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HETEROGENEITY OF Ia EXPRESSION ON NORMAL B CELLS, NEONATAL B CELLS, AND ON CELLS FROM B CELL-DEFECTIVE CBA/N MICE[1]

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The fluorescence-activated cell sorter (FACS) was used to examine the expression of Ia antigens on B cells obtained from adult spleen, lymph node, and Peyer's patch, and on splenic B cells of neonatal mice and mice with the CBA/N immune defect. Considerable heterogeneity of surface Ia antigen expression was seen among these different lymphocyte populations. Neonatal splenic B lymphocytes bore less surface Ia antigen than did adult splenic B lymphocytes; adult patterns were acquired after 3 weeks of age. Cells obtained from the different lymphoid tissues also showed considerable heterogeneity with regard to surface Ia antigen expression. Furthermore, the FACS profile of Ia^+ B lymphocytes from mice with the CBA/N immune defect was significantly different from that of B lymphocytes of normal adult or neonatal mice. These data suggest that surface Ia antigen distribution can be used as a marker to delineate B cell subpopulations.

Finally, in order to determine whether all immunoglobulin-positive (Ig^+) spleen lymphocytes in neonatal and adult mice also expressed surface Ia antigen, spleen cells lacking Ia antigens were purified on the FACS. On subsequent analysis by FACS, fewer than 5% of such cells were Ig^+, and the density of Ig on these cells was substantially less than that of the average population. These Ia^- cell populations also did not synthesize detectable amounts of immunoglobulin as assessed by SDS-PAGE techniques.

Although the great majority of murine B lymphocytes are known to express I-region-associated (Ia)[3] antigens on their surface (1, 2), there is still some uncertainty as to the existence of or the number of immunoglobulin $(Ig)^+$ cells that fail to express Ia antigens. Analysis of the immune responsiveness of adult spleen cells after treatment with anti-Ia antibody and complement (C) demonstrates pronounced diminution in both thymus-dependent (TD) (2–6) and T-independent (TI) antibody responses (4–7) in *in vitro* cell culture systems. Furthermore, similar treatment of neonatal spleen cell populations leads to marked reductions in responsiveness of these cells to the TI antigen trinitrophenylated *Brucella abortus* (TNP-BA) (7). These data suggest therefore that the majority of, if not all, antigen-sensitive B precursors of antibody-secreting cells express Ia antigens (Ia^+). Others, however, have reported the existence of Ig^+ Ia^- cells in neonatal spleen (8) and in adult bone marrow (9) as well as in adult spleen cell populations (10). It is possible that a heterogeneity in the amount of Ia antigen expressed on various B cell subpopulations might account for these discrepant findings. Indeed, heterogeneity in Ia expression between precursors responsive to TNP-Ficoll and TNP-BA has been shown (7).

In order to determine whether surface Ig $(sIg)^+$ sIa^- cells existed and to quantitate the density of surface Ia (sIa) antigen on individual B cells within populations of lymphoid cells, we utilized the technique of flow microfluorometry. Cells were incubated with fluorescein-conjugated anti-Ig to label Ig^+ cells or anti-Ia antiserum to label Ia^+ cells, and the frequency of Ig^+ and Ia^+ cells, as well as the density distribution of these antigens on such cells, was determined. In addition, Ia^- cells were derived from adult or neonatal spleens, and the frequency of Ig^+ cells in these populations determined. These Ia^- cells were also analyzed for surface membrane and newly synthesized Ig by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A small number of sIg^+ cells were found among the population of Ia^- cells isolated on the fluorescence-activated cell sorter (FACS); however, by FACS analysis these cells had extremely low densities of membrane Ig, which was not detected by using immunoprecipitation techniques.

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[3] Abbreviations used in this paper: FACS, fluorescence-activated cell sorter; Fl-RαMγ, fluorescein-conjugated rabbit anti-mouse γ-chain; Ia, I-region-associated; MHC, major histocompatibility complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sIg, surface Ig; T, thumus; TD, thymus-dependent; TI, thymus-independent; TNP-BA, trintrophenylated *Brucella abortus*; EDTA, ethylenediaminetetraacetic acid; Fl-RαMIg, fluorescein-conjugated rabbit anti-mouse Ig; sIa, surface Ia; NP-40, Nonidet P-40.

Considerable heterogeneity was observed in the expression of Ia antigen on B cells derived from immunologically normal adult mouse spleen, Peyer's patch, and lymph node. In addition, the density distribution of Ia antigens on B cells from immune defective CBA/N mice and from neonatal mice was considerably different from that of adult normal splenic B cells. These data support the view that very few if any Ig^+ Ia^- cells exist in the spleens of either adult or neonatal mice and demonstrate that the amount of Ia antigens on B cells is quite heterogeneous. It appears that the density of Ia may serve as a marker that delineates subpopulations of these cells.

MATERIALS AND METHODS

Animals. CBA/N and (CBA/N × DBA/2N)F_1 mice were obtained from the Small Animal Section, NIH; CBA/J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine; all other mice used were obtained from Dr. D. H. Sachs, National Cancer Institute, NIH.

Antisera. A.TH anti-A.TL was prepared as previously described (1). Preparation of fluorescein-conjugated $F(ab')_2$ fragment of rabbit anti-mouse γ chain (Fl-RαMγ) and of the fluorescein-conjugated $F(ab')_2$ of rabbit anti-mouse Ig (Fl-RαMIg) are described elsewhere (11). For absorption experiments, 0.1 ml of undiluted antiserum was absorbed with one spleen equivalent from the appropriate strain for 30 min at 4°C.

Fluorescence staining. Mouse spleen cell suspensions were prepared, treated with ammonium chloride lysing buffer, and washed twice in media consisting of RPMI-1640 supplemented with 10% fetal bovine serum and 0.2% NaN_3. For analysis of fluorescence profiles and frequency of Ig^+ or Ia^+ cells, 2×10^6 cells were pelleted and resuspended in 100 μl of medium containing appropriate dilutions of A.TH anti-A.TL serum. To determine the amount of A.TH anti-A.TL to be used in these studies, varying amounts (0.001 μl to 5 μl) of anti-Ia antiserum in a total volume of 100 μl were incubated with spleen cells for 30 min on ice. The cells were then washed two times in medium, resuspended in 100 μl of medium containing Fl-RαMγ, and incubated on ice for an additional 30 min. These cells were washed twice more and then analyzed on the FACS. The lowest amount of anti-Ia that gave an Ia fluorescence profile and frequency of Ia^+ cells similar to that obtained with 5 μl of antibody was the amount used in these studies.

Analysis and sorting of stained mouse cells. A FACS II (Becton-Dickinson, Mountain View, Calif.) was used as previously described (12). In general, the fluorescence intensity of 40,000 stained mouse spleen cells was determined for each sample. Cells were assigned to fluorescence intensity channels from 1 to 1000 on the basis of linearly increasing fluorescence intensity, and the number of cells in each fluorescence channel was determined. The median fluorescence intensity was determined in order to provide a measure of the average fluorescence of the cell population, and connotes that channel above and below which lay 50% of fluorescent positive cells.

In all the experiments to be reported, the number of Ia^+ cells represents the total number of cells that were positively stained with A.TH anti-A.TL serum followed by Fl-RαMγ minus the number that exhibited fluorescence when incubated with normal mouse sera and followed by Fl-RαMγ. The number of Ig^+ cells reflects the total number of cells that were positively stained by Fl-RαMIg minus the number that were positively stained by a fluorescein-conjugated rabbit anti-keyhole limpet hemocyanin antibody.

Radiolabeling and isolation of Ig from Ia^+ and Ia^- cells. CBA/J spleen cells were stained with A.TH anti-A.TL and Fl-RαMγ and separated into Ia^+ and Ia^- fractions with the FACS. Stained but unsorted cells, Ia^+ cells, and Ia^- cells were each washed three times in Hanks' balanced salt solution and were radiolabeled in one of two ways. Cells were cultured for 5 hr at 37°C at a density of 2.5×10^6/ml in an RPMI-1640 medium deficient in methionine and supplemented with 10% fetal calf serum, 20 mM glutamine, and 150 μCi/ml ^{35}S-methionine. Alternatively, surface membrane proteins were radiolabeled with ^{125}I by the lactoperoxidase method (13). After labeling, the cells were washed, solubilized in 0.5% Nonidet P-40 (NP-40) detergent in saline-EDTA[3]-Tris buffer, incubated for 30 min, and centrifuged for 30 min at 4000 × G. The lysates were then treated sequentially with 150 μl 10% (v/v) formalin-fixed protein A-bearing *Staphylococcus aureus* bacteria to precipitate residual complexes of Ia antigen and antibody, with 25 μg of polyspecific rabbit anti-mouse Ig antibodies plus 150 μl *S. aureus* to precipitate mouse Ig, and with 20 μl of antiserum against the major histocompatibility complex (MHC) antigens of the $H\text{-}2^k$ (C3H.Sw anti-C3H) plus 200 μl *S. aureus* to precipitate MHC molecules as an internal quantitative standard for Ig recovery. Eluted antigens were reduced in 5% mercaptoethanol and analyzed by electrophoresis in a discontinuous sodium dodecyl sulfate buffer system on 10% polyacrylamide gel slabs (SDS-PAGE) (14), after which the gels were processed for fluorography on Kodak XR-5 film (15). Quantitative radioactivity *vs* mobility plots were obtained by scanning on a Zenith SL-504 soft-laser scanning densitometer.

RESULTS

Analysis of the frequency and fluorescence profile of Ia^+ mouse spleen cells. When CBA/J spleen cells were stained with A.TH anti-A.TL serum and Fl-RαMγ, populations of Ia^+ and Ia^- cells were easily distinguishable. The positively staining cell population was defined as that which had a fluorescence intensity greater than that of cells stained under identical conditions with a normal mouse serum and Fl-RαMγ or irrelevant antiserum. For quantitative comparisons, the median fluorescent intensity, i.e., the fluorescence channel number above and below which lay 50% of a discrete population of stained cells, was used. Using this criterion, the frequency of sIa^+ CBA/J spleen cells ranged between 46 and 55%, and the median fluorescence of the Ia^+ cells was approximately 10- to 20-fold greater than the median fluorescence of sIa^- cells (Fig. 1). In most instances, the frequency of sIa^+ cells was equivalent to the frequency of sIg^+ cells. To determine the extent to which sIg^- cells were being stained by anti-Ia under the conditions studied, spleen cells were fractionated into populations enriched for B cells or depleted of B cells, with the use of anti-Ig-coated Petri dishes (16). The B cell-depleted population contained approximately 5% Ig^+ cells and a similar percentage of Ia^+ cells. The B cell-enriched population was 80% Ig^+ and 82% Ia^+.

In order to establish the specificity of the A.TH anti-A.TL serum, it was absorbed with B10.BR ($H\text{-}2^k$) or with B10.S ($H\text{-}2^s$) spleen cells. Absorption with B10.S spleen cells, which express no Ia determinants recognized in A.TH anti-A.TL antiserum, did not reduce the ability of this antiserum to stain sIa^+ CBA/J cells, whereas absorption with B10.BR, spleen cells which have the same Ia antigens as CBA spleen cells, completely abolished staining of CBA spleen cells (Table I). Furthermore, unabsorbed A.TH anti-A.TL serum had no significant activity when tested on SJL ($H\text{-}2^s$) spleen cells (Fig. 1). The frequency of Ig^+ cells was not affected by the absorption procedures.

Frequency of Ia^+ cells and heterogeneity of Ia expression

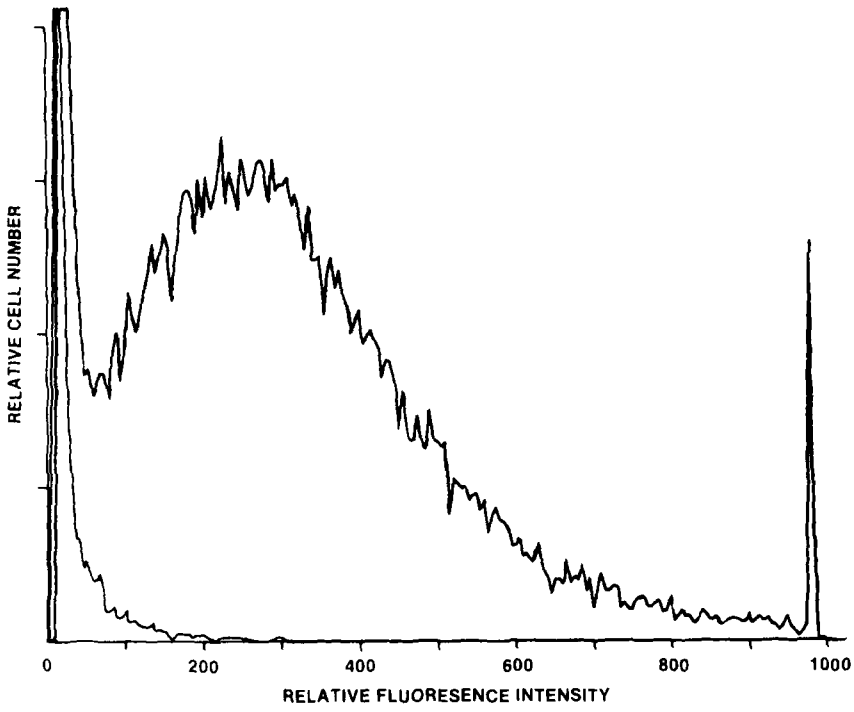

Figure 1. Spleen cells were obtained from CBA/J (——) or SJL (——) mice, incubated with A.TH anti-A.TL antiserum followed by Fl-RαMγ, and analyzed on the FACS.

on cells from different lymphoid tissues. In order to determine whether the distribution of Ia on the surface of B cells from various lymphoid organs parallels the expression of Ig on these cells, thymus, lymph node, Peyer's patch, and spleen cells of CBA/J mice were studied. Considerable heterogeneity existed in the FACS profiles of such cells (Table II; Fig. 2). From an examination of the median fluorescence intensity of sIa^+ cells, it was possible to distinguish between spleen cells that bore intermediate amounts of Ia antigens and cells from lymph node and Peyer's patch that had relatively greater amounts of Ia antigens. When spleen cells and Peyer's patch cells were separated by the FACS into brightly and dimly staining Ia^+ cells and subsequently evaluated for size by phase microscopy, no correlation between cell size and fluorescence intensity could be found. Thus, these differences in amounts of Ia antigens are not attributable to variations in the size of these cells. Furthermore, there was no direct relationship between the amount of surface Ig and Ia antigen on B cells from different organs. Peyer's patch cells, for example, stained more brightly with anti-Ia than did spleen cells, whereas they stained less brightly with Fl-RαMIg than did spleen cells. In all experiments reported here there was a very close correlation between the

TABLE I

FACS analysis of the frequency of surface Ia^+ spleen cells derived from H-2^k or H-2^s mice positively stained with A.TH anti-A.TL antiserum and Fl-RαMγ[a]

Donor of Spleen Cells	I-region Haplotype	Strain of Origin and I Region Haplotype of Spleen Cells Used for Absorption of A.TH anti-A.TL	I-region Haplotype	% Ia^+	% Ig^+
CBA/J	I^k			46	48
		B10.BR (I^k)	I^k	2	47
		B10.S (I^s)	I^s	44	44
SJL	I^s			0	52

[a] A.TH anti-A.TL were absorbed with B10.BR or B10.S by incubating 0.05 ml of antiserum with 10^8 spleen cells on ice for 30 min. Such absorption with the appropriate stain removes cytotoxic activity of this antiserum when tested in the presence of C.

TABLE II

Analysis of the frequency and fluorescence profile of Ig^+ and Ia^+ cells from various lymphoid tissues[a]

	Spleen	Peyer's Patch	Thymus	Peripheral Lymph Node
% Ig^+ cells	48	46	<1	13
Median channel no. of positive cells	206	166		296
% Ia^+ cells	42	41	<1	12
Median channel no. of positive cells	257	483		467

[a] CBA/J lymphocytes were obtained from spleen, Peyer's patch, thymus, or peripheral lymph node, incubated with A.TH anti-A.TL followed by Fl-RαMγ, and analyzed on the FACS.

frequencies of Ig^+ cells and the number of Ia^+ cells; in most experiments, the number of Ig^+ cells was 2 to 6% greater than the number of Ia^+ cells.

Analysis of the frequency and fluorescence profile of Ia^+ spleen cells taken from normal mice at various times after birth. Because a substantial fraction of neonatal Ig^+ cells have been reported to possess little or no sIa (8), we examined the relative expression of sIg and sIa in CBA/J spleen cells from mice at 2 days and 2, 3, and 8 weeks. The anti-Ia fluorescence staining profile of neonatal and 2- to 3-week-old spleen cells was heterogeneous displaying no distinct peak of positive cells (Fig. 3). As the animal ages, the anti-Ia spleen cell profile becomes more homogeneous and the cells fluorescence more brightly. A very close correlation between the frequency of Ia^+ cells and of Ig^+ cells in the spleen was maintained from the time of birth onwards. Indeed, when sIa^- cells from 2-day-old mice were prepared by sorting on the FACS, fewer than 5% were sIg^+ by staining with Fl-RαMIg and the density of Ig on these cells was extraordinarily low (Table III). Thus, even among a population of relatively immature B lymphocytes, we were able to identify few if any sIa^- sIg^+ cells.

Analysis of the frequency and fluorescence profile of Ia^+ cells from mice with the CBA/N immune defect. B lymphocytes

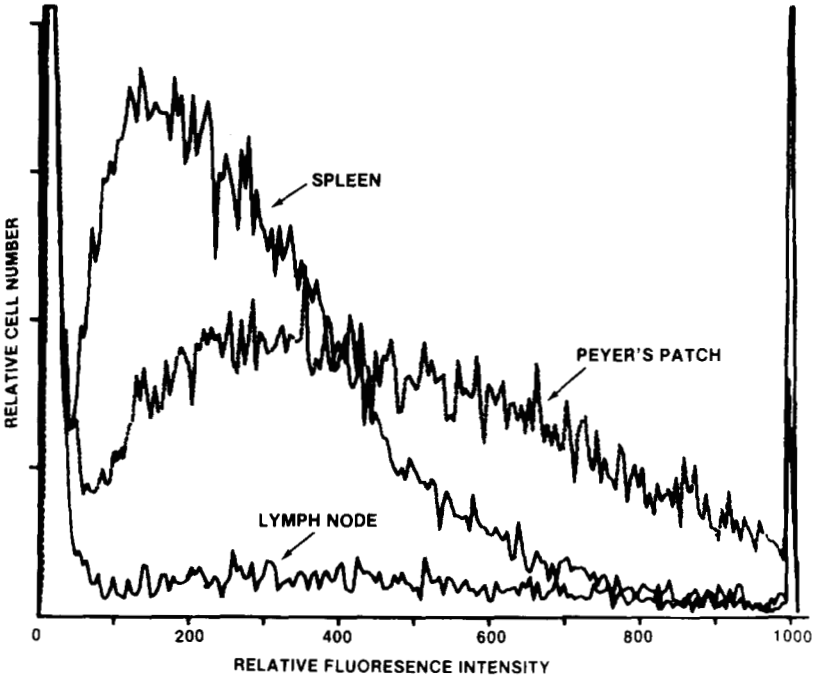

Figure 2. CBA/J lymphocytes were obtained from spleen, Peyer's patch, or lymph node, incubated with A.TH anti-A.TL followed by Fl-RαMγ, and then analyzed on the FACS.

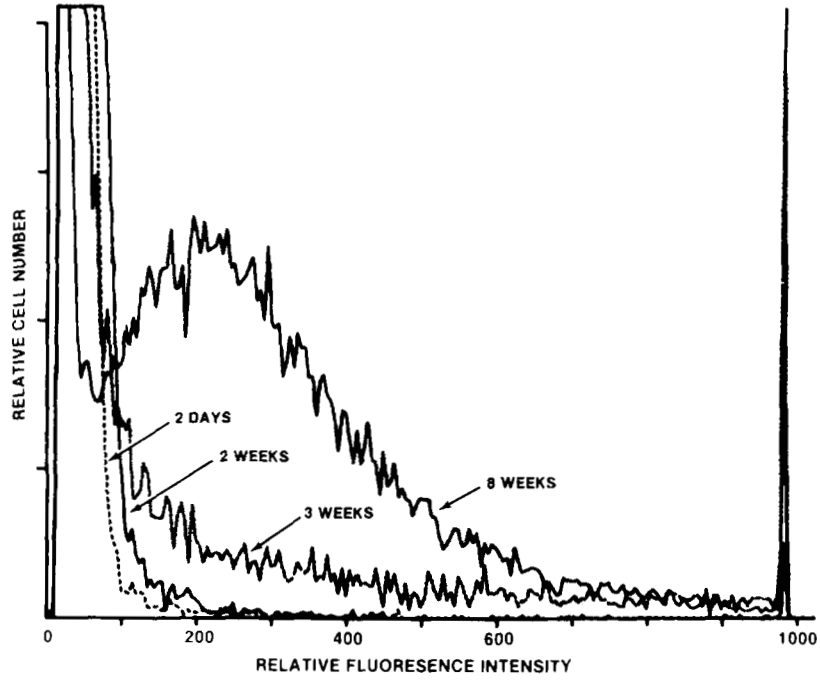

Figure 3. Spleen cells were obtained from CBA/J mice at 2 days, 2, 3, or 8 weeks after birth, incubated with A.TH anti-A.TL antiserum followed by Fl-RαMγ, and analyzed on the FACS.

TABLE III

FACS analysis of Ia⁻ and Ig⁻ spleen cells from 2-day-old mice incubated with A.TH anti-A.TL and Fl-RαMγ[a]

Fluorescent Stain	Unsorted Cells		Ig⁻ Sorted Cells		Ia⁻ Sorted Cells	
	Ig⁺	Ia⁺	Ig⁺	Ia⁺	Ig⁺	Ia⁺
% of total	18	15	2	7	4	2
Fluorescence intensity (median channel no. of positive cells)	171	256	83	110	98	179

[a] Pooled spleen cells from 2-day-old CBA/J mice were incubated with A.TH anti-A.TL antiserum followed by Fl-RαMγ, or with fluoresceinated rabbit anti-mouse Ig. Cells were then sorted into Ia⁻ or Ig⁻ cell pools. Such cells were then analyzed for sIa or sIg.

from immune defective CBA/N mice have some characteristics that are similar to those of B cells from neonatal mice as indicated by analysis of their B cell surface determinants (17) and their immune function (18), and it was therefore of interest to examine the pattern of Ia expression on B cells from mice with this defect. The staining profiles of such cells were considerably different from those of normal cells; the median fluorescence intensity of sIa⁺ cells was greater than that of normal cells (on the average, about 100 channel equivalents brighter), which predominantly reflected a decrease in the number of cells that were in the intermediate fluorescent intensity range (Table IV; Fig. 4). The preponderance of brightly staining cells among CBA/N cells was not due to the presence of larger-sized cells in mice with the CBA/N defect, inasmuch as the size of their cells was similar to normal cells as determined by sizing on the

Coulter counter. The fluorescence profiles of A.TH anti-A.TL stained lymphocytes obtained from the various lymphoid organs of mice with the CBA/N defect were also different from those of normal mice. In each of these organs, cells of intermediate Ia fluorescence intensity were diminished in amount, whereas the Ia^+ cells, which were of higher fluorescence intensity, were relatively enriched (Fig. 5).

FACS profiles of sIa^+ cells taken from mice with the CBA/N immune defect at various times after birth were compared with those of cells taken from normal mice at the same ages. It was apparent that the pattern of acquisition of sIa on spleen cells from (CBA/N × BALB/c)F_1 females and defective (CBA/N × BALB/c)F_1 males was similar until 3 to 4 weeks of age (Table V, Fig. 6). The abnormal profile characteristic of mice with the CBA/N defect developed at some time between 3 and 8 weeks of age.

Frequency of sIg^+, sIa^- cells in spleens of adult mice by FACS sorting and subsequent analysis of sIa^- cells. In order to examine the Ig phenotype of the adult splenic Ia^- cells, CBA/J spleen cells were stained with A.TH anti-A.TL serum and Fl-RαMγ, and separated into Ia^- and Ia^+ cell pools with the FACS. This population of Ia^- cells, which on reanalysis contained fewer than 1% residual sIa^+ cells, was then stained with Fl-RαMIg, and the frequency and the distribution of sIg^+ cells were determined. In three separate experiments, 5% or fewer of the Ia^- cells were also Ig^+, and the median fluorescence intensity of this small number of sIg^+ cells was quite distinct from and considerably lower than that of the original sIg^+ population (Table VI). In the converse experiments, spleen cells were also separated into Ig^- fractions by FACS sorting. When subsequently analyzed, fewer than 3% of the sIg^- cells were found to be Ia^+.

TABLE IV

Median fluorescence intensity of Ia^+ cells from normal CBA/J and (CBA/N × DBA/2)F_1 female or from B cell defective CBA/N and (CBA/N × DBA/2N)F_1 male mice[a]

Expt. No.	Mouse Strain	% Ia^+	Fluorescence Intensity of sIa^+ Cell
1	CBA/J	58	292
	CBA/N	47	382
2	(CBA/N × DBA/2N)F_1 ♀	42	308
	(CBA/N × DBA/2N)F_1 ♂	32	402

[a] Pooled spleen cells from CBA/J, CBA/N, and DBA/2N F_1 male and female mice were incubated with anti-A.TL antiserum followed by Fl-RαMγ and analyzed on the FACS.

An independent measure of Ig production in Ia^+ and Ia^- cells was obtained by radiolabeling and immunoprecipitating Ig from the respective cell populations. Surface Ia^+ and Ia^- cells obtained after sorting with the FACS, as well as unstained cells and cells stained for Ia but unsorted, were cultured with ^{35}S-methionine to radiolabel newly synthesized proteins. NP-40 detergent cell lysates were then prepared, and Ig and MHC-associated antigens were immunoprecipitated sequentially from the lysates and analyzed by SDS-PAGE. As shown in Figure 7, peaks of radioactivity with the mobilities of μ-chains, α- and/or γ-chains, and light chains were easily detected in reduced precipitates from lysates of cultures containing Ia^+ cells. However, only a trace amount of μ- and light chains was detected in the Ia^- fraction, and amounted to no more than 5% of that found in the Ia^+ fraction when normalized to the recoveries of MHC antigen from the respective fractions. In parallel studies, surface membrane proteins from these same cell preparations were radioiodinated by lactoperoxidase-catalyzed iodination, and immunoprecipitated Ig was also analyzed by SDS-PAGE. In this case, radiolabeled ^{125}I-μ, δ, and L chains were easily identified in the lysates of unstained cells, stained unsorted cells, and Ia^+ cells, but no Ig was detected in the sorted Ia^- fraction despite the recovery of approximately similar amounts of MHC antigen from the different cell preparations (data not shown). From preliminary cell dilution experiments it was known that a splenocyte-thymocyte mixture containing up to 5% typical sIg^+ cells could have gone undetected under the conditions used (unpublished observations).

DISCUSSION

It is now well established that most, if not all, of murine splenic B lymphocytes express sIa antigens (1, 2). However, several reports have described the existence of a small number

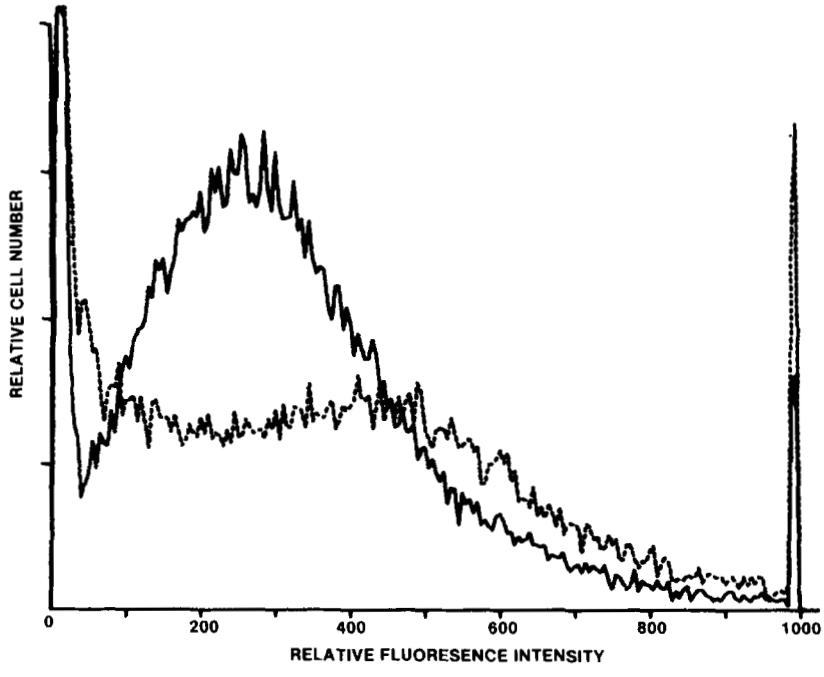

Figure 4. Spleen cells were obtained from (CBA/N × BALB/c)F_1 male (-----) or F_1 female (——) mice, incubated with A.TH anti-A.TL followed by Fl-RαMγ, and analyzed on the FACS.

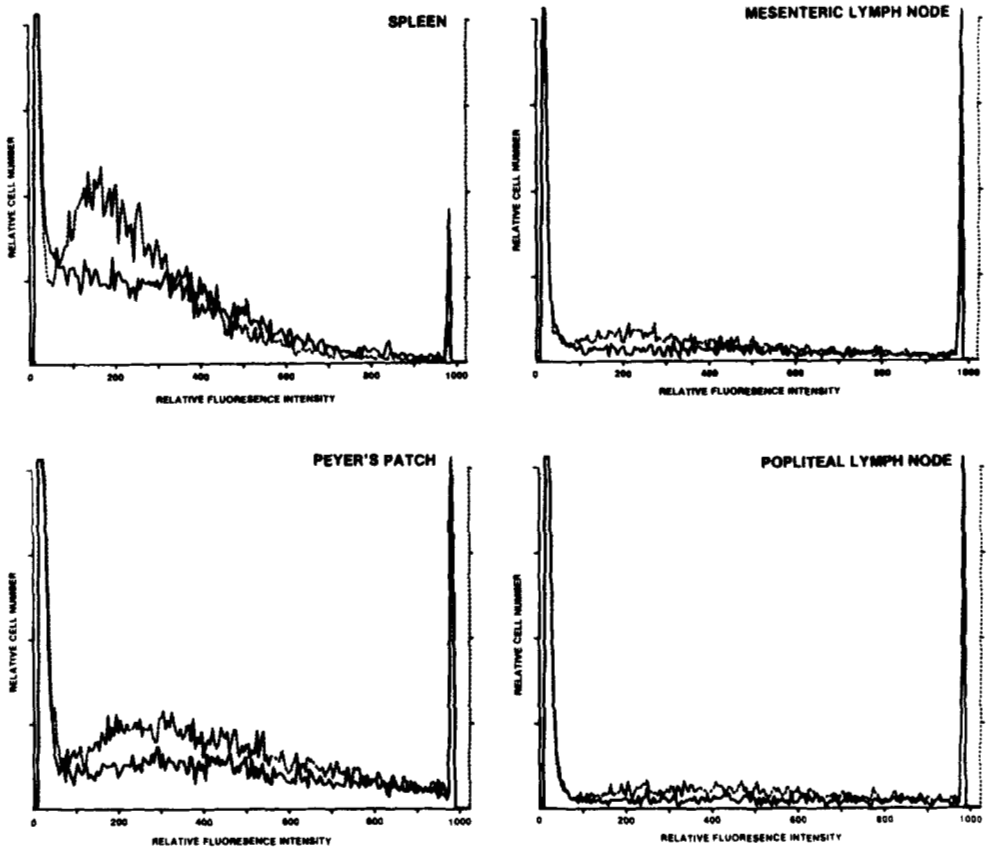

Figure 5. (CBA/N × BALB/c)F_1 male (——) or female (-----) lymphocytes were obtained from spleen, Peyer's patch, or lymph node, incubated with A.TH anti-A.TL followed by Fl-RαMγ, and then analyzed on the FACS.

TABLE V

FACS profile of Ia^+ spleen cells of (CBA/N × BALB/c)F_1 female and of B cell defective (CBA/N × BALB/c)F_1 male mice at various times after birth[a]

	Weeks			
	1	2	3	8
(CBA/B × DBA/2N)F_1 ♀				
% Ig^+	34	46	57	54
% Ia^+	36	43	57	44
Ia^+ median channel no.	138	123	172	306
(CBA/N × DBA/2N)F_1 ♂				
% Ig^+	22	32	39	38
% Ia^+	22	29	32	36
Ia^+ median channel no.	83	121	139	400

[a] Pooled spleen cells from (CBA/N × BALB/c)F_1 male and female mice at various times after birth were incubated with A.TH anti-A.TL antiserum followed by Fl-RαMγ and analyzed on the FACS.

of cells that bear membrane sIg but are resistant to killing by anti-Ia and C and presumably Ia lack antigens (5, 10). Similar conclusions were reached in studies that demonstrated that the expression of membrane Ig precedes the expression of Ia in murine neonatal spleen cells (8) and in adult bone marrow cells (9). The ability to identify and isolate Ig^+ Ia^- cell populations would be most useful in the study of B lymphocyte development.

The system we employed to study this problem relies on an indirect immunofluorescence staining assay for sIa combined with the very high sensitivity of rapid flow microfluorometry. The specificity of the A.TH anti-A.TL serum used for the identification of Ia antigens was established both by the ability of this serum to stain or kill cells expressing relevant but not irrelevant Ia specificities, and by absorption with spleen cells from MHC congenic strains.

In individual experiments, between 55 to 65% of adult spleen cells were Ia^+, and the frequency of Ig^+ cells averaged 4% higher. This difference between numbers of Ia and Ig-bearing cells was examined more closely by sorting spleen cells into sIa^+ and sIa^- fractions and then analyzing the Ia^- fraction for the presence of cells bearing Ig. Fewer than 5% of Ia^- cells were Ig^+ when stained with the rabbit anti-mouse Ig reagent and reanalyzed on the FACS. Even when neonatal spleen cells, a source allegedly enriched in Ig^+ Ig^- lymphocytes, were sorted into Ia^- fractions on the FACS and subsequently evaluated for sIg expression fewer than 5% were of such a phenotype. The density of Ig on Ig^+ cells in Ia^- populations was much less than that of Ig^+ cells in unsorted populations. In addition, the negligible recoveries of both biosynthetically and externally radiolabeled and immunoprecipitated Ig from the sorted adult Ia^- population is consistent with this frequency of Ig-bearing cells in Ia^- populations. These findings are in agreement with those of Ewert and Cooper (19), who were unable to demonstrate the existence of Ig^+ Ia^- cells in the avian model even at 10 days of incubation.

In order to investigate the heterogeneity of Ia distribution on B cells, we studied cells from various lymphoid organs. The frequency of sIa^+ cells from spleen, Peyer's patch, and lymph node was close to the frequency of Ig^+ cells, whereas the fluorescence intensity of Ia staining on Peyers's patch and lymph node cells was considerably greater than on splenic B cells. These reproducible differences in intensity of Ia staining presumably reflects variations in the overall density of sIa on the surface of these cells. This heterogeneity of Ia expression

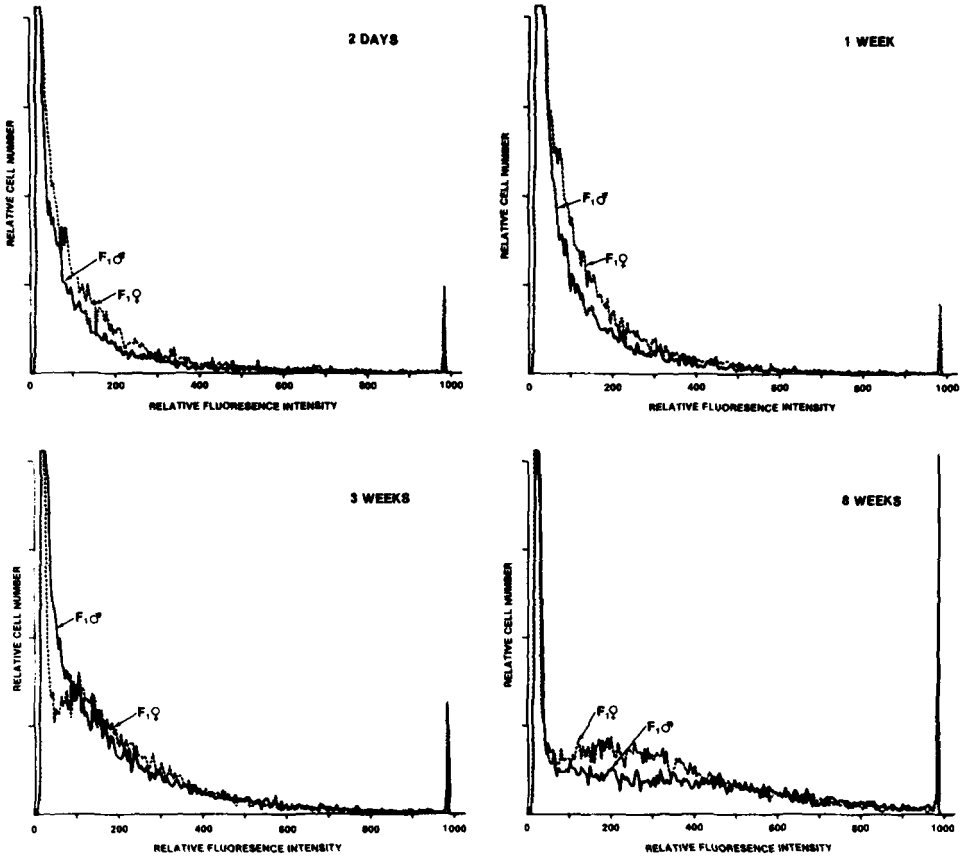

Figure 6. Spleen cells were obtained from (CBA/N × BALB/c)F_1 male (——) or female (-----) mice at 2 days, 1 week, 3 or 8 weeks after birth, incubated with A.TH anti-A.TL followed by Fl-RαMγ, and analyzed on the FACS.

TABLE VI

FACS analysis of Ia⁻ and Ig⁻ cells obtained by cell sorting and then analyzing with A.TH anti-A.TL or with a fluoresceinated anti-mouse Ig reagent[a]

Fluorescent Stain	Unsorted Cells		Ig⁻ Sorted Cells		Ia⁻ Sorted Cells	
	Ig⁺	Ia⁺	Ig⁺	Ia⁺	Ig⁺	Ia⁺
% of total	54	46	1	5	5	<1
Median channel no.	263	269	52	62	56	47

[a] CBA/J spleen cells were incubated with A.TH anti-A.TL antiserum followed by Fl-RαMγ or with fluoresceinated anti-mouse Ig. Cells were then sorted into Ia⁻ or Ig⁻ cell pools. Such cells were then reanalyzed for sIa or sIg.

may distinguish between B cells at different stages of maturation, as has recently been reported by Lala *et al.* (20), or it may delineate subpopulations of B cells with different functional properties. This observation is particularly significant in view of the fact that studies have shown that differences in the density of membrane Ig on splenic B cells may distinguish different B cell subpopulations (17, 21, 22). We also studied the ontogenetic expression of sIa on cells of neonatal mice in order to determine whether the heterogeneity of Ia expression was a property of cell maturation. Spleen cells from 2-day-old mice stained considerably duller with anti-Ia than adult Ia⁺ cells. With increasing time after birth, the splenic Ia⁺ cell profile became more homogeneous and stained more brightly. These findings are consistent with the concept that sIa antigens are present in low amounts on immature B cells and reach adult levels at 3 to 4 weeks of age. Recent findings reported by LaLa *et al.* (20) have also shown that B cell maturation in the adult animal is associated with a concurrent increase in sIa density.

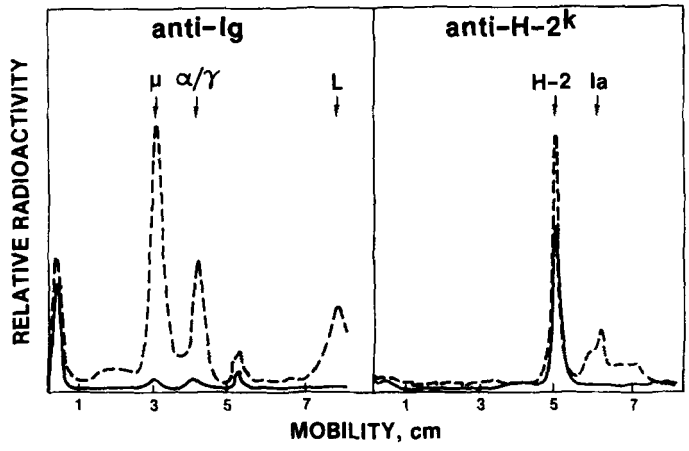

Figure 7. SDS-PAGE of biosynthetically labeled and reduced Ig and H-2 plus Ia molecules sequentially and quantitatively immunoprecipitated from lysates of FACS sorted sIa⁺ (-----) and sIa⁻ (——) cells. Three to 3.5×10^6 Ia⁺ cells and 2.5 to 3.0×10^6 Ia⁻ cells (all of the cells which recovered after sorting), along with stained, unsorted cells, and unstained cells were cultured under the same conditions and no differences were seen in the Ig and H-2 profiles of cells stained for Ia and unstained cells. Ig markers were radiolabeled and reduced mouse IgM and IgG myeloma proteins and this did not allow for a distinction to be made between cytoplasmic α- and γ-chains.

We have obtained similar profiles with hybridoma anti-Ia antisera with specificity to I-A and I-E/C, suggesting that the neonatal patterns were a consequence of quantitatively less sIa rather than a qualitative difference of expression of different Ia antigens (unpublished observations).

We also examined the heterogeneity of Ia expression on B

cells from CBA/N mice which have an X-linked B cell defect. These mice have provided a useful model for the study of B cell maturation and of the functions of B cell subpopulations. Mice bearing the CBA/N defect have multiple B cell defects (17, 23, 24). Functionally, they are unable to respond to some (25–27) but not all (28, 29) TI antigens, express responsiveness to TI antigens later in ontogeny than normal mice (30) and have been shown to be unresponsive to antigens whose response appears late in ontogeny (27, 31) and which requires a B cell that expresses the Lyb5 differentiation antigen (32). B lymphocytes from CBA/N mice were abnormal with respect to their cellular expression of Ia antigens. The frequency of cells expressing intermediate amounts of Ia is reduced, and a greater fraction of the Ia^+ cells is found in the very bright staining region. Whether this paucity of cells of intermediate sIa density in CBA/N spleens represents a phenotypic marker for these mice or is in fact responsible for their abnormal triggering defect remains unknown.

In conclusion, these studies demonstrate that sIa density may delineate B cells at different stages of maturation as well as different B cell subpopulations. A possible explanation for the divergent views in the literature on the existence of Ig^+ Ia^- cells may be the existence of cells that bear very few Ia determinants on their surface compared with the average B cell, which has many more sIa determinants. Such cells might be relatively more resistant to lysis with anti-Ia + C and should more properly be categorized as "Ia sparse" rather than Ia^-.

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