy of Dr. K. Ozato and Dr. D. H. Sachs from the American Type Culture Collection, Rockville, MD. RαMIg was used at a final concentration of 1:20. This antiserum is heavy and light chain specific and has a titer of >1:200 on normal spleen and lymph node (LN) B cells; the antibody is negative for purified T cells and lyses >95% of Thy-1$^-$ cells in LN and >80% of Thy-1$^-$ spleen cells (indistinguishable from the numbers of cells stained with fluoroscein-labeled RαMIg). The J11d antibody lyses >95% of Ig$^+$ cells in spleen and LN and also reacts with a proportion of "null" cells in spleen and marrow; the antibody does not react with mature T cells.

Cytotoxicity Assays. A one-step assay was used, using Ficoll gradient–separated cells (6). A mixture of mouse spleen–absorbed guinea pig serum (final concentration, 1:6) and rabbit serum (final concentration, 1:60) was used as a source of C′. Cell viability was measured by phase contrast microscopy.

Thoracic Duct Cannulation. Thoracic duct lymphocytes (TDL) were collected as described elsewhere (7).

Results

The simplest approach for studying the effects of thymectomy on *xid* B cell differentiation would be to examine the effects of neonatal thymectomy. This approach has two drawbacks. First, some T cells exit from the thymus before birth (8). Second, the "runting" syndrome that often develops in neonatally thymectomized mice can cause a sudden and unpredictable disappearance of B cells, presumably as the result of infection (7). The use of adult thymectomized, irradiated marrow–reconstituted mice largely avoids these two problems.

The approach we adopted was to transfer anti-Thy-1 plus C′–treated CBA/N (*xid*$^+$) vs. CBA/Ca (*xid*$^-$) marrow into adult thymectomized (ATx), irradiated (1,050 rad) (B6 × CBA/J)F_1 (*xid*$^-$) mice. Sham thymectomized (STx) mice were used as a control. The degree of donor cell chimerism developing in the recipients was established with the aid of host-specific anti-H-2 antibody. Chimeras were examined at 7–10 wk postreconstitution. The chimeras used in the experiments shown in Tables I–III were all in conspicuously good health.

B Cells in Spleen, LN, and TDL. With recipients of nondefective CBA/Ca marrow, thymectomy had little or no effect on the total number of Ig$^+$ B cells in spleen, LN, or TDL (Tables I and II, groups *A* vs. *B*). Very different results were found with recipients of *xid* CBA/N marrow. The control STx recipients of CBA/N marrow showed a three- to fourfold reduction of B cells relative to recipients of CBA/Ca marrow (groups *C* vs. *A*); a similar reduction in total B cell numbers is seen in normal (nonchimeric) *xid* mice relative to *xid*$^-$ controls (1).[1] ATx recipients of CBA/N marrow cells showed a much more profound deficiency of B cells. This deficiency was least obvious in the spleen, although considerable variation was observed between one batch of chimeras and another. In the experiment illustrated in Table I, total splenic Ig$^+$ B cells were fivefold less in CBA/N → ATx F_1 chimeras than in the STx controls. A less pronounced (two- to fourfold) reduction in splenic B cells was found in two other batches of chimeras. In a further two batches, however, Ig$^+$ cells were virtually absent in the spleen (Table I, footnote ‖).

[1] Sprent, J., and J. Bruce. Physiology of B cells in mice with X-linked immunodeficiency. I. Size, migratory properties, and turnover of the B cell pool. Manuscript in preparation.

TABLE I

*Numbers of T and B cells in Spleen and LN of Adult Thymectomized, Irradiated (B6 × CBA/J)F_1 Mice Reconstituted 9 Wk Previously with CBA/N vs. CBA/Ca Marrow**

Group	Cells transferred to irradiated (B6 × CBA/J)F_1 mice	Treatment of recipients	Cytotoxic indices with: α-Thy-1.2	RαMIg	α-I-A^k	J11d	α-D^b	Total number ($\times 10^{-6}$) of: Lymphoid cells	Thy-1^+ cells	Ig^+ cells
			Spleen							
A	CBA/Ca BM‡	STx	27§ (2)	57 (8)	69 (4)	71 (1)	3 (3)	152 (16)	41.7 (7)	86.2 (11)
B	CBA/Ca BM	ATx	−2 (6)	82 (4)	91 (1)	90 (4)	0 (1)	109 (8)	<1.0	88.8 (2)
C	CBA/N BM	STx	44 (11)	39 (10)	40 (8)	48 (3)	6 (1)	80.3 (28)	37.2 (19)	29.6 (5)
D	CBA/N BM	ATx	6 (6)	30‖ (12)	48 (6)	57 (6)	3 (2)	21.1 (5)	1.3 (0.3)	6.1 (4)
E	CBA/N BM + 25 × 10^6 CBA/N T cells¶	ATx	37 (6)	47 (7)	50 (10)	56 (9)	7 (3)	24.3 (4)	8.8 (0.8)	11.5 (4)
			Lymph nodes							
A	CBA/Ca BM	STx	55 (1)	45 (1)	46 (4)	44 (3)	5 (2)	48.7 (11)	26.7 (6)	21.9 (11)
B	CBA/Ca BM	ATx	4 (4)	92 (1)	95 (3)	94 (1)	3 (1)	21.5 (4)	0.8 (0.6)	19.8 (4)
C	CBA/N BM	STx	84 (1)	17 (5)	18 (1)	19 (2)	5 (6)	39.5 (7)	33.2 (5)	6.7 (1.3)
D	CBA/N BM	ATx	42	28	45	49	37	0.9 (0.3)	0.4	0.3
E	CBA/N BM + 25 × 10^6 CBA/N T cells¶	ATx	67 (11)	33 (7)	39 (11)	39 (7)	10 (7)	10.7 (0.9)	7.3 (2)	3.5 (0.4)

* Data based on individual counts in three to five mice per group. Mice were assayed over a 4-d period and were all from the same batch. Total cell counts refer to total numbers of live plus dead cells. LN cells were pooled from axillary, inguinal, cervical, and mesenteric nodes.

‡ Bone marrow (BM) cells were injected intravenously in a dose of 5 × 10^6 viable cells after pretreatment with monoclonal anti-Thy-1.2 antibody plus C′.

§ Arithmetic mean (SD) of data from three to five mice per group. In group *D*, the low LN cell yields required pooling the cells (from five donors) to measure cytotoxic indices. All cell populations were passed through Ficoll gradients to remove dead cells. Background lysis with cells treated with C′ alone was usually <5%.

‖ In another two batches of chimeras, Ig^+ cells in group *D* were virtually absent from the spleen (0–5%) as assessed either by immunofluorescence or by cytotoxicity. The spleen cells in these batches of mice were also Ia^- (<10%) and consisted mostly of large lymphoid cells; 50–70% of these cells were lysed by monoclonal J11d antibody.

¶ T cells were prepared from pooled LN of irradiated (1,000 rad) (B6 × CBA/J)F_1 mice reconstituted 6 mo before with T cell–depleted CBA/N marrow cells. LN cells from the chimeras were first passed over nylon wool columns and then treated with a cocktail of RαMIg and anti-D^b antibody plus C′. The surviving cells were >99.5% Thy-1^+ and contained no detectable Ig^+ cells or D^b-bearing (host) cells. T cells were injected intravenously 3 d after irradiation and marrow reconstitution.

LN and TDL of ATx recipients of CBA/N marrow were virtually acellular, and numbers of B cells were reduced by 20–25-fold relative to the STx controls (Tables I and II, groups *C* vs. *D*). This striking deficiency of B cells was a constant finding seen in five separate batches of chimeras (TDL were examined in only two batches). Interestingly, a high proportion (35–70%) of the LN and TDL from CBA/N → ATx F_1 chimeras were Thy-1^+ cells. Most of these cells could be lysed with anti-D^b antibody plus C′, and thus were presumably radioresistant T cells of host origin; in absolute terms, these cells were very few in number, i.e., $<10^6$ cells per mouse.

Influence of Mature T Cells. Group *E* of Tables I and II shows the effect of adding mature CBA/N T cells to CBA/N → ATx F_1 chimeras. The injected T cells (>99.5% Thy-1^+) were taken from LN of long-term CBA/N → F_1 chimeras,

TABLE II

*Numbers of T and B Cells in TDL of Adult Thymectomized, Irradiated (B6 × CBA/J)F_1 Mice Reconstituted 8 Wk Previously with CBA/N vs. CBA/Ca Marrow**

Group	Cells transferred to irradiated (B6 × CBA/J)F_1 mice	Treatment of recipients	Cytotoxic indices on untreated TDL			Cytotoxic indices on TDL pretreated with RαMIg + C′‡			Total number (×10^{-6}) of:		
			α-Thy-1.2	RαMIg	α-D^b	α-Thy-1.2	RαMIg	α-D^b	TDL	Thy-1^+ cells	Ig^+ cells§
A	CBA/Ca BM	STx	63 (10)	36 (5)	0 (1)	—	—	—	42.2 (8)	26.2 (5)	15.1 (4)
B	CBA/Ca BM	ATx	5 (4)	93 (3)	6 (6)	—	—	—	14.9 (2)	0.8 (0.5)	13.9 (2)
C	CBA/N BM	STx	84 (5)	16 (3)	9 (4)	99	2	4‖	28.8 (6)	24.3 (5)	4.7 (1)
D	CBA/N BM	ATx	64 (2)	35 (3)	58 (4)	96	2	85‖	0.7 (0.3)	0.5 (0.3)	0.2 (0.1)
E	CBA/N BM + 25 × 10^6 CBA/N T cells	ATx	65 (2)	38 (9)	16 (4)	98	0	20‖	6.5 (2)	4.3 (2)	2.4 (0.2)

* As for Table I. Mice were from the same batch as assayed in Table I; three to five mice per group. TDL were collected from 0 to 24 h postcannulation. BM, bone marrow.

‡ Aliquots of pooled TDL were treated with RαMIg plus C′ and then depleted of dead cells.

§ *P* values: group *A* vs. *B*, >0.05; *A* vs. *C*, <0.01; *C* vs. *D*, <0.01; *D* vs. *E*, <0.01.

‖ When other aliquots of TDL were pretreated with α-Thy-1 antibody plus C′ to yield purified B cells, <5% of these cells were lysed with α-D^b + C′.

thereby ensuring tolerance to host alloantigens (see Table I, footnote ¶). The T cells were injected in a dose of 25 × 10^6 per mouse 3 d after marrow reconstitution. This injection restored the T cell pool to only ~20% of normal (Tables I and II, groups *C* vs. *E*). Significantly, however, the injection of T cells substantially abrogated the deficiency of B cells, particularly in LN and TDL. B cells in these tissues were only twofold less than in the CBA/N → STx F_1 controls.

Differentiation of xid B Cells in Totally H-2-different Chimeras. The capacity of T cells to promote *xid* B cell differentiation might reflect direct H-2-restricted T-B interaction during ontogeny. If so, T cells differentiating in a thymus lacking the H-2 determinants expressed on the B cells would be expected to lose the capacity to help the differentiation of these B cells. Table III presents evidence against this notion. The table shows the number of B cells developing in irradiated (1,000 rad) (B6 × DBA/2)F_1 (bxd) mice reconstituted with *xid* marrow cells taken either from (*a*) totally H-2-different CBA/N mice (k → bxd) or (*b*) semisyngeneic (CBA/N × DBA/2)F_1 male mice (kxd → bxd). The numbers of B cells developing in the spleens of the two groups of recipients were very similar. It should be mentioned that T cells taken from the totally H-2-different CBA/N → (B6 × DBA/2)F_1 chimeras were markedly defective in providing sheep erythrocyte–specific T helper function for H-2-compatible CBA/Ca B cells, i.e., B cells expressing nonthymic H-2 determinants (data not shown).

Discussion

The data in this paper on thymectomized, irradiated, marrow-protected mice extend the earlier studies on *xid nu nu* mice (2, 3) and provide direct evidence that the differentiation of *xid* B cells is thymus-dependent, at least in part. The fact that addition of purified LN T cells largely overcame the B cell deficit suggests that mature T cells rather than thymic humoral factors control *xid* B cell development.

The T dependency of *xid* B cell differentiation was most conspicuous with LN

TABLE III

*Differentiation of CBA/N B Cells in Spleens of Totally H-2-different vs. Semisyngeneic Bone Marrow Chimeras**

Irradiated hosts	Marrow cells (male) used for reconstitution	Time after reconstitution (weeks)	No. of mice	Cytotoxic indices with:			Total number ($\times 10^{-6}$) of:		
				α-Thy-1.2	RαMIg	α-H-2^{bt}‡	Spleen cells	Thy-1$^+$ cells	Ig$^+$ cells
(B6 × DBA/2)F$_1$ (bxd)	CBA/Ca (k)	8	3	42§ (19)	30 (1.0)	6 (2)	105 (17)	47 (13)	32 (10)
		11–13	2	43 (11)	44 (6)	1 (1)	163 (6)	72 (6)	70 (20)
(B6 × DBA/2)F$_1$ (bxd)	CBA/N (k)	8	3	54 (15)	23 (5)	9 (2)	71 (15)	39 (16)	17 (9)
		11–13	3	61 (5)	34 (2)	4 (1)	109 (15)	60 (14)	31 (6)
(B6 × DBA/2)F$_1$ (bxd)	(CBA/N × DBA/2)F$_1$ (kxd)	8	3	46 (10)	31 (11)	5 (1)	63 (18)	29 (4)	20 (10)
		11–13	6	42 (5)	28 (9)	5 (2)	72 (19)	33 (4)	21 (7)

* Adult (10 wk) (B6 × DBA/2)F$_1$ mice were exposed to 1,000 rad and injected intravenously with 5×10^6 anti-Thy-1.2 + C′-treated bone marrow cells.

‡ CBA/J anti-B6 antiserum absorbed with DBA/2 spleen. This antiserum lysed >90% of normal (B6 × DBA/2)F$_1$ spleen cells.

§ Mean of data (SD) derived from individual mice.

and TDL B cells. Thus, B cells were virtually absent in the LN and TDL of CBA/N → ATx F$_1$ chimeras but reached nearly normal levels in the presence of mature T cells. The findings in the spleen were less striking. Thus, whereas some batches of ATx chimeras were almost devoid of Ig$^+$ cells in the spleen, others showed only a twofold reduction. These findings agree closely with the two studies in *xid nu nu* mice (2, 3). Although the LN of *xid nu nu* mice were markedly depleted of B cells in both of these studies, gross depletion of splenic B cells was seen in only one study (2), and then only in two of three experiments. In the case of the two batches of our own chimeras that showed a total absence of Ig$^+$ cells in the spleen, it is of interest that these mice looked less healthy than the other three batches of chimeras (which were in excellent condition). For this reason, we suspect that the extreme depletion of *xid* B cells seen in the spleens of some mice might be an artifact reflecting B cell destruction as a consequence of infection (7). Thus, the T dependency of *xid* B cells might only apply to certain B cell subsets, e.g., to recirculating B cells prominent in LN and TDL; the young B cells entering the spleen from the marrow might be T independent.

Speculating on the significance of T-dependent *xid* B cell differentiation begs the question of whether *xid* B cells have an exact counterpart in normal mice. This issue remains unresolved. If normal mice did contain a component of cells resembling *xid* B cells, thymectomy (or congenital athymia) would be expected to cause a significant decrease in total B cell numbers. Although no such reduction has been found, either in spleen, LN, or TDL (Tables I, II and reference 7), the possibility of a small (10%) reduction is difficult to exclude. Moreover, compensatory overproduction of other B cells might hide the disappearance of a T-dependent subset.

The alternative possibility is that *xid* B cells are a unique population that is not represented in normal mice. We favor this view in light of recent experiments on the effects of reconstituting irradiated mice with a mixture of *xid* and normal

marrow cells.[2] Unlike T cells, the *xid* marrow-derived B cells developing in these double chimeras are virtually undetectable by 6 mo postreconstitution. These findings are difficult to reconcile with the view that *xid* B cells are the equivalent of the (sizeable) population of Lyb-5$^-$ immature B cells in normal mice.

Why *xid* B cell differentiation should be T dependent is difficult to envisage. The data in Table III make it unlikely that T cells guide *xid* B cell differentiation via direct H-2-restricted cell interaction. The alternative possibility is that soluble T cell factors control *xid* B cell differentiation. One might test this idea by injecting ATx *xid* chimeras or *xid nu nu* mice with a cocktail of T cell factors, although careful studies on the requirement for inducing *xid* B cells to differentiate in vitro would probably be a more informative approach.

Summary

Evidence is presented that the in vivo differentiation of B cells expressing X-linked immunodeficiency (*xid*) is controlled by mature T cells. Normal (C57BL/6 × CBA/J)F_1 mice were thymectomized (ATx), heavily irradiated, and reconstituted with CBA/N (*xid*) or CBA/Ca (nondefective) marrow. In contrast to sham-operated mice, ATx recipients of *xid* marrow showed an almost total absence of Ig$^+$ B cells in lymph nodes (LN) and thoracic duct lymph at 2 mo postreconstitution; B cells were markedly reduced in the spleen in some mice but only moderately in others. Addition of mature T cells soon after marrow reconstitution substantially abrogated the B cell depletion. In control experiments with nondefective B cells, the number of B cells developing in ATx irradiated recipients of normal (*xid*$^-$) marrow cells was not detectably lower than in sham-operated recipients. These data imply that a subset of T-dependent B cells is either missing in normal mice or present in only very small numbers.

Received for publication 13 February 1984.

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