B-LYMPHOCYTE HETEROGENEITY: ONTOGENETIC
DEVELOPMENT AND ORGAN DISTRIBUTION
OF B-LYMPHOCYTE POPULATIONS DEFINED BY THEIR
DENSITY OF SURFACE IMMUNOGLOBULIN*

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The density of immunoglobulin (Ig) on the surface of mouse bone marrow-
derived (B) lymphocytes has recently been studied by two groups of investiga-
tors (1-3). Both groups attributed developmental significance to differences in
the density of Ig on the surface of these cells, but Osmond and Nossal concluded
that a substantially greater heterogeneity in the amount of Ig per B cell existed
in the immature population vs. the mature population, whereas Sidman and
Unanue indicated that immature cells have, on the average, more Ig than
mature cells. The techniques employed in these studies are very tedious and arerefore, poorly suited to a detailed analysis of the distribution of the density of
surface Ig on different populations of B lymphocytes. However, the density of
surface Ig on individual B lymphocytes derived from different sources is critical
to the understanding of antigen-B-lymphocyte interaction and activation. The
introduction of the technique of rapid flow microfluorometry (RFMF)1 and
electronic cell sorting makes possible a direct approach to the study of surface Ig
density on B lymphocytes and to an evaluation of its developmental and func-
tional significance.

Using this technology, we report here the identification among splenic B
lymphocytes of a large subpopulation of cells possessing a relatively low-to-
intermediate density of surface Ig when a fluorescein-conjugated (Fl) anti-
mouse Ig or anti-κ is used to detect Ig-bearing cells. Such B lymphocytes, as

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1970," and the principles outlined in the "Guide for the Care and Use of Laboratory Animals," U.S.
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1 Abbreviations used in this paper: FACS, Fluorescence Activated Cell Sorter; FCS, fetal calf
serum; FITC, fluorescein; Fl, fluorescein-conjugated; Mls, minor lymphocyte stimulating; nu/nu,
congenitally athymic; RFMF, rapid flow microfluorometry.
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detected by a peak in the fluorescence profile, are found in the spleen and lymph
nodes of adult mice. A similar peak is not detected when neonatal spleen or
adult bone marrow cells are examined. The development of this population of B
lymphocytes occurs during the first 4 wk of life and appears to be independent of
the influence of thymus-derived (T) lymphocytes or of antigenic stimulation,
since the fluorescence profiles of spleen cells of adult congenitally athymic (nu/
nu) mice and of mice raised under germfree conditions are indistinguishable
from those of normal adult animals. Parallel studies using Fl anti-\(\mu\) to identify
surface IgM failed to demonstrate the distinct B-lymphocyte subpopulation with
low-to-intermediate fluorescence intensity among adult spleen cells. Moreover,
the fluorescence profiles of Fl anti-\(\mu\)-labeled adult bone marrow and adult
spleen were similar to each other.

Materials and Methods

Animals. DBA/2N, C57BL/6N, and BALB/cAnN (germfree or conventionally reared) mice
were obtained from the Animal Production Section, Division of Research Services of the National
Institutes of Health, Bethesda, Md. CBA/J and C3H/HeJ mice were purchased from The Jackson
Laboratory, Bar Harbor, Maine. BALB/c nu/nu and +/nu littermates which were in the seventh
backcross generation were obtained from the Charles River Breeding Laboratories, Inc., Wilming-
ton, Mass. Mice were from 8 to 12 wk of age at the time of study unless otherwise noted. In all
instances, lymphoid organs from individual mice were studied, except when newborn to 3-wk-old
mice were analyzed, when the lymphoid organs of four to five individual mice were pooled.

Cell Suspensions. Spleens; Peyer's patches; popliteal, inguinal, and cervical lymph nodes; and
tibiae and fibulae were obtained from mice killed by cervical dislocation. Suspensions of spleen,
Peyer's patches, and lymph node cells were prepared by teasing of these organs with forceps into
RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) while bone marrow cells were
flushed from the shaft of the tibia or fibula using a 25 gauge needle and syringe. Cell aggregates
were dispersed by passage through a 26 gauge needle. Spleen and bone marrow cells were
centrifuged at 100g for 10 min, the supernatant fluid removed, and the pellet treated with 5 ml of
0.83% ammonium chloride at 4°C for 5 min to lyse red blood cells. Nucleated cells obtained by this
method were washed through fetal calf serum (FCS) (Grand Island Biological Co.) to remove red
blood cell ghosts.

Anti-Immunoglobulin Reagents. A polyspecific goat anti-mouse 7S globulin and an anti-
mouse-\(\mu\) were obtained from Meloy Laboratories Inc., Springfield, Va. (Lot nos. 40691 and 40643,
respectively). These antisera were digested with 2% (wt/wt) pepsin (Mann Research Labs, New
York) in acetate buffer (pH 4.0) for 18 h, and F(ab')2 fragments were further purified on a
Sephadex G-200 column (100 x 1 cm), dialyzed against a bicarbonate buffer (pH 9.0), and
concentrated to 4 mg/ml. F(ab')2 fragments were conjugated with fluorescein isothiocyanate (BBL,
Division of Becton, Dickinson & Co., Cockeysville, Md.) by overnight dialysis of 10 ml of protein
(10 mg) in a bicarbonate buffer (pH 9) against 1 mg of FITC in 1 ml of the buffer enclosed within a
dialysis bag. Fl F(ab')2 antisera with a molar ratio of protein to fluorescein of approximately 2:1
were isolated from proteins with a higher or lower protein-to-fluorescein ratio using DEAE-
cellulose column chromatography. The Fl F(ab')2 anti-Ig and anti-\(\mu\) will be referred to as the Fl
anti-Ig and Fl anti-\(\mu\), respectively. In addition to these preparations, a series of fluoresceinated
affinity column-purified antibodies was also used in these studies. These include goat anti-mouse
\(\gamma^\lambda, \gamma^\kappa, \alpha^-, \) and \(\mu\)-antibodies (4) and rabbit anti-mouse-\(\kappa\) antibody (a gift from Dr. R. Mage, NIH,
Bethesda, Md.).

In order to further analyze the specificity of the Fl anti-Ig, 0.8 mg (0.2 ml) of this reagent was
passed through a 1 ml column containing 300 \(\mu\)g each of IgG\(\kappa\), IgG\(\kappa\), and IgG\(\kappa\) myeloma
proteins bound to Sepharose. The column was eluted with 5 cm\(^3\) of borate buffer and the effluent
was dialyzed against saline and concentrated to the original protein concentration. The molar
ratio of protein to fluorescein of this material was similar to that of the original. As noted in the
Results section, absorption of the anti-Ig with these three mouse myeloma proteins abolished the
ability of this antisera to stain mouse spleen cells.
Lymphocyte Staining. Nucleated cells obtained from lymph node or ammonium chloride-treated bone marrow or spleen cell suspensions were washed twice with RPMI 1640 and adjusted to 50 x 10⁶ cells/ml with RPMI 1640 containing 5% FCS and 1 mg/ml of sodium azide. Preliminary studies indicated that 10 x 10⁶ cells in 200 μl would be optimally labeled for use with the Fluorescence Activated Cell Sorter (FACS) (Becton, Dickinson Electronics Laboratory, Mountain View, Calif.) with 20 μg of F1 anti-Ig; 10 μg of F1 anti-μ; or 2.5 μg of the affinity column purified anti-μ, anti-κ, anti-γ₁, anti-γ₂, and anti-α. The undigested F1 antibodies (anti-κ, anti-μ, anti-γ₁, anti-γ₂, and anti-α) were ultracentrifuged for 40 min at 100,000 g before their use. Cell suspensions were incubated with F1 antibodies at 4°C for 30 min, washed three times in cold RPMI 1640 (5% FCS, 1 mg/ml sodium azide), and resuspended in the same media to 10 x 10⁶ cells/ml.

Flow Microfluorometry. A detailed explanation of the principle of RFMF has been presented by Loken and Herzenberg (5). The frequency and fluorescence profile of cells stained with F1 antisera was determined after examining at least 100,000 viable cells. Cellular volume was determined by a Coulter Counter (Model B; Coulter Electronics Inc., Hialeah, Fla.) which was made available by Dr. C. Herman, NIH, Bethesda, Md.

Results

Fluorescence Profile of Murine Lymphocytes Stained with F1 Anti-Ig. The fluorescence profiles of unstained and F1 anti-Ig-stained CBA/J thymus and spleen cells are shown in Figs. 1a and b. The horizontal axis of these and all subsequent fluorescence profiles displays 1,000 linear channels of increasing fluorescence intensity toward the right, while the vertical axis displays the number of individual cells within each channel. Less than 0.2% of CBA/J thymus cells were stained by the F1 anti-Ig, indicating that this reagent was not detecting species-specific surface antigens found on mouse thymocytes. By contrast, when CBA/J splenic lymphocytes stained with F1 anti-Ig were studied by RFMF, 54.5% of the cells were found to be fluorescent. The fluorescence profile of these F1 anti-Ig-stained splenic B lymphocytes was characterized by a large peak of cells which has a low-to-intermediate degree of fluorescence intensity. Similar fluorescence profiles were obtained when DBA/2N (Fig. 2a), C57BL/6, BALB/c, and C3H/HeJ splenic lymphocytes were stained with F1 anti-Ig. Thus, the existence of a large number of cells with a low-to-intermediate degree of fluorescence intensity as a dominant feature of the fluorescence profile of adult splenic B lymphocytes appears to be a general phenomenon, as it is found in spleen cells from five different inbred mouse strains.

In order to further characterize the F1 anti-Ig, we stained C57BL/6 spleen cells with this reagent and compared the profile to that of C57BL/6 stained cells stained with F1 anti-κ. The fluorescence profiles of these cells after labeling with either F1 anti-Ig or F1 anti-κ were virtually identical and the frequency of Ig-positive cells were 44.7 and 46.5%, respectively. Moreover, after absorption of the F1 anti-Ig on a Sepharose column containing IgG₁, IgG₂a, and IgG₂b mouse myeloma proteins, as described in the Materials and Methods section, its capacity to label splenic B lymphocytes was abolished.

The large number of splenic B lymphocytes displaying a low-to-intermediate degree of fluorescence after staining with F1 anti-Ig could be accounted for by (a) the presence of cells of a uniform volume which contained a major population of cells with a low-to-intermediate density of surface Ig, (b) the presence of cells with a uniform density of surface Ig which contains a large population of cells with a low-to-intermediate cellular volume, or (c) populations of cells varying in...
Fig. 1. Fluorescence profiles of (a) CBA/J thymus cells, unlabeled (---) or labeled with F1 anti-Ig (—); (b) CBA/J spleen cells, unlabeled (---) or labeled with F1 anti-Ig (—).

Fig. 2. Fluorescence profiles of (a) DBA/2 spleen cells labeled with F1 anti-Ig, (b) DBA/2 spleen cells labeled with F1 anti-Ig and separated according to their relative fluorescence intensity as noted by the arrows, and (c) DBA/2 spleen cells labeled with F1 anti-Ig after separation. Fig. 3c is a composite picture of individual analytical runs of cells that were separated as shown in Fig. 3b.

In order to distinguish between these possibilities, spleen cells from DBA/2N mice were stained with F1 anti-Ig (Fig. 2a) and cells with low fluorescence intensity were separated from those with high fluorescence intensity using the FACS (Fig. 2b). When these cells were re-
examined with the FACS, the separation of low from high intensity fluorescence cells was confirmed (Fig. 2c). The average cellular volumes of these two populations were 119 and 128 $\mu$m$^3$ for the "bright" and low-to-intermediate fluorescence intensity B lymphocytes, respectively. This indicates that the distinct fluorescence intensities of these two populations does not reflect differences in their cellular volumes, but is secondary to differences in the density of Ig on their surfaces.

**Fluorescence Profiles of Bone Marrow, Lymph Node, or Peyer's Patch Lymphocytes Stained with Fl anti-\(\kappa\).** The fluorescence profiles of bone marrow, lymph node, and Peyer's patch lymphocytes stained with Fl anti-\(\kappa\) are shown in Figs. 3a–c, respectively. In order to facilitate the comparison of these profiles with those obtained with splenic lymphocytes, the scale (counts $\times$ 100) of the bone marrow, lymph node, and Peyer's patch plots was changed so that the areas under the Ig-positive portion of the curves (channels 80–1,000) would equal that of the splenic profile and the two profiles were plotted together. The frequency of Ig-positive cells in the bone marrow, lymph nodes, and Peyer's patches was 18.9, 18.4, and 55.2%, respectively. B lymphocytes derived from CBA/J lymph nodes (Fig. 3b) had a fluorescence profile which was very similar to the profile of splenic B lymphocytes. The profile obtained with Peyer's patch cells (Fig. 3c) demonstrates that a large fraction of the cells have a very low fluorescence intensity and are poorly separated from background. No fluorescence peak was seen when bone marrow cells (Fig. 3a) were labeled with Fl anti-\(\kappa\). Moreover, there were substantial numbers of very intensely stained B lymphocytes in bone marrow, and the relative frequency of bright cells in this organ was greater than in spleen.

**Fluorescence Profile of Neonatal Spleen Cells Stained with Fl Anti-Ig.** The absence of the fluorescence peak among bone marrow cells stained with Fl anti-Ig suggested that the cells within the low-to-intermediate fluorescence peak seen in the spleens and lymph nodes of adult mice represented a mature subpopulation of B lymphocytes. This was further studied by examining the fluorescence profile of spleen cells derived from 3-, 10-, 17-, and 30-day-old and adult BALB/c mice (Figs. 4a–e). In the first 17 days of life, the relative frequency of cells with a very low-to-low (channels 80–200) and low-to-intermediate (channels 200–400) fluorescence intensity among the Ig-positive cells (channels 80–1,000) increases so that at 17 days they represent 44.2 and 42.1% of the Ig-positive cells, respectively (Table I). These changes are associated with a marked increase in the overall frequency of Ig-positive cells (29.2 to 54.0%). After 17 days, the frequency of cells with a very low-to-low fluorescence intensity begins to decrease and the continued decline in this population associated with an increase in the relative frequency of cells with a low-to-intermediate fluorescence intensity results in the characteristic Fl anti-Ig-labeled adult profile (Fig. 4e).

**Fluorescence Profiles of nu/nu and Germfree BALB/c Splenic B Lymphocytes Stained with Fl Anti-Ig.** After labeling with Fl anti-Ig, the fluorescence profile and frequency of Ig-positive cells among spleen cells derived from conventionally or germfree-reared adult BALB/c mice were essentially identical (data not shown). The fluorescence patterns of nu/nu and +/nu spleen cells labeled with
Fluorescence Profiles of Lymphocytes Stained with Fl Anti-Heavy Chain Antibodies. The frequency of fluorescent cells after labeling adult CBA/J spleen cells with Fl anti-\(\gamma_1\), Fl anti-\(\gamma_2\), Fl anti-\(\alpha\), and Fl anti-\(\mu\) was <1, 3.8, <1, and 54.0%, respectively. Although the Fl anti-\(\mu\) stained an equivalent percentage of spleen cells when compared to Fl anti-\(\kappa\) (54 vs. 54.1%), a fluorescence peak was not seen after labeling with Fl anti-\(\mu\) (Fig. 6a).

When B lymphocytes from CBA/J adult bone marrow, lymph node, and Peyer's patches were stained with Fl anti-\(\mu\), the profiles obtained were generally similar to each other and to those observed with adult spleen cells (Figs. 6b–d). In no case, was a low-to-intermediate intensity fluorescence peak observed. However, the relative frequency of cells with a very low-to-intermediate density of surface IgM among the Ig-positive cells was greater in lymph node and Peyer's patches (86.0 and 89.4%, respectively) than spleen and bone marrow (74.2 and 71.7%, respectively). The frequency of Ig-positive lymphocytes, detected after labeling with Fl anti-\(\mu\), was 19.8, 16.6, and 33.8% when cells derived
from CBA/J, bone marrow, lymph nodes, or Peyer's patches were examined. These data are similar to that obtained when the cells from these organs were labeled with Fl anti-Ig, as noted above, except in the case of Peyer's patch cells, where Fl anti-κ labels 21.4% more cells than Fl anti-μ.

Finally, the fluorescence profiles of Fl anti-μ-labeled spleen cells from 3-, 10-, 17-, and 30-day-old and adult BALB/c mice are shown in Figs. 7a–e. These profiles are similar to one another in that they lack a fluorescence peak. However, the relative frequency of cells with a very low-to-intermediate fluorescence intensity among the positive cells increases considerably from 51.3% at 3 days to 67.2% at 17 days (Table II). The increase in this population of cells

Fig. 4. Fluorescence profiles of BALB/c spleen cells labeled with Fl anti-Ig from (a) 3-day-old, (b) 10-day-old, (c) 17-day-old, (d) 30-day-old, and (e) adult mice.
TABLE I

Frequency of Ig-Positive Lymphocytes Among BALB/c Spleen Cells Labeled with Fl Anti-Ig

| Figure | Age  | Ig-positive cells |
|--------|------|-------------------|
|        | days | %     |                  |
| 4a     | 3    | 29.2  | 39.9             | 36.6 |
| 4b     | 10   | 34.8  | 42.8             | 40.7 |
| 4c     | 17   | 54.0  | 44.2             | 42.1 |
| 4d     | 30   | 49.7  | 36.5             | 43.0 |
| 4e     | Adult| 44.0  | 23.2             | 50.8 |

* Fl anti-Ig-labeled lymphocytes appearing in channels 80-200 and 200-400 are designated as having a very-low density and low-to-intermediate density of surface Ig.

Fig. 5. Fluorescence profiles of spleen cells labeled with Fl anti-Ig from +/nu BALB/c (--), or nu/nu BALB/c (---) mice.

continues so that at 30 days of age these cells represent 73.4% of the total surface IgM-positive population, which is similar to the frequency of these cells in the spleen cells of adults (Fig. 7e). Thus, as B lymphocytes mature in the spleens of neonatal mice, the relative density of their surface IgM decreases.

Discussion

In this study, we have utilized the technology of RFMF to determine the relative density of surface Ig on B lymphocytes derived from different lymphoid organs of adult mice and from the spleens of neonatal mice in different stages of their maturation. Splenic B lymphocytes derived from adult DBA/2, BALB/c, CBA/J, C57BL/6, and C3H/J mice labeled with either Fl anti-Ig or Fl anti-κ had a fluorescence profile which was characterized by a large peak of cells with a relatively homogenous intensity of fluorescence. The cells within this peak have
a low-to-intermediate density of total surface Ig. Moreover, since Fl anti-γ2 antibody labeled only 3.8% of splenic lymphocytes, it is unlikely that cells bearing passively acquired cytophilic antibody of the IgG2 class contribute to any extent to this peak. The characteristic profile observed after labeling adult spleen cells with Fl anti-Ig was also seen when adult spleen cells derived from either nu/nu mice or from mice reared under germfree conditions were examined. Thus, the development of the large, relatively homogenous population of splenic B lymphocytes, which have a low-to-intermediate density of surface Ig, appears to be independent of either antigenic stimulation or T-lymphocyte cooperation. Moreover, the absence of the low-to-intermediate fluorescence peak among adult bone marrow cells and neonatal spleen cells and its presence in adult spleen and lymph node cells suggest that the cells with a low-to-intermediate density of surface Ig represent a distinct, mature subclass of B lymphocytes.

The fluorescence profiles of Fl anti-μ-labeled cells derived from adult mouse bone marrow, adult spleen, or neonatal spleen lacked the peak seen when Fl anti-Ig-labeled adult spleen cells were examined. Nonetheless, a clear difference in the fluorescence profiles of Fl anti-μ-labeled adult and neonatal spleen was noted. On the other hand, the Fl anti-μ profiles of adult bone marrow and adult spleen were not distinguishable. Thus, the relative immaturity of adult bone

**Fig. 6.** Fluorescence profiles of (a) CBA/J spleen cells unlabeled (---) or labeled with Fl anti-μ (--), (b) CBA/J bone marrow cells unlabeled (---) or labeled with Fl anti-μ (--), (c) CBA/J lymph node cells unlabeled (---) or labeled with Fl anti-μ (--), and (d) CBA/J Peyer's patch cells unlabeled (---) or labeled with Fl anti-μ (--).
Fluorescence profiles of BALB/c spleen cells labeled with Fl anti-μ from (a) 3-day-old, (b) 10-day-old, (c) 17-day-old, (d) 33-day-old, and (e) adult mice.

Fig. 7. Fluorescence profiles of BALB/c spleen cells labeled with Fl anti-μ from (a) 3-day-old, (b) 10-day-old, (c) 17-day-old, (d) 33-day-old, and (e) adult mice.

marrow cells, which is observed after comparing the fluorescence profiles of Fl anti-Ig-labeled adult bone marrow and adult spleen cells, is not detected when such cells are labeled with Fl anti-μ.

The frequency of IgM-bearing B lymphocytes within the spleen, lymph node, and bone marrow of mice is similar to the frequency of cells from these lymphoid organs detected by Fl anti-Ig or Fl anti-κ. Other authors using conventional fluorescence microscopy have reported that the frequency of IgM-bearing lymphocytes is substantially less than that of Ig-bearing B lymphocytes in these organs (6). This apparent discrepancy is most likely explained by the ability of the FACS to detect those cells with a level of fluorescence which is just above


### Table II

**Frequency of IgM-Positive Lymphocytes Among Spleen Cells from BALB/c Mice Labeled with Anti-μ**

| Figure | Age | IgM-positive cells | Frequency of cells, among IgM-positive cells, with a fluorescence intensity of 80-400* |
|--------|-----|-------------------|----------------------------------------------------------------------------------|
| 7a     | 3   | 33.6              | 51.3                                                                              |
| 7b     | 10  | 35.8              | 59.2                                                                              |
| 7c     | 17  | 52.1              | 67.2                                                                              |
| 7d     | 30  | 45.1              | 73.4                                                                              |
| 7e     | Adult | 42.9              | 75.7                                                                              |

* Cells appearing in channels 80-400 are designated as having a very-low-to-intermediate density of surface IgM.

that of unlabeled cells and which might not be detected as Ig-positive cells using conventional microscopy. This is not an important problem with F1 anti-Ig- or F1 anti-κ-labeled spleen or lymph node cells because the relative frequency of cells with a very low level of fluorescence intensity is quite low. However, such cells represent a larger fraction of cells labeled by F1 anti-μ. On the other hand, we did observe a large difference in the frequency of cells labeled by F1 anti-κ and by F1 anti-μ in Peyer’s patch cell suspensions, as has been previously reported by Guy-Grand and his co-workers (7).

A number of observations suggest that the development of the peak of splenic B lymphocytes is associated with the acquisition by these cells of the surface membrane protein which is the putative homologue of human surface IgD (subsequently referred to as IgD) (8). The appearance of the adult splenic F1 anti-Ig-labeled fluorescence profile and of an adult ratio of surface IgM to IgD occurs after the first 30 days of life and their development is seen in both germfree and nu/nu mice. In addition, the differences between the profiles of F1 anti-Ig-labeled bone marrow and spleen, and the similarities seen after labeling these cells with F1 anti-κ can be best explained by the absence of surface IgD on the surface of bone marrow cells, as noted by Vitetta et al. (8). The decrease in the fraction of F1 anti-Ig-labeled very-low-to-low fluorescence intensity cells and the increase in the frequency of F1 anti-μ-labeled very-low-to-low fluorescence intensity cells in developing mice is, therefore, most likely due to the acquisition of surface IgD by maturing splenic B lymphocytes which bear very-low-to-low densities of surface IgM.

It is not clear from our data if a population of B lymphocytes that exclusively expresses IgD exists in lymph node or spleen. However, if such a population is present in these organs, then it must represent a relatively small fraction of Ig-bearing B lymphocytes, as the frequency of IgM-positive cells was only slightly less than that of cells detected with F1 anti-Ig. It is possible that a population of B lymphocytes exists with a relatively high IgD-to-IgM ratio but which still bears sufficient IgM to be detected by the FACS. Of course, unanticipated cross-reactions between IgM and IgD may exist and may lead to an overestimate of
the relative frequency of IgM-bearing cells. In Peyer's patches, on the other hand, there may be a substantial number of cells which bear IgD only.

The development of a large population of splenic B lymphocytes with a low-to-intermediate density of surface Ig also coincides temporally with the acquisition of the antigens responsible for the minor lymphocyte-stimulating (Mls) mixed lymphocyte reactions and the appearance of complement receptor lymphocytes (9, 10). These data indicate that the development of Mls-defined antigens, the complement receptor, surface IgD, and large numbers of lymphocytes with a low-to-intermediate density of surface Ig serve as markers of a mature subpopulation of B lymphocytes. Moreover, they show that the acquisition of immunocompetence by B lymphocytes is not simply a function of expression of large amounts of immunoglobulin on the cell membrane.

Summary

The density of total Ig and of IgM, IgG1, IgG2, and IgA on the surface of adult murine splenic B lymphocytes was measured using the technique of rapid flow microfluorometry. In addition, the density of total surface Ig and of IgM on B lymphocytes derived from adult bone marrow, lymph nodes, and Peyer's patches, and from neonatal spleen was determined. Adult spleen and lymph node B lymphocytes are characterized by the presence of a large population of cells with a low-to-intermediate density of total surface Ig, which is seen as a peak in the fluorescence profiles when these cells are labeled with fluorescein-conjugated (Fl) anti-Ig. This peak is not detected when neonatal spleen or adult bone marrow are examined; the development of this peak among spleen cells occurs during the first 4 wk of life. Although the characteristic fluorescence intensity peak is not seen when adult spleen cells are labeled with Fl anti-μ, changes in the density of surface IgM do occur during the first few weeks of life and are detected as a decrease in the frequency of cells which have relatively large amounts of surface IgM. The differences seen in the fluorescence patterns of adult spleen cells labeled with Fl anti-Ig and Fl anti-μ may be due to the appearance of IgD on the surface of mature splenic B lymphocytes. This is supported by the similarity of the fluorescence profiles of adult bone marrow cells stained with Fl anti-Ig and Fl anti-μ, as the latter population of cells is reported to lack surface IgD.

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References

1. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. I. Quantitative radioautographic studies of antiglobulin binding by lymphocytes in bone marrow and lymphoid tissues. Cell. Immunol. 13:117.
2. Osmond, D. G., and G. J. V. Nossal. 1974. Differentiation of lymphocytes in mouse bone marrow. II. Kinetics of maturation and renewal of antiglobulin-binding cells studied by double labeling. Cell. Immunol. 13:132.

3. Sidman, C. I., and E. R. Unanue. 1975. Development of B lymphocytes. I. Cell populations and a critical event during ontogeny. J. Immunol. 114:1730.

4. Finkelman, F. D., J. A. van Boxel, R. Asofsky, and W. E. Paul. 1976. Cell membrane IgD. Demonstration of IgD on human lymphocytes by enzyme-catalyzed iodination and comparison with cell-surface Ig of mouse, guinea pig and rabbit. J. Immunol. 116:1173.

5. Loken, M. R., and L. A. Herzenberg. 1975. Analysis of cell populations with a fluorescence-activated cell sorter. Ann. N. Y. Acad. Sci. 54:163.

6. Warner, N. L. 1974. Membrane immunoglobulins and antigen receptors on B and T lymphocytes. Adv. Immunol. 19:67.

7. Guy-Grand, D., C. Griscelli, and P. Vassalli. 1974. The gut-associated lymphoid system: nature and properties of the large dividing cells. Eur. J. Immunol. 4:435.

8. Vitetta, E. S., U. Melcher, M. McWilliams, M. E. Lamm, J. M. Phillips-Quagliata, and J. W. Uhr. 1975. Cell surface immunoglobulin. XI. The appearance of an IgD like molecule on murine lymphoid cells during ontogeny. J. Exp. Med. 141:206.

9. Ahmed, A., I. Scher, and K. W. Sell. 1976. Functional studies of the ontogeny of the M-locus product: a surface antigen of murine B lymphocytes. In Leukocyte Membrane Determinants Regulating Immune Reactivity. V. P. Eijssvoogel, D. Roos, and W. P. Zeijlemaker, editors. Academic Press, Inc., New York. 189.

10. Gelfand, M. C., G. J. Elfenbein, M. M. Frank, and W. E. Paul. 1974. Ontogeny of B lymphocytes. II. Relative rates of appearance of lymphocytes bearing surface immunoglobulin and complement receptors. J. Exp. Med. 138:1125.