B-LYMPHOCYTE HETEROGENEITY: DEVELOPMENT AND CHARACTERIZATION OF AN ALLOANTISERUM WHICH DISTINGUISHES B-LYMPHOCYTE DIFFERENTIATION ALLOANTIGENS*

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There is now considerable evidence to indicate that heterogeneity exists among thymus-independent (B) lymphocytes (1–4). One particularly important model in which to analyze the functions of B-lymphocyte subpopulations has been provided by the CBA/N strain of mice which has an X-linked immune defect (5–8). Extensive studies of this strain and of F_1 hybrids which are hemizygous for the CBA/N X chromosome suggest that the immune defect reflects a maturational arrest, with a consequent deficit in or absence of a late developing B-lymphocyte subpopulation (2, 9–12). We reasoned that the absence of this subpopulation of cells from CBA/N mice should make possible the development of alloantisera specific for a differentiation antigen (or antigens) unique to these late developing B cells. Such sera would have obvious value for the delineation of functions and characteristics of B-lymphocyte subpopulations from normal strains.

In this communication we describe a cytotoxic alloantiserum which recognizes a B-cell differentiation antigen. This serum was produced by immunization of C57BL/6 mice with DBA/2 spleen cells and was made specific by absorption with thymus and spleen cells derived from (CBA/N ♀ × DBA/2 ♂)F_1 male mice, which are hemizygous for the defective X chromosome and which display the immune defect. We show that the serum interacts with a subclass of B cells characterized by: (*a*) a late appearance in ontogeny, (*b*) the possession of the complement receptor, (*c*) a relatively low ratio of surface IgM to surface IgD, and (*d*) low-to-intermediate amounts of total surface Ig.

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Materials and Methods

Mice. DBA/2N, CBA/N, C57BL/6N, and (CBA/N ♀ × DBA/2 ♂)F_1 mice were obtained from the Division of Research Services of the National Institutes of Health, Bethesda, Md. Adult mice of 8–12 wk of age were used in these experiments. They were killed by cervical dislocation.

Immunization Procedures. Adult male C57BL/6 mice were immunized with 50 × 10^6 DBA/2 spleen cells emulsified in complete Freund's adjuvant (final vol 0.2 ml) by footpad injection. They were boosted by intraperitoneal inoculation with 25 × 10^6 DBA/2 spleen cells in media at weekly intervals thereafter, for a total of 4 wk. Sera were collected 4 days after the last boost.

Cytotoxicity Assay. A trypan blue exclusion microcytotoxicity technique described previously was used in this study (13). RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) was used as media. Doubling dilutions of antiserum or normal C57BL/6 serum (50 μl; ultracentrifuged at 100,000 *g* for 40 min before their use) were added to 10^4 spleen cells (50 μl). The cell suspension was incubated at 4°C for 30 min and then washed twice. 100 μl of a 1:8 dilution of rabbit serum, which had been absorbed with mouse liver powder, was added as a source of complement (C). The cells were incubated at 37°C for 30 min, and the percent cytotoxicity was judged by scoring 200 cells after adding 50 μl of a 0.4% solution of trypan blue.

The chromium release assay was performed as described previously (7). ^{51}Cr was supplied as $Na^{51}CrO_4$ (Amersham/Searle Corp., Arlington Heights, Ill.). Assays were done in triplicate and the net specific ^{51}Cr ± SE was determined by the following formula:

$$\%\ \text{net chromium released} = \frac{\text{radioactivity released by antisera and C} - \text{radioactivity released by NMS and C}}{\text{radioactivity released by freeze-thawing} - \text{radioactivity released by NMS and C}} \times 100$$

Absorption Procedures. 2 × 10^9 (CBA/N ♀ × DBA/2 ♂)F_1 male thymocytes were used to absorb 1 ml of heat-inactivated (56°C, 30 min) C57BL/6 anti-DBA/2 antiserum by slowly rotating the resultant cell suspension at 4°C for 1 h. After removal of the thymocytes by centrifugation, F_1 male spleen cells, in increments of 0.5 × 10^9, were used for absorption until the antiserum was no longer cytotoxic for F_1 male spleen cells. This generally required a total of 2 × 10^9 F_1 male spleen cells in addition to the initial absorption with 2 × 10^9 F_1 male thymocytes.

Isolation of B Lymphocytes with the Fluorescence-Activated Cell Sorter. A fluorescein-conjugated $F(ab')_2$ goat anti-mouse Ig (Fl anti-Ig)[1] was used to stain mouse spleen cells as noted previously (2). The Fluorescence-activated cell sorter (FACS; Becton-Dickinson Electronics Laboratory, Mountain View, Calif.) was used to separate Ig-negative (Ig^-) cells (channels 0–80) from Ig-positive (Ig^+) cells (channels 80–1,000) (Fig. 1). In addition, Ig^+ cells with a relatively low density of surface Ig (channels 80–420) were separated from Ig^+ cells with a relatively high density of surface Ig (channels 420–1,000).

Separation of Complement Receptor Lymphocytes. Ig^+ spleen cell suspensions (5 × 10^6/ml), obtained from the FACS, were mixed with an equal vol (0.4 ml) of sheep erythrocytes coated with anti-Forssman antibody and complement (EAC) reagent (14) for 45 min at 37°C. The mixture was then layered on Ficoll-Hypaque (specific grade 1.094) and centrifuged for 30 min at 450 *g*. The cells at the interface were collected, washed, and an aliquot was incubated with EAC to determine the frequency of complement receptor (CR) lymphocytes. Since less than 3% of these cells were CR^+, this population was considered to be enriched in CR^- Ig^+ lymphocytes. The cells in the pellet were treated to remove erythrocytes (2), washed with media, and regarded as a source of CR^+ Ig^+ lymphocytes.

Analysis of Lymphocyte Surface Immunoglobulin Class. Spleen cell surface molecules were labeled with ^{125}I (1.5 mCi ^{125}I/10 × 10^6 cells in 1 ml of phosphate-buffered saline [Amersham/Searle]) using the lactoperoxidase catalyzed procedure for radioiodination as previously described (9). Cells were lysed with Nonidet P-40 (0.5% in phosphate-buffered saline) and immunoglobulin precipitated with a goat anti-mouse immunoglobulin (Meloy Laboratories, Springfield, Va.) and a *Staphylococcus aureus* of the Cowan I strain (15). ^{125}I-labeled Ig was solubilized, reduced, and

[1] *Abbreviations used in this paper:* CR, complement receptor; EAC, sheep erythrocytes coated with anti-Forssman antibody and complement; FACS, fluorescence-activated cell sorter; LAD, lymphocyte-activating determinant; Mls, minor lymphocyte stimulating locus; NMS, normal mouse serum.

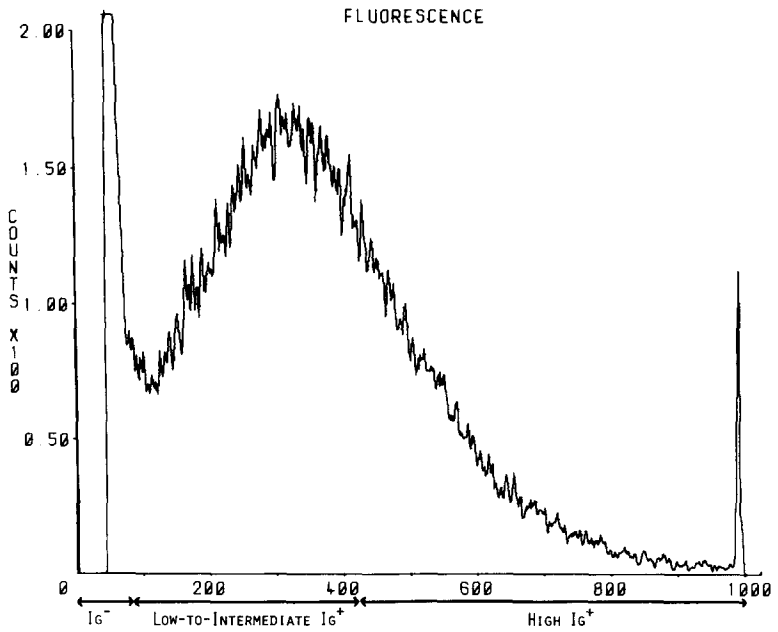

FIG. 1. The fluorescence profile of adult DBA/2 spleen cells stained with a fluorescein-conjugated $F(ab')_2$ goat anti-mouse Ig. Separation of cells into Ig^- (channels 0–80), Ig^+ (channels 80–1,000), low-to-intermediate density surface Ig^+ (channels 80–420), and high density of surface Ig^+ (channels 420–1,000) are noted. The fluorescence intensity of lymphocytes is plotted on the x axis (channels 0–1,000), and the number of cells at each intensity is plotted on the y axis.

analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis as previously described (9). Each electrophoretic profile represents 10×10^6 cpm ^{125}I of solubilized cell-surface molecules.

Results

Cytotoxicity of C57BL/6 Anti-DBA/2 Antiserum. In an effort to isolate alloantibodies directed at differentiation antigens expressed by B lymphocytes absent from (or present in reduced numbers in) mice with the CBA/N immune defect, C57BL/6 anti-DBA/2 sera were extensively absorbed with lymphoid cells from phenotypically abnormal (CBA/N ♀ × DBA/2 ♂)F_1 male mice. 1 ml of antiserum was absorbed with a total of 2×10^9 F_1 male thymocytes, as noted in the Materials and Methods section. This preparation remained cytotoxic for 100% of F_1 male, female, and DBA/2 spleen cells (Table I). Continued absorption with increasing numbers of F_1 male spleen cells reduced the cytotoxicity of the antiserum for F_1 male cells to less than 6% while the serum continued to lyse approximately 26% of F_1 female and DBA/2 spleen cells.

In separate experiments, the titers at which the absorbed antiserum remained cytotoxic for DBA/2 and F_1 female cells were determined both by trypan blue exclusion and by ^{51}Cr-release assays. As shown in Fig. 2, a 1:4 dilution of antiserum resulted in a net cytotoxicity, by trypan blue exclusion, of 25.3% for F_1 female cells and 18.2% for DBA/2 cells; similar results were obtained with a 1:8 dilution. In a series of trypan blue exclusion experiments, conducted with different preparations of the absorbed antiserum, the net

TABLE I
*Cytotoxicity of C57BL/6 Anti-DBA/2 Antisera**

Number of F_1 male cells used for absorption	Source	Net percent spleen cell cytotoxicity‡		
		F_1 ♂	F_1 ♀	DBA/2
–	–	>95	>95	>95
2×10^9	Thymocytes	>95	>95	>95
0.5×10^9	Spleen	>95	>95	>95
1.0×10^9	Spleen	46.2	>95	>95
1.5×10^9	Spleen	12.5	73.9	62.5
2.0×10^9	Spleen	<6	39.5	44.5
3.0×10^9	Spleen	<6	38.2	29.2
4.0×10^9	Spleen	<6	34.5	26.8

* 1 ml of antisera was absorbed with the indicated number of F_1 male cells. Cytotoxicity was measured by the trypan blue dye exclusion technique.

‡ The maximum percent cytotoxicity caused by a dilution (1:2–1:32) of the absorbed C57BL/6 anti-DBA/2 antiserum minus the maximum percent cytotoxicity caused by a dilution (1:2–1:32) of NMS. The percent cytotoxicity of the NMS, complement control ranged from 6.0 to 7.9.

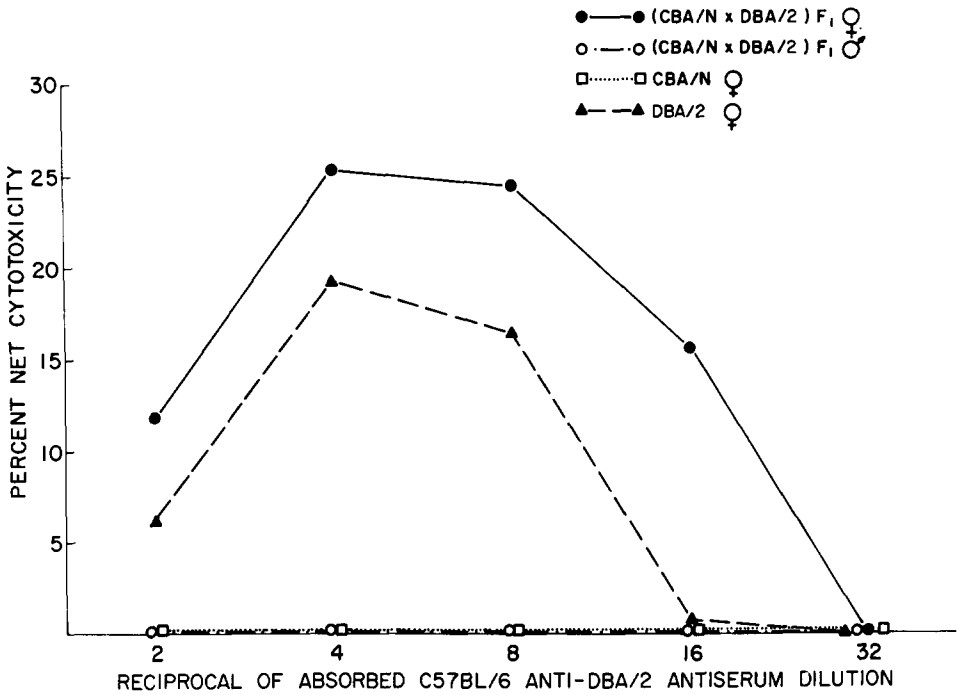

FIG. 2. Cytotoxicity titer of C57BL/6 anti-DBA/2 absorbed serum for spleen cells from CBA/N female, DBA/2 female, and (CBA/N ♀ × DBA/2 ♂)F_1 male and F_1 female mice as measured by the trypan blue dye exclusion technique.

maximum percent cytotoxicity for F_1 female and DBA/2 spleen cells ranged from 23.7 to 31.6% and from 18.2 to 36.0%, respectively. Using a ^{51}Cr-release assay, comparable results were obtained (Fig. 3).

Analysis of Cell Type Lysed by Absorbed Antisera. The absorbed serum appeared to identify an alloantigen present on spleen cells of phenotypically

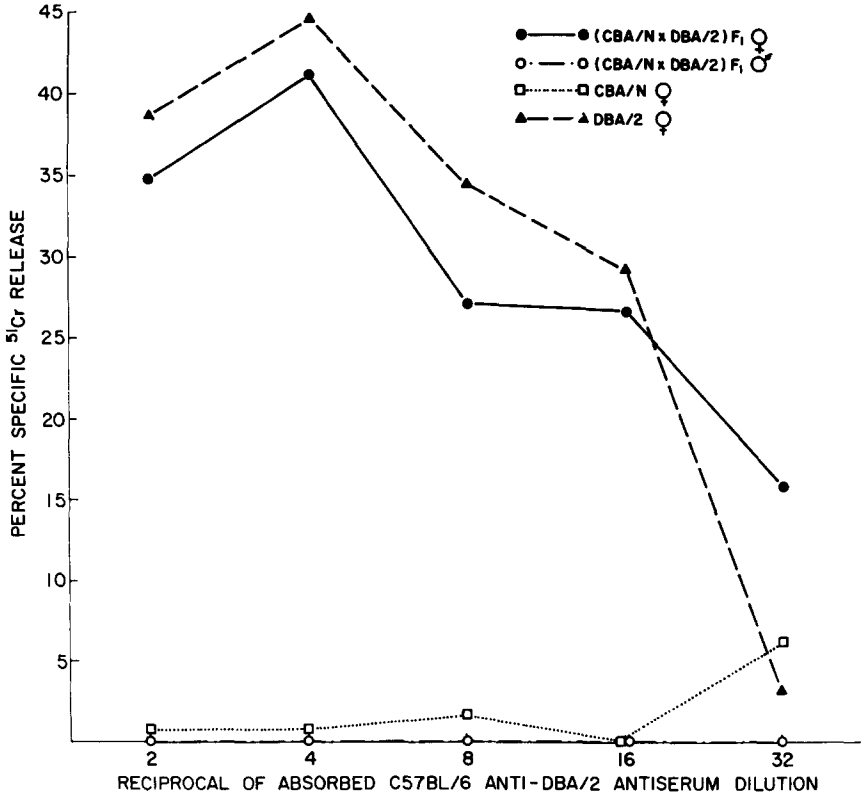

FIG. 3. Cytotoxicity titer of C57BL/6 anti-DBA/2 absorbed serum for spleen cells from CBA/N female, DBA/2 female, and (CBA/N ♀ × DBA/2 ♂)F_1 male and F_1 female mice as measured by the ^{51}Cr-release assay.

normal F_1 female mice and absent (or markedly diminished) on spleen cells of immunologically defective F_1 male mice. Since the F_1 male mice appear to lack a mature or late developing population of B lymphocytes, we attempted to determine whether this cell population in immunologically normal mice bore the alloantigen recognized by the absorbed antiserum. Initially, we incubated spleen cells of adult F_1 male, F_1 female, and C57BL/6 mice with Fl anti-Ig and separated fluorescence positive cells (Ig^+) from fluorescence negative cells (Ig^-) with the FACS (Fig. 1). When adult F_1 male spleen cells were separated in this manner, 6.0% or fewer of Ig^+ or Ig^- cells were lysed by the absorbed antiserum and C (Table II). Similarly, only 7.4% of Ig^- cells from F_1 female animals were lysed. By contrast, the percentage of cells lysed by the absorbed antiserum was increased considerably when Ig^+ F_1 female spleen cells were compared to unsorted F_1 female spleen cells (59.8 vs. 31.6%) (Table II). Further separation of adult Ig^+ F_1 female spleen cells into CR^+ and CR^- populations resulted in an Ig^+ CR^+ population that was very susceptible to lysis (75.8%) and an Ig^+ CR^- population that was not (5.3%). As anticipated from the fact that the serum was prepared in C57BL/6 mice, it showed no cytotoxicity for C57BL/6 spleen cells.

Scher et al. previously demonstrated that Ig^+ cells which bear a low-to-

TABLE II
*Cytotoxicity of Absorbed Antisera for FACS-Separated Spleen Cells**

Strain	Net percent cytotoxicity‡				
	Unsorted	Ig^-	Ig^+	Ig^+§	
				CR^-	CR^+
C57BL/6	0.0	0.0	0.0	–	–
(CBA/N ♀ × DBA/N ♂)F_1 ♂	3.5	6.0	4.5	8.8	7.4
(CBA/N ♀ × DBA/N ♂)F_1 ♀	31.6	7.4	59.8	5.3	75.8

* Cytotoxicity was measured by the trypan blue dye exclusion technique.
‡ The maximum percent cytotoxicity caused by a dilution (1:2–1:32) of the absorbed C57BL/6 anti-DBA/2 antiserum minus the maximum percent cytotoxicity caused by a dilution (1:2–1:32) of NMS. Cells were labeled with Fl anti-Ig and passed through the FACS before treatment with the absorbed antisera. Ig^+ and Ig^- cells were separated using the FACS.
§ Ig^+ Cr^- and Ig^+ Cr^+ cells were isolated as described in the Materials and Methods section.

intermediate density of surface Ig are relatively deficient in adult CBA/N and F_1 male mice (10). To determine if the B-lymphocyte antigen detected by the absorbed antiserum was associated with this subpopulation of B lymphocytes, F_1 female spleen cells were separated into those bearing low-to-intermediate from those bearing high densities of surface Ig by sorting with the FACS (Fig. 1). Among lymphocytes bearing a low-to-intermediate density of surface Ig, 81.2% were lysed by the absorbed antiserum, while cells bearing a high density of surface Ig were only lysed to an extent of 21.2%. Thus, the majority of cells with a low-to-intermediate density of surface Ig possess the alloantigen.

Ratio of Surface IgM to IgD in F_1 Female Cells After Killing with Absorbed Antisera. Splenic B lymphocytes of neonatal mice and those of adult CBA/N and adult (CBA/N ♀ × DBA/2 ♂)F_1 male mice have been shown to have a high ratio of surface IgM to the putative mouse homolog of human IgD (hereafter referred to as IgD) when compared to splenic B lymphocytes of normal adult mice (9, 16). To examine the ratio of surface IgM to IgD on those B lymphocytes of F_1 females which were not susceptible to lysis by the absorbed antiserum, we treated F_1 female spleen cells with the absorbed antiserum and C. Live cells were recovered by Ficoll-Hypaque centrifugation, labeled with ^{125}I using the lactoperoxidase technique, solubilized with 0.5% NP-40 in phosphate-buffered saline, and the surface Ig precipitated as noted in the Materials and Methods section. Fig. 4 illustrates the sodium dodecyl sulfate-polyacrylamide gel electropherogram of ^{125}I-labeled membrane immunoglobulin obtained from F_1 female spleen cells treated with normal mouse serum (NMS) and C (Fig. 4 B) or with absorbed antiserum and C (Fig. 4 A). The ratio of surface IgM to IgD in the NMS- and C-treated cells is approximately one, as has been demonstrated previously when the spleens of adult mice are examined (9, 16). By contrast, the cells that remained after lysis with the absorbed antiserum and C had a very high ratio of surface IgM to IgD.

Ontogeny of Alloantigen-Bearing B Lymphocytes in DBA/2 Mice. The results presented thus far indicate that the alloantigen(s) recognized in the absorbed antiserum is present on a subpopulation of B lymphocytes deficient or absent in immune defective CBA/N and F_1 male mice. Previous studies have

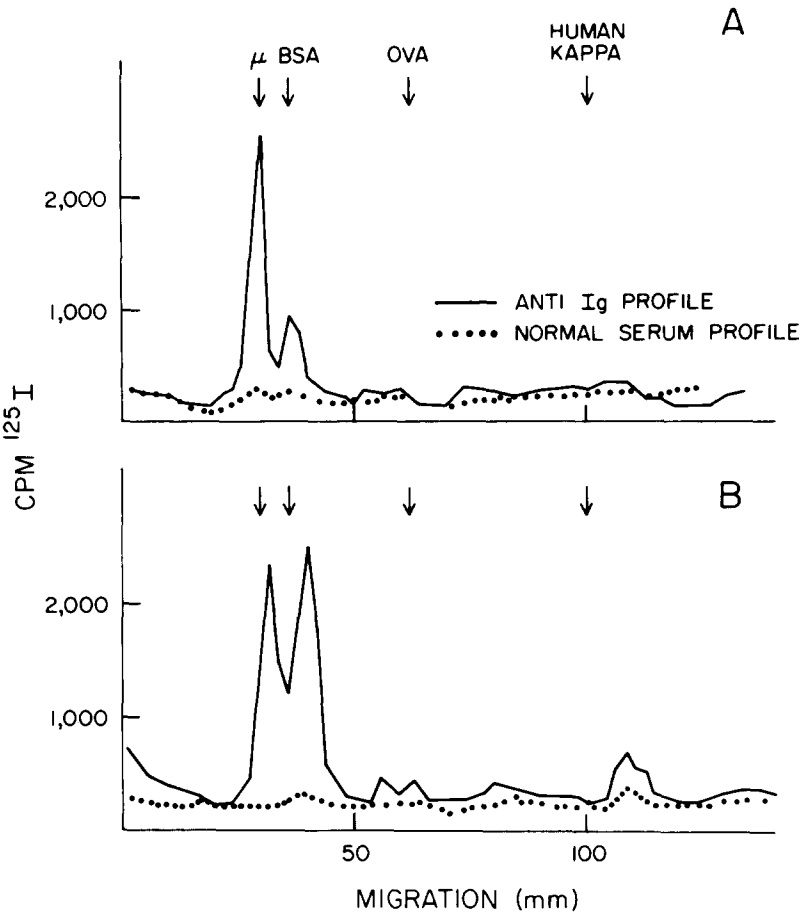

FIG. 4. SDS-polyacrylamide gel electropherogram of ^{125}I-labeled membrane immunoglobulin specifically precipitated from (CBA/N ♀ × DBA/2 ♂)F_1 female spleen cells pretreated with (A) absorbed C57BL/6 anti-DBA/2 serum + C. (B) NMS + C.

indicated that this subpopulatpon develops relatively late in the course of maturation in normal mice. Consequently, we examined the ontogeny of alloantigen-bearing cells in DBA/2N mice, an immunologically normal strain. Among spleen cells of 14-day-old DBA/2 mice, only 5.7% were lysed by the absorbed antiserum, in contrast to the lysis of 34.5% of adult (>8 wk) spleen cells by this serum (Table III). Furthermore, with FACS-purified cells only 20.6% of Ig^+ spleen cells derived from 14-day-old DBA/2 mice were lysed, whereas 66.7% of adult Ig^+ cells were lysed. This indicates that the increase during maturation of cells capable of being lysed by the absorbed serum represents expansion of a subpopulation of Ig^+ lymphocytes bearing the alloantigen and provides further evidence that the differentiation antigen is expressed on a mature subpopulation of B lymphocytes.

Discussion

Previous work in our laboratory has demonstrated that CBA/N mice and F_1 male mice derived from crosses of CBA/N female mice with normal male mice

TABLE III
*Cytotoxicity of Absorbed Antisera of FACS-Separated DBA/2 Spleen Cells**

Age of DBA/2 donor	Frequency of Ig^+ cells	Net percent cytotoxicity‡		
		Unsorted	Ig^-	Ig^+
14 days	16.1	5.7	2.7	20.6
21 days	54.1	18.3	2.8	31.4
35 days	57.1	31.4	9.6	73.9
Adult	59.2	34.5	7.2	66.7

* Cytotoxicity was measured by the trypan blue dye exclusion technique.

‡ The maximum percent cytotoxicity caused by a dilution (1:2–1:32) of the absorbed C57BL/6 anti-DBA/2 antiserum minus the maximum percent cytotoxicity caused by a dilution (1:2–1:32) of NMS. Cells were labeled with Fl anti-Ig and passed through the FACS before treatment with the absorbed antisera. Ig^+ Ig^- cells were separated using the FACS.

have a deficiency in a subpopulation of B lymphocytes. This subpopulation of cells develops late in neonatal life and can be distinguished from other B lymphocytes by the presence of the following surface membrane characteristics: (*a*) the complement receptor (14), (*b*) the minor lymphocyte stimulating (Mls) coded lymphocyte-activating determinants (LADs) (12–17), (*c*) a low density of IgM (2), (*d*) a low-to-intermediate density of total Ig (2), and (*e*) a relatively low ratio of surface IgM to IgD (9). The apparent absence of this B-lymphocyte subpopulation in CBA/N mice presented an opportunity to develop an antiserum directed against differentiation antigens unique to these cells (2, 9–12). Initially both CBA/N anti-CBA/J, and (CBA/N ♀ × DBA/2 ♂)F_1 male anti-F_1 female sera were prepared. Unfortunately, both these antisera were not cytotoxic. Therefore, we adopted the strategy of producing a polyvalent antiserum and rendering it specific for the B-lymphocyte alloantigen by extensive absorption with thymus and spleen cells from immune defective mice. Thus, C57BL/6 mice were immunized with DBA/2 spleen cells and the resulting serum absorbed with thymus and spleen cells from (CBA/N ♀ × DBA/2 ♂)F_1 male mice. This absorbed serum lysed fewer than 6% F_1 male spleen cells, but was cytotoxic for 25–35% of DBA/2 F_1 female spleen cells. The small percent of F_1 male spleen cells lysed by the absorbed antisera may reflect the existence of a small number of alloantigen-bearing B lymphocytes in the immune defective mice. More likely, it reflects the presence of antisera against DBA/2 histocompatibility antigens, which were not completely removed during the absorption procedure.

Analysis of the lysis of normal lymphoid cells by the absorbed antiserum showed that approximately 50–70% of Ig^+ adult F_1 female or DBA/2 spleen cells were susceptible to lysis, but that only 20% of Ig^+ spleen cells of immature mice (14-day-old DBA/2) were lysed. Therefore, the determinants detected by the absorbed antiserum were found on a late developing B-lymphocyte subpopulation. This is what was predicted before the testing of the antiserum, knowing the cell type which was absent from the populations of immune defective cells used for absorption. Furthermore, the Ig^+ cells which are killed by the absorbed antiserum have a relatively low density of surface Ig, a low ratio of surface IgM to IgD, and a large percentage of them bear the CR. These are characteristics that distinguish mature (or late developing) B-lymphocyte subpopulations from immature B cells.

These findings indicate that there is an allotypic differentiation antigen (or antigens) on a subpopulation of B lymphocytes which can be identified by making use of a mouse strain which has a defect in the development of this population. Strain distribution analyses are underway to further characterize this new allotypic antigen. Preliminary experiments using anti-Ly 4.1 sera indicate that such sera lyse all of the Ig^+ cells derived from both (CBA/N ♀ × DBA/2 ♂)F_1 male and female mice (Ahmed, A., I. Scher, and W. E. Paul. Unpublished observations). Thus, the alloantigen reported in this communication must be distinct from Ly 4.1. Tentatively, we propose to designate this B-cell differentiation antigen as Lyb 5.1.

Summary

CBA/N mice and F_1 male mice, which are hemizygous for the CBA/N X chromosome, have an immune defect which is associated with the absence (deficiency) of a subpopulation of mature or late developing B lymphocytes. This characteristic was utilized to develop an antiserum that was specific for this subclass of B cells. C57BL/6 mice were immunized with DBA/2 spleen cells, and the resulting antisera was absorbed with lymphoid cells derived from immunologically abnormal (CBA/N ♀ × DBA/2 ♂)F_1 male mice. The absorbed antisera was cytotoxic for a subpopulation of lymphocytes that was present in the spleens of adult DBA/2 and (CBA/N ♀ × DBA/2 ♂)F_1 female mice.

The cells killed by the absorbed antisera were Ig-bearing, complement receptor-bearing B lymphocytes, which had a low-to-intermediate density of total surface Ig. Moreover, the cells remaining after treatment of adult (CBA/N ♀ × DBA/2 ♂)F_1 female spleen cells with the absorbed antisera and C had a high ratio of surface IgM to IgD. The development of this cytotoxic alloantisera, which is specific for a late developing (mature) subpopulation of B lymphocytes, will allow the functional characterization of subclasses of B lymphocytes.

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