IDENTIFICATION OF A B-CELL SURFACE STRUCTURE INVOLVED IN ANTIGEN-DEPENDENT TRIGGERING: ABSENCE OF THIS STRUCTURE ON B CELLS FROM CBA/N MUTANT MICE*

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The CBA/N mouse, a mutant of CBA/H, carries an X-linked recessive defect, reflected in part by its inability to respond to Type III pneumococcal polysaccharide, a "thymus-independent antigen" (1). This defect has been studied extensively by Scher and his colleagues (2–10), who have confirmed that the defective gene is X linked and recessive and have also shown that the defective phenotype is expressed in B lymphocytes. This defective phenotype is associated with two major abnormalities of immune function: (*a*) inability to produce significant antibody responses to certain thymus-independent antigens (1, 2, 5, 9) and (*b*) failure to produce at least some sets of high affinity antibodies after immunization with sheep erythrocytes (11).

Since all CBA/N T-cell responses which have been examined are normal (3), and there is no evidence that abnormal suppressor T-cell activity accounts for these defective B-cell responses (2), it has been suggested that these abnormal immune responses reflect a B-cell maturation defect (10). Analyses of the surface properties of CBA/N B cells have indicated that CBA/N B cells (*a*) fail to develop *M*-locus determinants (10), (*b*) express abnormally low concentrations of surface C3 receptors (10), (*c*) lack B cells which bear low to intermediate amounts of immunoglobulin on their surfaces (6, 7), and (*d*) have an increased ratio of IgM to IgD chains on their cell membranes (8). Thus CBA/N B cells have surface and functional characteristics of immature B cells.

These considerations suggest that CBA/N mice may lack a mature subclass of B cells which expresses characteristic surface structures, analogous to surface Ly^+ components which are selectively expressed on distinct T-cell subclasses (12). To test this possibility, we have immunized CBA/N F_1 mice carrying the defective X chromosome with normal parental spleen cells in an attempt to serologically define this B-cell subclass. The resulting antiserum defines a cell surface component expressed selectively on approximately 50% of H cells. This

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surface component is directly involved in triggering of B cells to produce antibody.

Materials and Methods

Animals. C57BL/6 (B6),[1] BALB/c, A/J, DBA/1, and CBA/HT6 (CT6) mice, 8–10 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine. CBA/N mice were originally obtained from the NIH and maintained at Yale by R. K. Gershon. CBA/N ♀ mice were mated with BALB/c ♂ to produce normal F_1 ♀ and defective F_1 ♂ hybrid offspring.

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. Macrophage-depleted spleen cells were prepared by incubating spleen cells with carbonyl-iron on a rotator at 37°C for 1/2 h; the cells were then settled on a magnet and the supernatant cells removed. Purified T lymphocytes were obtained from nylon wool columns, according to the method used by Julius et al. (13); 80–90% of the recovered cells were $Thy1^+$ and 5–10% were Ig^+. Non-T cells were obtained by treating spleen cells twice with anti-Thy1 plus complement (C). Highly purified B cells were also obtained by elution of spleen cells from a Rabbit anti(α)-mouse-Fab coated Sephadex G-200 column (14); these cells were 96–99% Ig^+ by immunofluorescent criteria.

Analysis of Binding Activity of Sera. 2.5×10^6 lymphocytes were incubated with 50 μl of test or control sera at various dilutions for 1/2 h at 4°C in the presence of 0.2% Na azide. After washing three times, the cells were then incubated with a fluorescein-conjugated goat α-mouse mIgG (GαMIgG) kindly provided by Dr. L. A. Herzenberg, Stanford Medical School, Stanford, Calif. Background levels of spleen cell fluorescence, as indicated by the numbers of fluorescent cells treated with control serum, followed by fluorescein isothiocyanate (FITC) GαMIgG, were less than 3%. All sera were ultracentrifuged at 40,000 rpm for 20 min to remove aggregated material.

Analysis of Functional Activity of Test and Control Sera. Mice were immunized intravenously (i.v.) with sheep erythrocytes (SRBC) or horse erythrocytes (HRBC) alone or in combination with varying dilutions of test or control serum; 4–8 days later the mice were killed and the number of spleen plaque-forming cells (PFC) to sRBC or HRBC was determined in a modified Cunningham assay (15). In some experiments, "B" mice, i.e. thymectomized, lethally irradiated mice restored with T-cell-deficient bone marrow (for details see reference 15), were immunized with SRBC and test or control sera.

Results

I. Production of Antiserum. Since the defective X-linked gene is recessive, we immunized defective (CBA/N ♀ × BALB/c ♂) F_1 ♂ and normal (CBA/N × BALB/c) F_1 ♀ mice with BALB/c spleen cells to produce a test and a control serum, respectively. Immunization was achieved by an initial intraperitoneal (i.p.) injection of 10^7 BALB/c spleen cells along with 6×10^9 pertussis organisms followed by weekly injections with progressively increasing numbers of spleen cells in increments of 10^7/wk. After 5 wk, the ♂ and ♀ F_1 animals were bled to produce a test and control serum, respectively. The test antiserum was called αLyb3 for reasons discussed below, and see reference 16.

To determine the Ig class of the functional antibody, the antiserum was treated with 2-mercaptoethanol (0.1 M 2-mercaptoethanol in a ratio 1:1) and alkylated with iodoacetamide (0.1 M ICH_2CONH_2 in a ratio 1:1). Neither binding activity nor functional activity of the test serum were eliminated after this treatment. Absorption of the antiserum with various lymphocyte popula-

[1] *Abbreviations used in this paper:* α, anti; BM, bone marrow; B6, C57BL/6; CT6, CBA/HT6; FITC, fluorescein isothiocyanate; GαMIgG, goat α-mouse IgG; HRBC, horse erythrocytes; PFC, plaque-forming cells; TNP, trinitrophenyl.

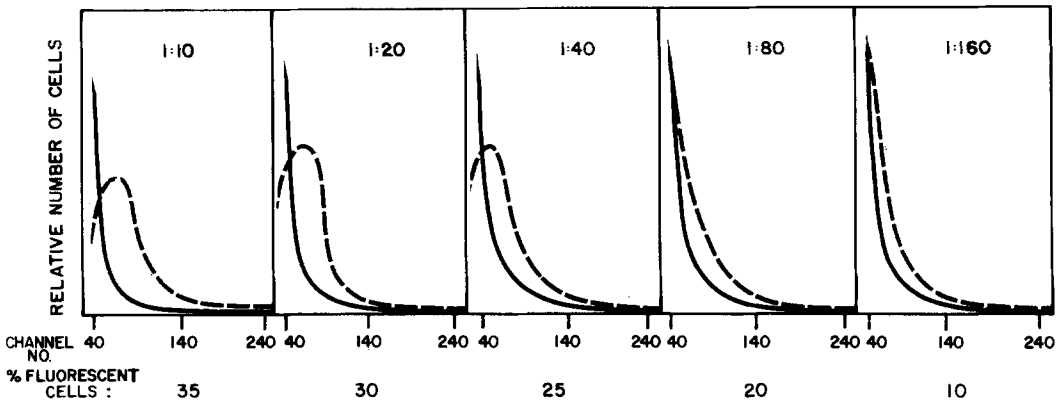

FIG. 1. Titration of αLyb3 on BALB/c spleen cells. Fluorescent profiles and percent specific fluorescent staining of BALB/c spleen cells, incubated with various dilutions of αLyb3 or control serum followed by labeling with FITC-GαMIgG, are shown. (---), αLyb3 serum; (—), control serum.

tions was performed at 4°C for 1 h at a concentration of 50–100 × 10^6 cells/40 μl serum.

II. Analysis of Reactivity of the Test and Control Sera

(a) DOSE RESPONSE OF ANTISERA (FIG. 1). No cytotoxicity was apparent after spleen cells from BALB/c mice were exposed to different concentrations of the test antiserum in the presence of a highly active rabbit C. To determine whether the serum specifically reacted with BALB/c cells but did not fix C, spleen cells were incubated with varying dilutions of test or control serum and then labeled with FITC-GαMIgG (which reacts directly with <2% of B cells). This approach demonstrated that the test serum reacted with approximately 20–30% of the spleen cells in dilutions as high as 1:40, compared with control serum which was completely unreactive. Therefore, in the following experiments, the test and control sera were used at a final dilution of 1:20.

(b) REACTIVITY OF TEST AND CONTROL SERUM AGAINST DIFFERENT LYMPHOID SUBPOPULATIONS (TABLE IA). The test serum but not the control serum reacted with approximately 30% of whole spleen and macrophage-depleted spleen cells, 46% of purified B cells and 0–4% of bone marrow (BM) cells; T cells and thymocytes were completely negative. Further Fluorescence Activated Cell Sorter analysis showed that the fluorescent cells were found mainly within the smaller B lymphocytes (results not shown). Since these data indicate that the (CBA/N × BALB/c) F_1 ♂ αBALB/c spleen serum reacts selectively with a portion of B cells, and does not react with T cells (or nonlymphoid cells, results not shown), we have provisionally termed the antiserum αLyb3 for ease of discussion, although we do not yet know whether this serum defines a single discrete component on this B-cell subclass.

(c) ONTOGENY OF Lyb3 CELLS (TABLE I B). Reactivity of the αLyb3 serum against spleen cells from B6 mice of different ages was determined. No reactivity was noted against cells from newborn mice; at 18 days 19% of the cells were $Lyb3^+$ while maximum reactivity (30%) was noted using spleen cells from mice 110 days of age.

TABLE I
Characterization of Lyb3$^+$ Cells

A. %Lyb3$^+$ cells in different BALB/c lymphocyte populations

Cell population	% Lyb3^{+*}
Unseparated spleen cells	30
Macrophage-depleted spleen cells	30
B cells	46
BM cells	2
Thymus cells	0
T cells	0

B. Ontogeny of Lyb3 surface component

Age of spleen cell donor (days)	% Lyb3^{+*}
2	9
18	19
56	30
110	33

C. Strain distribution of Lyb3 surface antigen

Strain	*H-2* haplotype	% Lyb3^{+*}
CBA/N	*k*	0
CBA/T6	*k*	25
BALB/c	*d*	30
B6	*b*	30
A/J	*a*	25
DBA/1	*q*	25

* Each figure represents the mean of three to six individual mice per group. SE of the mean was less than 10% for all groups.

(d) REACTIVITY OF αLyb3 SERUM AGAINST SPLEEN CELLS OF DIFFERENT STRAINS (TABLE IC). We compared the proportion of Lyb3$^+$ spleen cells obtained from B6, BALB/c, CT6, DBA/1, and A/J and CBA/N mice. B6 and BALB/c spleen populations contained 30% Lyb3$^+$ cells; CT6, DBA/1, and A/J mice about 25%; and CBA/N 0–5%. The reactivity of αLyb3 against spleen cells from CBA/N mice (5%) was reduced to undetectable levels by preincubating the cells with aggregated normal mouse serum to block Fc receptor binding; such pretreatment had no influence on the reactivity of αLyb3 serum with spleen cells from other strains.

III. Ability of αLyb3 Serum to Trigger B Cells to Produce Specific Antibody after In Vivo Immunization with Low Doses of Antigen

(a) αLyb3 ENHANCEMENT OF PFC RESPONSES OF NONMUTANT, Lyb3$^+$ MOUSE STRAINS. To determine whether the reaction of this serum with the Lyb3 component(s) on the surface of B cells altered the immune reactivity of these cells, we examined the PFC response of BALB/c mice injected 4 days previously with 5×10^5 SRBC alone or in combination with graded doses of αLyb3 or control serum. Mice injected with SRBC alone or in combination with graded doses of control serum produced about 500 PFC per spleen (5–15 PFC per 10^6 cells), whereas mice injected with SRBC and optimal concentrations of αLyb3 serum

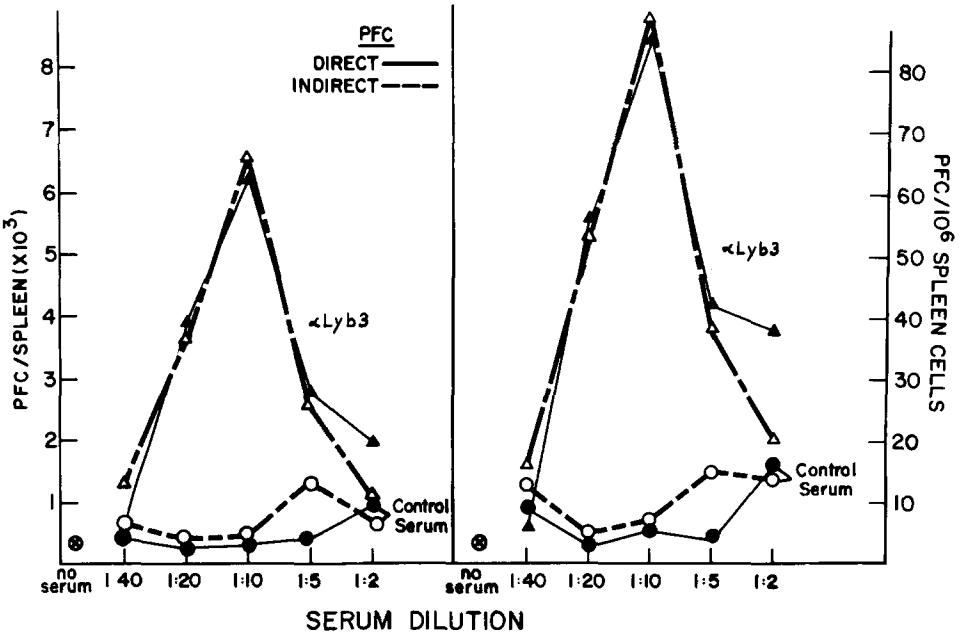

FIG. 2. In vivo dose response of αLyb3 serum. BALB/c mice were injected i.v. with 5×10^5 SRBC plus varying doses of αLyb3 or control serum and their anti-SRBC response measured on day 4. Each point represents the mean PFC response of four to six mice from a representative experiment of a series of four.

produced 15 to 20-fold more anti-SRBC PFC (Fig. 2). Injection of higher doses of antiserum resulted in progressively decreasing responses, probably reflecting either an opsonization effect or the presence of excessive amounts of αLyb3 on the surface of B cells. Similar enhancement of αSRBC responses of CT6 and B6 mice were also observed. In contrast, i.v. injections of varying doses of αLyb3 serum failed to enhance the anti-SRBC PFC response of CBA/N mice, indicating that the enhancement effect was clearly due to a specific reaction of the serum with a component which is absent in the CBA/N mice (Fig. 3). Finally, studies of the time-course of this enhanced PFC response also indicated that the peak response occurred on day 6, and by day 8 the response had declined to normal levels (Fig. 3).

(b) αLyb3 ENHANCEMENT IS ANTIGEN SPECIFIC (TABLE II). Mice injected with αLyb3 serum without antigen did not produce significant PFC responses to either SRBC or HRBC. In addition, the enhanced PFC response of mice immunized with low doses of either SRBC or HRBC plus αLyb3 was specific for the immunogen. These findings indicate that αLyb3 serum acts synergistically with antigen to produce enhanced antigen-specific PFC responses.

(c) ABSORPTION OF ENHANCING ACTIVITY OF αLyb3 SERUM BY DIFFERENT LYMPHOCYTE POPULATIONS (TABLE III). Absorption of αLyb3 with purified B6 B cells eliminated subsequent in vivo enhancing activity, whereas absorption of αLyb3 with thymocytes, purified T cells, or BM cells did not. These findings (*a*) confirm the conclusion of section II b, that αLyb3 serum reacts selectively with B cells and (*b*) indicate in addition that the enhancing effects of the serum reflect a

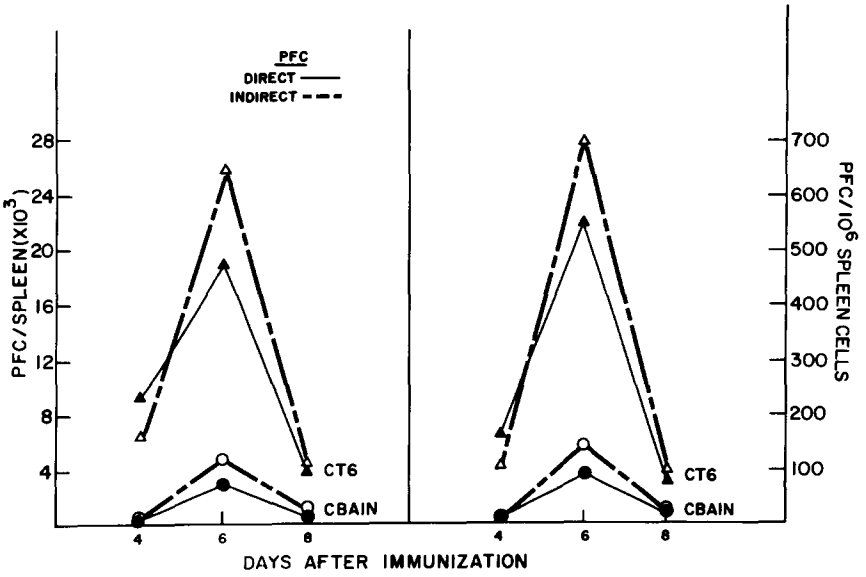

FIG. 3. Time-course and specificity control of the enhanced PFC response. CT6 and CBA/N mice were injected with 5×10^5 SRBC plus 20 μl αLyb3 or control serum and their anti-SRBC PFC response was measured on days 4, 6, and 8. Each point represents the mean PFC response of two to three mice.

TABLE II
Antigen Specificity of αLyb3-Induced B-Cell Activation

Dose of immunizing antigen	αLyb3 sera (20 μl)	SRBC PFC/spleen*		HRBC PFC/spleen*	
		Direct	Indirect	Direct	Indirect
No antigen	+	0	0	0	0
5×10^5 SRBC	+	21,450	19,500	65	0
5×10^5 HRBC	+	390	390	18,070	16,900

* Anti-SRBC and anti-HRBC PFC responses, 4 days after i.v. immunization with the indicated dose of RBC. Mean response of three mice per group.

direct interaction between αLyb3 and a component on the surface of B cells.

Analysis of the Role of Lyb3 in Antigen-Dependent B-Cell Triggering. The data presented in section III suggest that stimulation of the Lyb3 surface component on antigen-reactive B cells allows more efficient triggering of these cells by antigen. This implies that the CBA/N mouse, which lacks Lyb3$^+$ B cells, might be expected to respond poorly to relatively low doses of antigen. We therefore compared the PFC responses of CBA/N mice and CT6 age- and sex-matched mice (the latter are identical to the CBA/N as judged by indefinite mutual acceptance of skin grafts, B. Huber, unpublished observation). CBA/N mice were unable to produce significant PFC responses to relatively low doses of antigen, in contrast to CT6 (Fig. 4 A).

To test the notion that the enhancing effects of αLyb3 serum upon the PFC response of the normal strain (CT6) would be particularly decisive when the

TABLE III

*Removal of αLyb3 Enhancing Activity by Absorption of Sera with B Cells But Not Thymocytes or T Cells**

αLyb3 antiserum	Cell population used for absorption	Direct anti-SRBC PFC (mean + range of three experiments)
μl		
–	–	423 (260–535)
20	–	8,320 (6,240–9,620)
20	Thymus	6,825 (6,110–7,540)
20	T	5,590 (5,200–5,980)
20	BM	5,135 (3,380–6,890)
20	B	875 (260–1,690)

* 40 μl antiserum were absorbed on ice with 10^8 cells for 1 h.

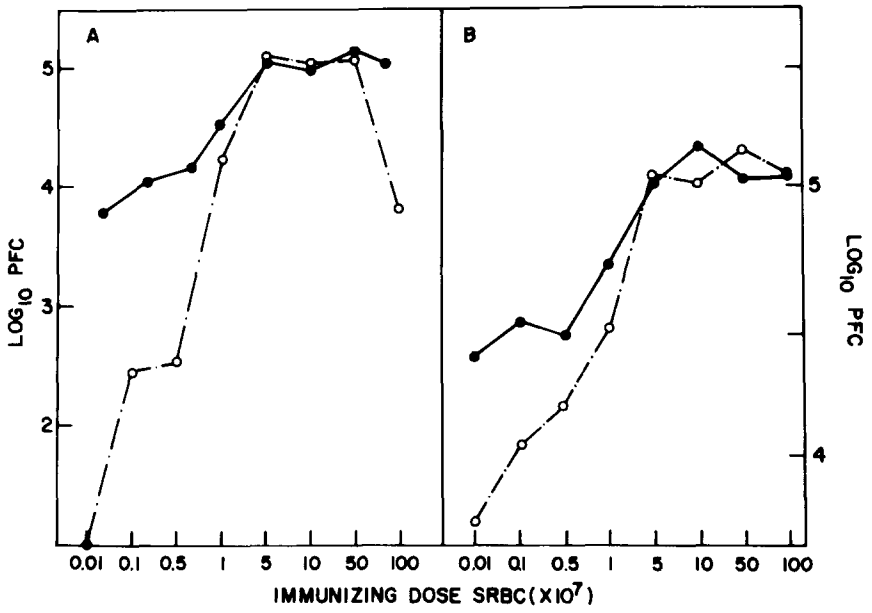

FIG. 4. PFC response of CBA/N and CT6 mice to gradually increasing doses of SRBC. The total (developed) PFC responses of CBA/N (○) and CT6 (●) 7 days after i.v. injection of increasing doses of SRBC. Each point represents the mean response of two to four mice. (B) Enhancing activity of αLyb3 in combination with gradually increasing doses of SRBC in CT6 mice. Total (developed) PFC responses of CT6 mice 6 days after i.v. administration of 20 μl of αLyb3 (●) or control serum (○) in combination with increasing doses of SRBC. Each point represents the mean response of two to four mice.

signal provided by antigen and T cells is relatively low, we examined the enhancing effects of αLyb3 in the presence of (*a*) different concentrations of antigen and (*b*) in the presence of limiting numbers of T cells.

The administration of αLyb3 serum markedly enhanced the reponse of CT6 mice to low doses of SRBC (Fig. 4 B). By contrast, the response of these mice to large doses of SRBC was not significantly enhanced by the administration of αLyb3 serum. These findings are consistent with the idea that increased effi-

TABLE IV
The Effect of αLyb3 on B cells in the Absence of Detectable T-Cell Function

A. Primary αSRBC PFC responses in "B" mice*

Immunization (no. SRBC × 10^6)	Direct PFC/spleen	
	Control serum (range)	αLyb3 (range)
0.5	0	0
3	0	520 (500–560)
10	0	675 (630–720)
100	90 (0–180)	1,680 (960–2,400)

B. Secondary αSRBC responses of SRBC-primed B cells in lethally irradiated syngeneic hosts‡

Recipient challenge SRBC	Indirect PFC/spleen	
	Control serum (range)	αLyb3 (range)
None	0	0 (30)
10^7 SRBC	150 (120–180)	1,560 (1,240–1,980)

* Mean PFC response of "B" mice (see Materials and Methods) 6 days after i.v. injection of 20 μl of control or αLyb3 serum + SRBC; three mice per group.

‡ Mean PFC response of irradiated (850 R) hosts given 12 × 10^6 "B" cells (obtained after αThy1 + C treatment of spleen cells) from donors primed 4 mo previously with 10^7 SRBC plus αLyb3 or control serum. Mean PFC response of four mice per group on day 4 is shown.

ciency of the antibody response in the presence of αLyb3 serum is most apparent when the antigen-induced signal is suboptimal.

The ability of αLyb3 to substitute for T-helper activity in the induction of a primary and secondary response was also tested. B mice (see Materials and Methods) did not produce significant αSRBC after priming with up to 10^8 SRBC plus control serum; the administration of αLyb3 to these mice resulted in the production of a significant PFC response (Table IV A).

Moreover, B cells from antigen-primed donors could adoptively transfer a secondary antibody response to lethally irradiated hosts only if co-transferred with Lyb3 plus antigen (Table IV B). No response was seen if the primed B cells were transferred with antigen alone or with αLyb3 alone.

Discussion

The studies of Scher and his co-workers have indicated a B-cell maturation defect in CBA/N mice (see introduction). Our work confirms and extends these findings. We have shown that serum produced by immunization of defective F_1 mice with nondefective parental cells reacts predominantly with a subclass of B cells from mice of many *H-2* genotypes. Although a possible additional reaction with macrophages has not been ruled out, the serum does not react with thymocytes or T cells (and cannot be absorbed by brain cells, liver cells, or

kidney cells, data not shown). Since this antiserum, provisionally termed αLyb3, does not react with BM cells and reacts only weakly with splenic B cells from young mice, it is likely that it defines a cell surface differentiation component(s) expressed selectively on mature B cells.

In view of Lyb3 expression on the surface of B cells from all mouse strains tested, it is likely that this component is specified by genes expressed during the differentiation of B cells of all mouse strains. The present studies do not indicate the location of the structural gene(s) coding for this component and, since it is likely that the present method of immunization has defined a constant portion of this structure, these data do not bear upon whether Lyb3 is expressed as different alleles in different mouse strains.

The reaction of αLyb3 with B cells from all strains tested (except CBA/N) markedly enhanced the development of antigen-specific PFC in the presence of suboptimal doses of antigen. This enhancement reflected a direct interaction between Lyb3 antiserum and a component on the surface of B cells, since enhancing activity was removed by absorption of the serum with B cells, but not T cells, thymocytes, or BM cells. Enhancement is very likely due to stimulation of $Lyb3^+$ B cells, rather than removal of "suppressive" B cells by opsonization, in view of (*a*) the loss of enhancement with increasing doses of αLyb3 and (*b*) the finding that similar enhancing effects can be produced in vitro (data not shown).

Since, (*a*) Lyb3 antiserum does not induce a "polyclonal" PFC response when administered without antigen, and (*b*) the enhancement of antibody produced is specific for the immunizing antigen, it is likely that it interacts with a B-cell surface structure that is directly involved in antigen-dependent B-cell triggering. Possibly this surface component(s) normally acts to increase the efficiency of specific B-cell triggering by antigen and/or T-cell factors. This possibility is illustrated most directly by the finding that the CBA/N mutant, lacking $Lyb3^+$ B cells, fails to respond to doses of antigen that elicit substantial responses in the nonmutant CBA/HT6 strain.

Lyb3 may represent the "constant portion" of the B-cell antigen receptor or may be distinct from the Ig antigen receptor. Since, (*a*) the administration of αLyb3 serum can partially replace the requirement for T cells in both primary and secondary responses to a thymus-dependent antigen (see Results, section IV), (*b*) CBA/N mice do not generate normal profiles of T-cell-dependent high affinity antibodies after immunization with SRBC (11), and (*c*) the αLyb3 serum described here has no anti-T-cell activity (see Results, sections II b and III c), it is possible that this serum reacts with a previously undefined cell surface component(s) on a subclass of B cells which acts as a receptor for a certain T-cell signal(s). This signal would result in more efficient triggering of a portion of B cells by antigen, as well as the subsequent generation of high affinity antibody.

The idea that a special subclass of mature B cells is required for the PFC response to low concentrations of antigen and for the production of high affinity antibody is most clearly suggested from the work of Goidl and Siskind (17). These authors showed that, in the presence of identical numbers of adult T cells and in the same adult environment, splenic B cells from mice less than 7 days of age do not produce high affinity antibody, whereas B cells from older mice (i.e., greater than 10 days of age) do. Their work suggests that between 1 and 2 wk of age B cells undergo a maturational event which may allow a portion of B cells to

respond differently to T-cell signals. This maturational step may be reflected by the development of a B-cell subclass expressing the Lyb3 component (which also first appears in the 2nd wk of life on B cells of normal animals [see Results, section II c]).

B cells of CBA/N mice also do not respond normally to certain "thymus-independent" antigens (e.g., trinitrophenyl(TNP)-Ficoll) but do respond to others (e.g., TNP-lipopolysaccharide). The present studies do not directly indicate whether cells of the Lyb3$^+$ subclass are responsible for reactivity to the TNP-Ficoll category of thymus-independent antigens. Possibly the defective response of CBA/N mice to this category of antigens may reflect an arrest of normal B-cell maturation, resulting in an absence of a mature Lyb3$^+$ B-cell subclass which can respond without T-cell help to certain thymus-independent antigens.

Taken together, these considerations suggest that αLyb3 serum may prove extremely useful in understanding the molecular basis of B-cell triggering. In addition, some practical implications of αLyb3 activity deserve comment. Injections of relatively small quantities of this serum along with weakly immunogenic doses of antigen result in a substantial (10- to 20-fold) increase in specific antibody formation. It is therefore possible that αLyb3 serum will prove useful as a potent stimulus of specific antibody production against relatively weak antigens such as Ig idiotypes, minor histocompatibility products, tumor-associated determinants, and cell surface differentiation antigens.

Summary

CBA/N mice have an X-linked B-cell maturation defect which is reflected in part in an absence or dysfunction of a subclass of mature B cells. We have immunized the defective ♂ offspring of the mating (CBA/N ♀ × BALB/c ♂) with BALB/c spleen cells. The resulting antiserum (αLyb3) selectively reacts with a component on the surface of a portion of B cells from a panel of *H-2* different mouse strains. Binding of αLyb3 serum to this B-cell subclass results in substantial (10- to 20-fold) enhancement of the antibody response to low doses of SRBC. Both binding and enhancing activity are removed by absorption with B cells from B6 and BALB/c, but not CBA/N mice. Absorption of the serum with bone marrow cells, T cells, or thymocytes from Lyb3$^+$ strains does not remove activity.

Since the enhanced plaque-forming cell (PFC) responses are specific for the immunizing antigen, and since no PFC response is produced by injection of the antiserum alone, this enhancement probably reflects a second signal produced by specific interaction between antibody and the surface Lyb3 component. Moreover, this signal can partially replace the requirement for T cells in the production of antibody to a "thymus-dependent" antigen. These findings (taken in conjunction with the previously described immune defects in CBA/N mice and other studies of B-cell maturation) suggest to us that Lyb3 is a cell surface component expressed selectively on a mature B-cell subclass. This component is important in B-cell triggering by antigen and fails to develop in CBA/N mice, due to a dysfunction of a regulatory gene on the CBA/N X chromosome.

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