NEGATIVE SELECTION IN VIVO REVEALS EXPRESSION OF STRONG *Mls* DETERMINANTS IN MICE WITH X-LINKED IMMUNODEFICIENCY

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Although typical mixed lymphocyte reactions (MLR)[1] are directed predominantly to H-2 determinants, high primary MLR in mice can also be elicited by gene products of the minor lymphocyte-stimulating (*Mls*) locus located on chromosome 1 (1). Of the four described *Mls* alleles, *a b c d*, Mls^a and Mls^d determinants elicit the strongest response. Since these two sets of determinants cross-react extensively (and are probably indistinguishable), we shall refer to them as "$Mls^{a,d}$ determinants" (2).

$Mls^{a,d}$ determinants are of interest for at least three reasons. Firstly, in our hands the T cell response to these determinants does not exhibit H-2 restriction (3, 4) (although others disagree with this viewpoint [5]). Secondly, from studies on karyotypically unstable hybridomas prepared from a dual-reactive T cell clone, we have preliminary evidence that the receptors for allo-H-2 and $Mls^{a,d}$ determinants are encoded on different chromosomes.[2] Thirdly, the expression of $Mls^{a,d}$ determinants appears to define one of the two subsets of B cells, namely the "mature" B cells, which develop late in ontogeny and express Lyb 5 surface alloantigens (6); immature (Lyb 5^-) B cells do not express $Mls^{a,d}$ determinants. $Mls^{a,d}$ determinants are thus a potentially useful marker for probing the functional differences between mature and immature B cells, and for addressing the key issue of whether these two subsets of cells represent distinct lineages or different stages of differentiation of the same lineage.

Much of the evidence on the differences between mature and immature B cells has come from studies on B cell development in CBA/N mice, i.e., mice expressing X-linked immunodeficiency (*xid*) (6). These mice contain immature

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[1] *Abbreviations used in this paper:* FCS, fetal calf serum; LN, lymph node; MLR, mixed lymphocyte reactions; MLS, minor lymphocyte-stimulating; PFC, plaque-forming cells; TDL, thoracic duct lymphocytes; TNP, trinitrophenyl; *xid*, x-linked immunodeficiency.

[2] T cell hybridomas with dull reactivity for $Mls^{a,d}$ and allo H-2 determinants can selectively lose one specificity. Webb, S. R., J. Hu Li, I. MacNeil, P. Marrack, J. Sprent, and D. B. Wilson. Manuscript in preparation.

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B cells but show a profound deficiency of the mature subset of B cells. Responsiveness to TI-2 antigens, a hallmark of mature B cells, is nearly totally absent in *xid* mice. Likewise, the expression of Mls determinants is held to be negative in these mice (6). For the *xid* mouse model to be relevant to normal B cell development, however, it is important to know whether (1) the B cells in *xid* mice are the precise counterpart of the immature set of B cells in normal mice and (2) whether the deficiency of the mature B cell subset in *xid* mice indeed reflects an absence of these cells rather than aberrant differentiation. These questions have yet to be resolved.

Our approach to the second of these two questions has been to reinvestigate the claim that *xid* mice fail to express Mls determinants. The results show that by a variety of parameters, including negative selection of T cells through irradiated hosts, even very young *xid* mice express $Mls^{a,d}$ determinants. If $Mls^{a,d}$ expression is a valid marker for mature B cells, then these data imply that xid^+ mice do contain cells that manifest at least some of the properties of mature B cells.

Materials and Methods

Animals. (CBA/N × DBA/2)F_1 males and females were bred at the Institute for Cancer Research, Philadelphia. The (C57BL/6(B6)XCBA/J)F_1, BALB/c, CBA/J, CBA/Ca, and P/J mice were all purchased from The Jackson Laboratory, Bar Harbor, ME.

Primary Mixed Lymphocyte Responses (MLR). The medium used for all cell cultures was RPMI 1640 with 10% fetal calf serum (FCS), 5% NCTC 109, glutamine, 5×10^{-5}M 2-mercaptoethanol, and antibiotics. $1-2 \times 10^5$ lymph node cells or thoracic duct lymphocytes (TDL) were cultured in flat-bottom microtiter plates with 5×10^5 irradiated (1,500 rad) spleen cells as stimulators. The cultures were pulsed on day 3 or day 4 with 0.5 μCi [^{3}H]-thymidine and harvested ~18 h later.

T Cell Clone. The T cell clone, D7, was produced and characterized as described previously (4). Briefly, B10.D2 lymph node (LN) cells were cultured for 14 d with irradiated (3,000 rad) DBA/2 spleen cells. The viable cells were restimulated with irradiated DBA/2 spleen cells and cloned by limiting dilution 24 h later in the presence of 10% interleukin 2 (IL-2)–containing rat concanavalin A supernatant. Clones were assayed by culturing 2×10^4 cloned cells with $3-5 \times 10^5$ irradiated spleen stimulator cells in round-bottom microtiter plates. These cultures were pulsed with [^{3}H]thymidine on day 2 and harvested on day 3.

This particular clone proliferates in response to spleen cells from all $Mls^{a,d}$ positive strains tested including: DBA/2, CBA/J, D1.LP, AKR/J, NZB, and SM. The clone does not respond to spleen cells from any of 13 $Mls^{a,d}$ negative strains tested of various H-2 haplotypes and backgrounds.

Negative Selection. The procedure for negative selection was similar to that described previously (3). 10^8 viable lymph node cells were injected intravenously into mice given 750 rad of irradiation 3 h before. Thoracic duct cannulae were inserted in the recipients ~ 15 h after injection of cells and TDL were collected between 18–40 h post injection. Under these conditions, 90–98% of the cells collected are small T lymphocytes of donor origin.

Plaque-forming Cells (PFC) to TNP. Direct anti-trinitrophenyl (TNP) PFC were assayed according to Rittenberg and Pratt (7) following immunization of mice with 20 μg TNP_{54} aminoethylcarbamylmethyl-Ficoll (TNP-Ficoll).

Results

Anti-Mls MLR Elicited by Cells from xid Mice of Various Ages. Fig. 1 compares the capacity of spleen cells from Mls^a-positive (CBA/N × DBA/2)F_1 male (xid^+)

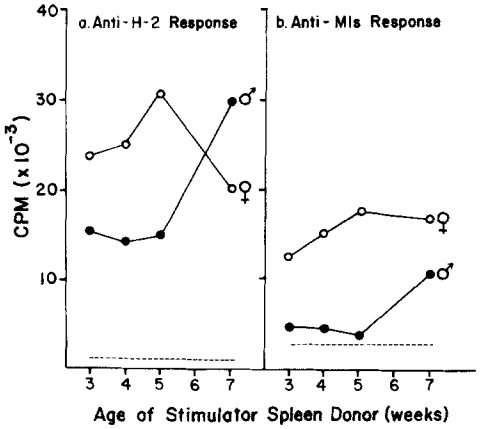

FIGURE 1. (*a*) For the anti-H-2 response, (B6 × CBA/J)F_1 lymph node cells were cultured with irradiated spleen cells from (CBA/N × DBA/2)F_1 male or female mice of the ages indicated on the abscissa. (*b*) To test the ability of mice of various ages to stimulate an anti-$Mls^{a,d}$ response, (C3H/HeJ × BALB/c)F_1 lymph node cells were the responding population. ^{3}HTdR incorporation was measured on day 4. Each point represents the mean of the data (measured in triplicate cultures) obtained from two separate stimulator spleen donors. The dotted line shows the background response obtained when each of the two responder populations was cultured with autologous spleen cells.

and female (*xid*$^-$) mice of various ages to stimulate anti-H-2 and anti-$Mls^{a,d}$ MLR. (B6 × CBA/J)F_1 (*H-2*^{bxk}, *Mls*^{bxd}) LN cells were used to measure anti-H-2 (anti-H-2^d) responses; (C3H/HeJ × BALB/c)F_1 (*H-2*^{kxd}, *Mls*^{cxb}) LN cells detected anti-$Mls^{a,d}$ responses. In the case of anti-H-2 responses (Fig. 1 *a*), strong responses were observed to both male and female F_1 stimulators of all ages tested (3, 4, 5, and 7 wk). The response to young (3–5 wk) F_1 male stimulators was somewhat lower than to female stimulators in the experiment illustrated, but this was not a constant finding in other experiments. In contrast to anti-H-2 MLR, there was a marked difference in the capacity of young male vs. female F_1 spleen cells to elicit anti-$Mls^{a,d}$ responses (Fig. 1 *b*). Whereas female mice of all ages stimulated strong anti-$Mls^{a,d}$ MLR, young (3–5 wk) male mice gave only minimal (<twofold) responses. Interestingly, however, older (7 wk) male mice generated appreciable anti-$Mls^{a,d}$ responses.

Similar data were observed in several other experiments. Young (<5 wk) male F_1 mice consistently evoked only low (<twofold) anti-Mls responses, whereas older mice (8–30 wk) invariably generated responses which, though always lower than to female cells, were 5–20-fold above background. To confirm the existence of the *xid* defect in the male F_1 mice, each individual spleen cell donor in the experiment shown in Fig. 1 was given TNP-Ficoll (20 μg intraperitoneally) 6 d before. Whereas spleens from the female donors all gave high anti-TNP responses (30,000–50,000 plaque-forming cells (PFC)/spleen), anti-TNP responses were completely undetectable (<100 PFC/spleen) in the male donors of all ages tested (see Materials and Methods).

The responder cells in the above experiments were normal LN cells. Table I illustrates that similar results were obtained with a well-characterized $Mls^{a,d}$-specific T cell clone. It can be seen that the clone gave definite responses with

TABLE I

*Response of an Mls^{a,d}-Specific T Cell Clone, D7, to Male vs. Female (CBA/N × DBA/2)F$_1$ Spleen Stimulators**

Stimulators			Age	Response of D7 T cells: ^{3}HTdR uptake ($\times 10^{-3}$) on day 3
Strain	H-2, Mls	Sex		
			wk	
(C3H/HeJ × BALB/c)F$_1$	kxd, cxb	Male	8	0.3‡
(CBA/N × DBA/2)F$_1$	kxd, ?xb	Female	7	14.6
(CBA/N × DBA/2)F$_1$	kxd, ?xb	Female	30	19.6
(CBA/N × DBA/2)F$_1$	kxd, ?xb	Male	7	4.5
(CBA/N × DBA/2)F$_1$	kxc, ?xb	Male	30	12.1
DBA/2	d, a	Male	8	12.4

* The characteristics of the D7 clone are discussed in the Materials and Methods section.
‡ Arithmetic mean of triplicate cultures; SD were within 20% of the mean.

7- and 30-wk-old F$_1$ male stimulators, the response being somewhat less with 7-wk mice.

Negative Selection of T Cells to Mls^{a,d} Determinants in xid Mice. Previous studies have demonstrated that T cells filtered from blood to thoracic duct lymph through irradiated hosts undergo specific negative selection to host Mls^{a,d} and H-2 determinants (3). Since very young F$_1$ male mice elicit only minimal anti-Mls^{a,d} MLR, would such mice fail to induce negative selection to Mls determinants? As shown in Table II, BALB/c (*H-2^d*, *Mls^b*)(*d,b*) LN cells filtered through irradiated (750 rad) 10-wk-old male and female F$_1$ hosts were almost totally depleted of reactivity to stimulators bearing host H-2 determinants (CBA/Ca)(*k,b*), host Mls^a determinants (DBA/2)(*d,a*) and stimulators expressing a combination of host H-2 and "cross-reactive" Mls^d determinants (CBA/J)(*k,d*); responsiveness to third-party H-2 alloantigens (P/J) was retained. Similar findings were observed with filtration through 3-wk female F$_1$ mice. With filtration through 3-wk male F$_1$ mice, however, anti-Mls responses were greatly reduced but not abolished; responses to host H-2 determinants were completely undetectable. Another experiment gave similar results.

Xid Mice Are Tolerant to Self Mls^{a,d} Determinants. In two experiments, one of which is shown in Table III, LN cells from male and female F$_1$ mice gave little if any response to Mls^{a,d} determinants. Responder cells from 10-wk male and female F$_1$ mice were both totally unreactive to Mls^a determinants on DBA/2 stimulators. Young (2 wk) mice responded slightly better to DBA/2 than to syngeneic (autologous) stimulators. This "response," however, was minimal, no higher with F$_1$ male than female responders and largely attributable to the lower background counts seen with young autologous stimulators.

Discussion

Collectively, the data in this paper indicate that *xid$^+$* (CBA/N × DBA/2)F$_1$ male mice do express Mls^{a,d} determinants, despite the total inability of these mice to respond to the TI-2 antigen, TNP-Ficoll. At 7–30 wk of age, F$_1$ male mice invariably stimulated conspicuous anti-Mls^{a,d} MLR, either with normal LN cells or a T cell clone as responders. These responses were usually in the range of

TABLE II

*Negative Selection of BALB/c LN Cells in Irradiated Male vs. Female (CBA/N × DBA/2)F$_1$ Mice**

Irradiated hosts for negative selection of BALB/c LN	Age of selection host	^{3}HTdR Uptake (× 10^{-3}) by negatively selected T cells stimulated against:				
		BALB/c (d, b)	DBA/2 (d, a)	CBA/Ca (k, b)	CBA/J (k, d)	P/J (p, ?)§
	wk					
BALB/c¶	8	1.3‡	90.6	56.8	273.9	56.8
(CBA/N × DBA/2)F$_1$ female¶	10	0.6	1.2	0.6	0.8	32.3
(CBA/N × DBA/2)F$_1$ male #1	10	0.7	2.1	0.2	0.3	54.7
(CBA/N × DBA/2)F$_1$ male #2	10	0.6	1.6	0.4	0.5	29.0
(CBA/N × DBA/2)F$_1$ female¶	3	0.9	2.8	0.4	2.2	49.3
(CBA/N × DBA/2)F$_1$ male #1	3	2.0	10.0	1.1	16.6	123.9
(CBA/N × DBA/2)F$_1$ male #2	3	1.4	13.4	1.0	22.5	69.8
(CBA/N × DBA/2)F$_1$ male #3	3	1.2	16.6	1.0	24.3	98.1

* BALB/c LN cells were filtered from blood to thoracic duct lymph through irradiated mice (see Materials and Methods) and then tested in mixed-lymphocyte culture against the stimulator strains listed.

‡ As for Table I.

§ Although the Mls allele of P/J is unknown, this strain does not stimulate our Mls^{a,d}-specific T cell clone; hence the allele is presumably either Mls^b or Mls^c.

¶ Cells used in MLR were pooled from two selection hosts.

TABLE III

Self Tolerance of (CBA/N × DBA/2)F$_1$ Male and Female Mice to Mls^{a,d} Determinants

Responders (LN cells)*		Age of responders	^{3}HTdR Uptake (× 10^{-3}) Against:		
			Autologous	DBA/2 (d, a)	P/J (p, ?)§
		wk			
(CBA/N × DBA/2)F$_1$	male	2	2.1‡	5.9	71.3
(CBA/N × DBA/2)F$_1$	female	2	3.5	5.6	54.3
(CBA/N × DBA/2)F$_1$	male	10	5.0	4.5	32.9
(CBA/N × DBA/2)F$_1$	female	10	5.2	4.7	32.5
BALB/c	female	2	1.6	17.5	72.3
BALB/c	female	10	4.6	19.7	82.6

* Responders were pooled from two donors for the 2-wk mice and from single donors for the 10-wk mice.

‡ As for Table I.

§ See Table II.

20–50% of the response given by female F_1 mice, but reached near normal levels with old (30-wk) mice. Somewhat different results were obtained with young *xid*$^+$ F_1 male mice. At 3–5 wk, *xid*$^+$ mice stimulated only very low anti-Mls^{a,d} responses. Interestingly, however, T cell filtration through these mice reduced the anti-Mls^{a,d} response by 90%. Moreover, even 2-wk *xid*$^+$ mice exhibited self tolerance to Mls^{a,d} determinants.

Before considering the implications of these findings it is useful to consider the literature on the expression of Mls determinants in *xid*$^+$ mice. The literature is fragmentary. With regard to "weak" Mls determinants, Ahmed and Scher (8) originally reported that CBA/J (*k,d*), C3H/N(*k,c*), and CBA/N (*k,?*) responder cells all gave low (~threefold) MLR to *xid*$^-$ (CBA/N × C3H/N)F_1 female spleen cells, but no response to *xid*$^+$ male F_1 stimulators. These findings are not easily interpreted. In the case of strongly-stimulatory Mls^{a,d} determinants, Ryan et al. (9) with blood leukocytes and Glasebrook et al. (10) with an Mls^{a,d}-specific T cell clone both reported that *xid*$^-$ (DBA/2 × CBA/N)F_1 male spleen cells were much better stimulators than *xid*$^+$ reciprocal (CBA/N × DBA/2)F_1 male spleen cells. Though low in magnitude, however, the anti-Mls^{a,d} responses elicted by the *xid*$^+$ stimulators were clearly above background in both studies.

It is worth stressing that the precise age of the stimulator cell donors in the above three studies was not stated. This is an important point because Ahmed et al. (11) clearly established that the expression of Mls^{a,d} determinants by normal *xid*$^-$ mice occurs quite slowly during ontogeny. Spleen cells do not develop the capacity to elicit anti-Mls^{a,d} responses until day 10 of age, and generate normal responses only after 4–5 wk.

With this fact in mind, the simplest explanation of the present data is that *xid*$^+$ mice do express Mls^{a,d} determinants, but at a much slower tempo than normal mice. If the expression of Mls^{a,d} determinants denotes the presence of the mature subset of B cells, however, one would expect to find a parallel slow emergence of the functions attributable to mature B cells in *xid*$^+$ mice. By and large the literature does not support this prediction, particularly in the case of responsiveness to T1-2 antigens (reviewed in reference 6): very old (>1 year) *xid*$^+$ mice sometimes do show responsiveness to TNP-Ficoll, but the responses are low and are not a general finding. This raises the crucial issue of whether *xid*$^+$ B cells have a counterpart in normal mice. Although this is a popular view, there is accumulating evidence to the contrary, including the failure of *xid*$^+$ B cells, but not Lyb 5$^-$ B cells from normal mice, to respond to certain preparations of LPS (12). One may also mention the odd dependency of *xid*$^+$ B cells on the thymus during B cell formation (references 13, 14, and Sprent, unpublished), and recent findings that *xid*$^+$ B cells (but not T cells) gradually disappear in double irradiation chimeras prepared with a mixture of *xid*$^+$ and *xid*$^-$ B cells (Sprent, unpublished). Such findings support the opposing view that *xid*$^+$ B cells are intrinsically abnormal: the cells are crippled and have no exact counterpart in normal mice. The cells grow and differentiate slowly, eventually achieve some of the characteristics of mature B cells, e.g., the expression of Mls^{a,d} determinants, but never resemble normal immature or mature B cells.

The notion that the Mls^{a,d}-positive cells in *xid*$^+$ mice are qualitatively abnormal is consistent with recent observations of Ryan et al. (9). These workers made the

interesting discovery that preinjecting normal xid^- mice with anti-δ antibody dramatically increased the capacity of their spleen cells to stimulate anti-$Mls^{a,d}$ responses. Significantly, there was no enhancement of the small (twofold) response stimulated by xid^+ spleen cells.

How can one account for the finding that spleen cells from young (3 wk) xid^+ male F_1 mice stimulated only minimal anti-$Mls^{a,d}$ MLR but, when they were used as hosts for negative selection, the anti-$Mls^{a,d}$ response of normal T cells was reduced by 90%? Two explanations can be considered. The first is that young xid^+ mice have only a quantitative and not a qualitative deficiency of Mls-positive cells. According to this idea the presence of even small numbers of $Mls^{a,d}$-positive cells is sufficient to obtain marked negative selection but only minimal MLR. The second possibility is that very young xid^+ B cells do express $Mls^{a,d}$ determinants but not in a form sufficient to cause the responding T cells to proliferate, e.g. because of abnormally low expression of $Mls^{a,d}$ determinants by young xid^+ B cells or because these cells might lack an essential co-factor required for T cell stimulation. According to this idea T cells recognize (bind to) the B cells but fail to become activated. Though in need of direct support, this notion is consistent with the finding that young xid^+ mice are unequivocally $Mls^{a,d}$-positive only in terms of causing T cell selection, a manifestation of short-term (1 d) contact of T cells with antigen, and not in causing T cells to proliferate.

Direct evidence for covert expression of $Mls^{a,d}$ determinants by young xid^+ B cells might be detected by a T cell binding assay (15) and/or IL-2 production by $Mls^{a,d}$-specific T hybridomas (Webb, unpublished). These questions are currently under study. It is worth pointing out that if young xid^+ B cells (or neonatal normal B cells) were indeed $Mls^{a,d}$-positive by these parameters, the notion that $Mls^{a,d}$ expression is a marker for mature B cells would obviously require modification.

Summary

Evidence is presented that mice with X-linked immunodeficiency (*xid*) express strong $Mls^{a,d}$ determinants, a putative marker of the mature subset of B cells. Although young (3–5 wk) (CBA/N × DBA/2)F_1 male (xid^+) mice stimulated only very weak mixed lymphocyte reactions (MLR) to $Mls^{a,d}$ determinants, older mice (>7 wk) regularly elicited conspicuous responses, despite being totally unresponsive to TNP-Ficoll.

Expression of $Mls^{a,d}$ determinants by xid^+ mice was also detected by the procedure of negative selection in vivo. Thus BALB/c T cells were totally depleted of $Mls^{a,d}$ reactivity after blood to lymph recirculation through 10-wk old irradiated xid^+ (CBA/N × DBA/2)$_1$ male mice. Significantly, a marked (90%) reduction in the anti-$Mls^{a,d}$ response also occurred with T cell filtration through 3-wk xid^+ mice, i.e., mice that elicit only minimal primary MLR; filtration through 3-wk xid^- normal female mice led to near-complete (99%) negative selection.

Collectively these data indicate either, (*a*) that xid^+ mice contain appreciable numbers of cells with at least some of the properties of mature B cells, or (*b*) that the expression of $Mls^{a,d}$ determinants is not restricted to mature B cells. In either

case, B cells from *xid* mice cannot be viewed as a simple model for immature normal B cells.

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