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Immunology

Antigenic marker on a functional subpopulation of B cells, controlled by the *I-A* subregion of the *H-2* complex

(X-linked B cell defect/CBA/N mutant mouse)

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ABSTRACT CBA/N mice have an X-linked recessive defect that results in the absence of a subpopulation of B cells carrying the Lyb3 surface marker. It has been shown previously that this marker is present on a mature subset of B cells in all mouse strains. In this paper the $Lyb3^+$ B cell population in C57BL/6 mice was analyzed further. This subset of B cells selectively expresses a surface marker controlled by the *I-A* subregion of the *H-2* complex. A cytotoxic antiserum recognizing this marker was raised by immunizing defective (CBA/N × C57BL/6)F_1 ♂ mice with C57BL/6 spleen cells. This antiserum also contained noncytotoxic, non-strain-restricted anti-Lyb3 antibodies. The possible functional relevance of this surface marker is discussed.

Different approaches have been used to delineate the pathway of B lymphocyte differentiation and to define functional B cell subsets. The heterogeneity of B cells is reflected in their physical properties—e.g., size distribution (1) and charge (2)—as well as in their expression of surface markers, such as immunoglobulin isotypes (3), I region associated antigens (4), complement receptor (5), and Pc-1, a plasma cell antigen (6). The appearance of the various Ig isotypes during ontogeny (7) and the differential expression of IgD during the development of a memory response (8, 9) have been analyzed in detail. Since the discovery of Ia antigens there have been numerous reports on their presence on lymphocyte subpopulations: thus, Ia antigens are expressed on functional subsets of T cells (10, 11) and macrophages (12). However, Ia antigens are expressed on virtually all B cells, appearing very early in ontogeny (13).

A different approach to define B cell subsets has been reported in which an antiserum was raised against a differentiation marker using the CBA/N (N) mutant strain (14). The functional defects of these mice have been well characterized by many investigators (15–17); the mice lack a subpopulation of B cells that appears late in ontogeny and responds to thymus-independent type 2 antigens (18). Because the defect is X-linked and recessive, the results of an N ♀ by BALB/c ♂ cross will yield defective F_1 ♂ and normal F_1 ♀ offspring. By immunizing defective F_1 ♂ with BALB/c spleen cells it was possible to raise an antiserum, anti-Lyb3, that reacted exclusively with a subset of B cells in all mouse strains except N. It was also shown that Lyb3 is a receptor for triggering signal(s) (14, 19).

In order to detect a possible polymorphism of Lyb3, the strain combination used for the immunization was changed: instead of BALB/c ♂, C57BL/6 ♂ were mated with the N ♀ mice. The antiserum raised in this strain combination defines a marker present on the $Lyb3^+$ cell that is strain restricted. The gene(s) coding for this marker maps in the *I-A* region of the *H-2* complex. Thus a marker coded by the I region has been found to be differentially expressed on a functional subpopulation of B cells.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), BALB/c, DBA/2, AKR, SJL, and CBA/T6 mice, 8–10 weeks of age, were purchased from the Jackson Laboratory. N mice and F_1 hybrid progeny of crosses between N and B6 were bred in the animal facilities of Tufts University School of Medicine from breeding stock originally obtained from the National Institutes of Health. The B10 congenic and recombinant mice were a generous gift of Susan Bear, who maintained all the lines at Tufts University School of Medicine from breeding stock kindly provided by D. Schreffler, Washington University, and L. Stimpfling, McLaughlin Research Institute. All other mice used in these studies were a generous gift of Martin Dorf, Harvard Medical School.

Preparation of Lymphocyte Subpopulations. Whole spleen cell suspensions were prepared by teasing the spleen with forceps in a petri dish containing phosphate-buffered saline plus 5% fetal calf serum and then flushing the suspension through a nylon gauze. Erythrocytes were lysed with Tris/ammonium chloride. T lymphocytes were obtained from whole spleen cells by passage over nylon wool columns, according to the method described by Julius *et al.* (20); 80–90% of the recovered cells were Thy 1^+ and 5–10% were Ig^+ when tested by immunofluorescence. B lymphocytes were obtained either by treating spleen cells twice with rabbit anti-mouse-brain serum (αBAT) (21) plus complement (C) or by eluting the nylon-wool-adherent cells; 85–90% of the cells from both preparations were Ig^+. For cytotoxicity assays, the cells were treated with low ionic strength buffer (22) to lyse the dead cells, resulting in 95–98% live cells.

Antisera. (A × B10.A)F_1 αB10.A(5R) antiserum was obtained from Research Resource Branch, National Institute of Allergy and Infectious Disease, and its αK^b activity was absorbed with EL-4 tumor cells. (LP × A.TH)F_1 αA.TL (αIa^k), (C3H × LG/ckc)F_1 αC3H.OH (αIa^d) and (LP × A.TL)F_1 αA.TH (αIa^s) antisera were generous gifts of Martin Dorf. αBAT was prepared according to Golub (21). Anti-lymphocyte serum was prepared by immunizing rabbits with mouse spleen cells.

Microcytotoxicity Assay. Medium for the whole assay was Hanks' balanced salt solution plus 0.1% bovine serum albumin plus 10 mM Hepes buffer. Cells at a concentration of 5×10^5/10 μl were incubated for 20 min on ice with 25 μl of antiserum or normal mouse serum (NMS) at various dilutions in V-bottom microtiter plates (Linbro, Titertek). Then 35 μl of preabsorbed rabbit C at a dilution of 1:6 (final dilution 1:12) was added and the plate was incubated at 37°C on a rocker for ½ hr. The C

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Abbreviations: B6, C57BL/6; N, CBA/N; C, complement; NMS, normal mouse serum; α, anti-; αBAT, anti-mouse-brain serum; NαB6, (N × B6)F_1 ♂ anti-B6; MHC, major histocompatibility complex.

reaction was stopped by addition of 80 μl of cold medium and the viable cells were counted with phase contrast microscopy. The percent specific killing was calculated as:

$$\frac{(\%\ \text{dead cells with antiserum} - \%\ \text{dead cells with NMS}) \times 100}{100\% - \%\ \text{dead cells with NMS}}.$$

Background killing in the NMS control was between 5% and 15%; killing with antilymphocyte serum (1:5) was 100%.

RESULTS

Characterization of Antiserum. The (N × B6)F_1 ♂ anti-B6 serum (NαB6) was first screened for noncytotoxic, non-strain-restricted anti-Lyb3 activity in a functional enhancement assay, as described (14). Most batches of antisera contained anti-Lyb3 antibodies. In addition, some batches contained cytotoxic antibodies which lysed 25–30% of B6 spleen cells in dilutions up to 1:80 (Fig. 1). The specificity of this cytotoxic reaction was determined in absorption experiments (Fig. 2). Aliquots of antisera (10 μl) were absorbed with increasing numbers of spleen cells and then tested for lysis of B6 spleen cells in a microcytotoxicity assay. B6(5×10^6) and, to a lesser but significant degree, (N × B6)F_1 ♀ (10^7) spleen cells removed the cytotoxic activity completely, whereas (N × B6)F_1 ♂ spleen cells were unreactive, even at the highest concentration (10^7).

Strain Distribution. (See Table 1.) The NαB6 serum killed B6 but not BALB/c or CBAH/T6 spleen cells. To examine this in more detail, mice with the various *H-2* haplotypes and their congenic partners on the B10 and C3H background were tested. Spleen cells from all mice with the H-2^b haplotype and F_1s thereof were positive (25–35% specific killing), regardless of the background, whereas all mice expressing different H-2 haplotypes were negative. As a positive control the various strains were tested with the relevant anti-Ia sera, which lysed 90–95% of all B cells (45–67% of whole spleen cells). Antilymphocyte serum plus C killed 100% of the spleen cells. Confirming the absorption experiments (Fig. 2), (N × B6)F_1 ♀ spleen cells were positive and (N × B6)F_1 ♂ spleen cells were negative.

Mapping of the Gene(s) Coding for H-2^b Specific Marker. (See Table 2.) H-2^b recombinant mice were used to define the region within the major histocompatibility complex (MHC) that codes for the marker recognized by the NαB6 serum. All strains expressing I^b alleles to the left of *I-J* of the MHC were positive

Table 1. Strain distribution

Strain	H-2 haplotype	% specific cytolysis of spleen cells ± SD*		
		NαB6		αIa†
		1:20	1:40	1:50
B6	*b*	25 ± 9	25 ± 8	45 ± 3
B10	*b*	30 ± 5	31 ± 7	47 ± 6
A.By	*b*	35 ± 2	31 ± 2	52 ± 3
C3H.SW	*b*	36 ± 10	32 ± 4	67 ± 11
LP/J	*b*	32 ± 4	33 ± 2	52 ± 5
(B6 × A)F_1	*b/a*	32 ± 6	nt	52 ± 2
(B6 × C3H)F_1	*b/k*	25 ± 4	20 ± 5	44 ± 2
(N × B6)F_1 ♀	*b/k*	28 ± 3	19 ± 6	46 ± 2
(N × B6)F_1 ♂	*b/k*	5 ± 2	3 ± 2	53 ± 13
BALB/c	*d*	2 ± 3	−5 ± 5	47 ± 3
B10.D2	*d*	1 ± 2	0	50 ± 4
CBAH/T6	*k*	1 ± 1	4 ± 2	46 ± 2
CBA/N	*k*	0	0	43 ± 3
C3H/HeJ	*k*	0	−1 ± 2	58 ± 5
B10.BR	*k*	2 ± 1	1 ± 2	50 ± 4
A/J	*a*	−8 ± 4	nt	49 ± 2
B10.A	*a*	2 ± 2	nt	50 ± 3
A.CA	*f*	1 ± 2	0	nt
C3H.Q	*q*	2 ± 1	0	nt
C3H.JK	*j*	0	−1 ± 2	nt
C3H.NB	*p*	4 ± 2	−1 ± 2	nt
B10.SM	*v*	4 ± 2	2 ± 1	nt
SJL	*s*	0	nt	45 ± 10

nt, Not tested.

* Calculated by the formula given in *Materials and Methods* to control for nonspecific lysis by NMS plus C; anti-lymphocyte serum (1:5) plus C killed 100% of the cells.

† αIa: αIa^b, αIa^d, and αIa^s were used at 1:50 for the relevant *H-2* haplotypes.

[B10.A(5R)], whereas strains expressing *b* alleles to the right only of *I-A* were negative [B10.A(4R)]. Because B10.MBR (K^b, I-A^k) was negative, it can be concluded that at least one gene coding for the marker maps within the *I-A* region of the MHC, but we cannot exclude the possibility that a second gene in the *I-B* region is necessary for expression of the marker. Lack of the relevant recombinant mice does not allow a precise definition of the genes involved.

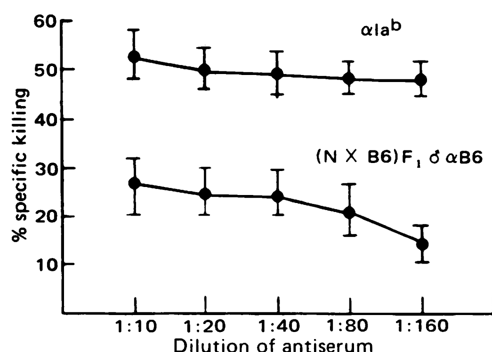

FIG. 1. NαB6 serum was titrated on B6 spleen cells in a microcytotoxicity assay. A standard αIa^b serum was used as a positive control. Anti-lymphocyte serum (1:5) killed 100% of the spleen cells (not shown). % specific killing was calculated according to the formula given in *Materials and Methods* to control for nonspecific lysis by NMS + C.

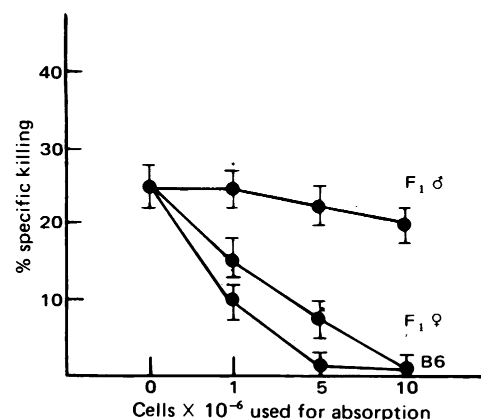

FIG. 2. NαB6 serum (10 μl) was absorbed with increasing numbers of spleen cells for 1 hr on ice. The absorbed sera were then tested at a dilution of 1:20 on B6 spleen cells (see legend to Fig. 1).

Table 2. Mapping of gene(s) coding for marker

Strain	*H-2* haplotype	*H-2* region formula *K*	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*	% specific cytolysis of spleen cells ± SD*
C57BL/10	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	30 ± 5
B10.A(18R)	*i18*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	?|	*d*	32 ± 6
B10.A(3R)	*i3*	*b*	*b*	*b*	*b*|	*k*	*d*	*d*	*d*	*d*	35 ± 9
B10.A(5R)	*i5*	*b*	*b*	*b*|	*k*	*k*	*d*	*d*	*d*	*d*	31 ± 2
B10.A(15R)	*h15*	*k*	*k*	*k*	*k*	*k*	*d*	*d*	*d*|	*b*	1 ± 1
B10.A(4R)	*h4*	*k*	*k*|	*b*	*b*	*b*	*b*	*b*	*b*	*b*	0
B10.GD	*g2*	*d*	*d*	?	?|	*b*	*b*	*b*	*b*	*b*	2 ± 1
B10.MBR$_{Sx}$†	*bq1*	*b*|	*k*	*k*	*k*	*k*	*k*	*k*	*k*	*q*	−1 ± 2

* As for Table 1.

† Recombinant mouse derived from C57BL/10 and B10.AKM crosses kindly provided by D. H. Sachs, National Institutes of Health.

Characterization of the Lymphoid Subpopulation Expressing the Marker. Because the NαB6 serum consistently lysed 25–30% of whole spleen cells, a further analysis of the target cell in B6 mice was necessary (Table 3). Fifty to sixty percent lysis was obtained with splenic B cells (>90% Ig$^+$), whereas splenic T cells and thymocytes were not lysed. Because bone marrow cells were negative, the possibility of a pre-B cell or immature B cell as a target can be excluded. In addition, EL-4 tumor cells, which express H-2^b antigens but lack detectable Ia antigens (23), were not lysed by NαB6 serum and C. These negative results were confirmed in sensitive absorption experiments (Fig. 3). Aliquots of antisera (10 μl) were absorbed with increasing numbers of various cell populations and then tested for cytotoxic activity on B6 spleen cells. Up to 10^7 thymocytes, bone marrow cells, or EL-4 cells were completely ineffective in removing the cytotoxic antibody, whereas B6 spleen cells gave a characteristic absorption profile—namely, 5×10^6 spleen cells removed all the cytotoxic activity.

DISCUSSION

The X-linked recessive defect in CBA/N mice has been extensively studied by many investigators (15–18). These mice fail to develop a functional B cell subset expressing the differentiation antigens Lyb3 (14), Lyb5 (24), and Lyb7 (25). Whereas Lyb3 is found on a subpopulation of B cells in all strains, Lyb5 and Lyb7 show independent allelic distributions, neither of which are H-2 linked.

In this paper the discovery of another surface marker, selectively expressed on the Lyb3$^+$ subset of B cells in B6 mice, is reported.

A cytotoxic antiserum was raised that reacted with 25–30% of spleen cells in H-2^b mice regardless of the non-H-2 background. No crossreaction was seen with spleen cells of any other H-2 haplotype, which indicates that the antiserum recognizes a "private" specificity, unique for the H-2^b haplotype. F_1 hybrids of crosses between H-2^b mice and mice with other H-2 haplotypes were positive. Therefore the gene coding for this marker is expressed in a dominant or codominant fashion. The H-2^b recombinant mice showed a clear pattern of reactivity. All strains expressing *b* in the *I-A* and *I-B* regions were positive [see B10.A(5R)], whereas all strains expressing *b* only to the right of *I-A* were negative [see B10.A(4R)]. These results indicate that at least one gene coding for the marker maps within the *I-A* region of the MHC. A second gene in the *I-B* region could be necessary for expression of the marker, by analogy to the *Ir-GLφ* genes α and β, which map in the *I-C* and *I-A* subregions, respectively, and are both necessary for an immune response to GLφ (26).

Analysis of lymphocyte subpopulations showed that the marker defined here has the same tissue distribution as Lyb3. Because both antigens are expressed on 50% of B cells in the normal (N × B6)F_1 ♀ mice and are absent in the defective F_1 ♂ mice, it is likely that they are expressed on the same B cell subset in H-2^b mice.

EL-4 tumor cells do not express the surface antigen recognized by the NαB6 serum. Because they express the whole repertoire of H-2K^b specificities, but do not express detectable

Table 3. Characterization of lymphoid subpopulation in B6 mice

Lymphoid subpopulation	% specific cytolysis ± SD* at various antisera dilutions 1:10	1:20	1:40
Whole spleen	28 ± 4	25 ± 9	25 ± 8
B-cell-enriched (αBAT + C)†	60 ± 5	50 ± 4	50 ± 4
B-cell-enriched (nylon wool adherent)†	50 ± 2	45 ± 6	45 ± 8
T-cell-enriched (nylon wool effluent)†	2 ± 1	0	−1 ± 2
Bone marrow	1 ± 2	−1 ± 2	2 ± 2
Thymocytes	−2 ± 1	−3 ± 2	0
EL-4 tumor cells	1 ± 2	0	−4 ± 2

* Calculated by the formula given in *Materials and Methods* to control for nonspecific lysis of NMS + C.

† See *Materials and Methods*. Eighty-five to ninety percent of both B-cell-enriched populations were lysed with αIa^b serum + C.

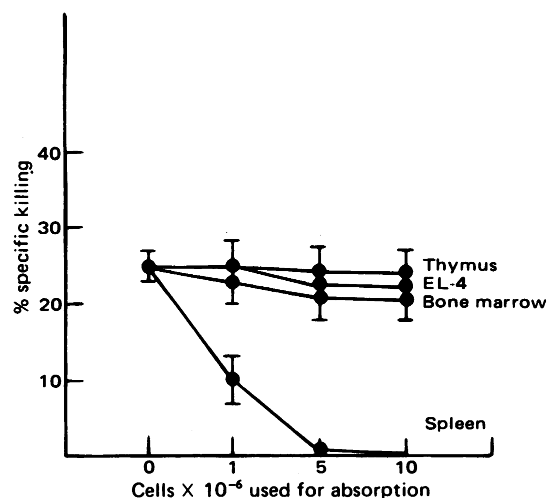

FIG. 3. NαB6 serum (10 μl) was absorbed for 1 hr on ice with increasing numbers of cells from different tissues. The absorbed sera were then tested at a dilution of 1:30 on B6 spleen cells (see legend to Fig. 1).

Ia antigens (23), this is an indication that the marker may be an Ia-like molecule. However, conventional αIa serum directed against specificities in the *I-A* and *I-B* subregions did not discriminate between spleen cells from defective F_1 ♂ and normal F_1 ♀ mice: the same degree of lysis was obtained in both cell preparations (Table 1). These results strongly indicate that the marker recognized by the NαB6 serum is a specificity different from previously described Ia antigens. It will be of interest to absorb conventional α-Ia sera with spleen cells from defective F_1 ♂ mice to test for remaining antibodies with specificity for a B cell subset. It may be that conventional alloimmunizations evoke little or no antibody to this B cell marker. Immunoprecipitations from radioiodinated spleen cells should be useful in studying the molecular nature of the new antigen and its relationship to the defined Ia specificities coded for by the *I-A* region of the MHC.

The fact that this new surface marker is encoded by a gene within the *I-A* region of the *H-2* complex and is selectively expressed on a functional subpopulation of B cells leads to speculation as to its direct functional relevance. Cell interaction genes controlling optimal T–B cell cooperation have been mapped to the *I-A*, *I-B*, or both subregions of the MHC (27–29). Accessory cells (macrophages ?) are the antigen-presenting cells in the T–B cell interaction, and it has been shown that they, too, require identity of the *I-A* region with T and B cells for effective interaction to take place (28, 29). In addition, both macrophages and B cells express the immune response genes, mapped in the *I-A* region, which determine high or low responsiveness to poly(L-Tyr,L-Gln)-poly(DL-Ala)--poly(L-Lys) (29). Because it is known that a subset of macrophages (12) and dendritic cells (30) express Ia antigens, it will be interesting to examine if they also express the surface marker recognized by the NαB6 serum. It should be informative to test the ability of the specific antiserum to deplete relevant subsets of B cells and accessory cells or to block these various cell interactions.

The expression of both Lyb3 and the surface marker defined here is controlled by a gene on the X chromosome (*xid*) (31), because they both fail to develop in the defective F_1 ♂ mice. *xid* inhibits either the total development of a whole subset of B cells or only the specific appearance of these B cell differentiation markers. If the latter case is true, one might expect a structural or organizational relationship between the genes coding for these markers.

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