

PRE-B AND B CELLS IN RABBITS

Ontogeny and Allelic Exclusion
of Kappa Light Chain Genes*By ANTHONY R. HAYWARD,‡ MARGARET A. SIMONS,§ ALEXANDER R.
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Mononuclear cells containing small amounts of cytoplasmic IgM (cIgM⁺)¹ but lacking stable surface immunoglobulin (sIg) appear in both mouse and human fetal liver before the earliest sIg⁺ B lymphocytes (1-3). These cIgM⁺·sIg⁻ cells, called pre-B cells, are the least mature members of the B-cell lineage so far identified in mammals. Well defined allotypic markers on the kappa light chains of rabbit immunoglobulins (b-allotypes) provide a means for the further analysis of pre-B and B-cell development (4). The expression of immunoglobulin molecules by rabbit B cells is controlled by regulatory and/or structural genes for the variable and constant regions of heavy, kappa, and lambda chains occurring as separate clusters at three unlinked, complex loci. The particular immunoglobulin polypeptide chains synthesized by each cell result from the activation of selected members of the linked gene clusters. In heterozygous rabbits, individual plasma cells synthesize Ig of only one of the alternative allotypes (5-6), apparently using genes inherited from one parent but not both. The mechanism of this allelic exclusion has not been established and the stage of B-cell differentiation at which it occurs is uncertain because of the conflicting results of tests for allotype exclusion by B lymphocytes.

When blood lymphocytes from b⁴b⁵ rabbits were treated with ¹²⁵I anti-b4 and anti-b5 (7), or studied by combined immunofluorescence and radioautography (8), the majority of B lymphocytes had only one sIg allotype detectable but a small percentage

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¹ Abbreviations used in this paper: cIg, cytoplasmic immunoglobulin; EAC, erythrocyte-antibody complement; FITC, fluorescein isothiocyanate; F/P, fluorescein: protein molar ratio; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; P/R, protein: rhodamine ratio; RITC, tetramethylrhodamine isothiocyanate; sIg, surface immunoglobulin.

apparently had both (8). The presence of Fc receptors on B lymphocytes which bind Ig not synthesized by the cell can complicate studies of this type. Indeed, Jones et al. (9) found by immunofluorescence that up to 63% of blood lymphocytes from adult b^5b^8 rabbits and 16% of b^4b^5 heterozygote lymphocytes had sIg of both allotypes. After pronase treatment to remove sIg and in vitro culture only 3–7% of the cells synthesized both allotypes. In contrast Schoenberg and Wolf (10) detected 8–30% of double allotype-positive cells by a rosetting method after overnight culture of pronase-stripped lymphocytes from b^4b^6 heterozygotes. Linthicum and Sell (11), using immunoelectron microscopy, found both allotypes on half of the sIg⁺ blood lymphocytes of b^4b^6 rabbits. They considered their technique capable of distinguishing endogenous from passively absorbed sIg. Recently Bessinger et al. (12) found that 22–31% of sIg⁺ blood lymphocytes of b^4b^5 rabbits had both allotypes and that double allotype-positive cells reappeared when pronase-stripped cells were cultured overnight. The proportions of double allotype-positive cells in other tissues was much lower. In these experiments the sIg allotypes were detected by rosetting with antibody-coated erythrocytes or bacteria. The differences between immunoelectron microscopy and rosetting results on one hand and fluorescence on the other remain unexplained.

In the present study the ontogeny of pre-B and B cells in rabbits has been examined to determine the sites of B-cell origin and the ontogeny of isotype diversity. In addition we have looked for allotype inclusion during B-cell development.

Materials and Methods

Rabbits and Cell Preparations. Outbred pregnant does and young rabbits were obtained from Pine Tree Rabbitry, Birmingham, Ala. Animals of known allotype came from colonies kept at the National Institutes of Health, or were a gift of Dr. Louise Adler, St. Jude's Hospital, Memphis, Tenn. The duration of pregnancy was determined from the day of mating. Animals were exsanguinated during ether or barbiturate anesthesia and the blood was defibrinated to remove platelets. Tissues were dissected immediately and single cell suspensions in Hanks' balanced salt solution with 5% fetal calf serum (HBSS) were prepared by teasing or with a ground glass tissue homogenizer. Bone marrow samples from many of the allotype-defined animals were collected into heparinized HBSS by puncture of the tibia during brief anesthesia. Cell clumps and debris were allowed to settle. The lymphocyte-containing supernates were centrifuged on Ficoll-Hypaque gradients for 15 min at 4°C and 600 g. Blood lymphocytes were separated on Ficoll-Hypaque gradients after preliminary sedimentation with an equal vol of 3% dextran to remove most of the erythrocytes.

Preparation and Labeling of Antibodies. Heavy chain specific antisera were raised in goats. IgM from the euglobulin fraction of serum of rabbits hyperimmunized with killed *Brucella abortus*, was purified by chromatography on Sepharose 6B followed by zone electrophoresis. IgG and secretory IgA were purified from serum and colostrum, respectively, as described earlier (13, 14). The purified immunoglobulins were coupled to Sepharose 2B by the cyanogen bromide method (13) for use as immunoadsorbents. The anti-heavy chain sera were absorbed on columns of the heterologous classes, then eluted as purified antibodies from the column of the corresponding class.

Anti-b4 and anti-b5 were raised by immunizing homozygous b6 rabbits with purified b4 or b5 IgG matched for other allotypes (15). F(ab')₂ fragments of the anti-b4 and anti-b5 were prepared by pepsin digestion of the globulin fraction precipitated by 50% saturated (NH₄)₂SO₄. Globulins were incubated at pH 4.5 with 2 mg pepsin/100 mg protein (Worthington pepsin lot PM 2 BA, Worthington Biochemical Corp., Freehold, N. J.) for 18 h at 37°C. Digests were dialyzed at 4°C against borate-saline, pH 7.8, before affinity purification. The anti-b4 was first passed over a Sepharose-b5 IgG column. The eluate was then adsorbed onto a Sepharose-b4 IgG column from which the antibody was eluted with glycine-HCl, pH 2.8. The reverse order of adsorption and elution was used to prepare F(ab')₂ fragments of anti-b5.

Purified anti-heavy chain and anti-allotype antibodies were conjugated to fluorescein isothiocyanate (FITC) or to tetramethylrhodamine isothiocyanate (RITC) as previously described (16). The molar F/P ratios ranged between 2.2 and 3.5 and the P/R ratios between 0.9 and 1.6. The concentration of fluorochrome-conjugated antibodies was 0.5 mg/ml for staining viable lymphocytes and 0.1 mg/ml for fixed preparations.

Immunofluorescence Staining. Viable lymphocytes aspirated from density gradients were washed three times. Samples of about 10^6 cells were incubated with fluorochrome-labeled antibodies for 20 min at 4°C for detection of sIg. The cells were then washed three times and cytocentrifuge preparations were made. These were fixed at -20°C in 95% ethanol-5% acetic acid and rinsed in phosphate-buffered saline before staining for cytoplasmic Ig for 15 min with antibodies conjugated with the contrasting fluorochrome. For detection of two immunoglobulin determinants on the surface of B lymphocytes, viable cells were stained sequentially with FITC and RITC conjugates with an intervening wash. Cytocentrifuge preparations were fixed as indicated above. Stained slides were washed in several changes of phosphate-buffered saline (PBS) and kept overnight in PBS at 4°C before mounting in Elvanol for fluorescence and phase contrast microscopy as previously described (17). Attention was paid to maintaining optimal UV illumination, and UV lamps were not used past their recommended life span. At least 200 cells were counted in all preparations; 500 cells were counted where the frequency of positives was between 2 and 5%. The entire preparation (5,000-20,000 cells) was scanned if the frequency of positive cells was less than 2%. Preparations stained for pre-B cells with anti- μ or anti-allotype reagents were counted by two or more independent observers.

Complement Receptor Test. Complement receptors were identified by rosetting with erythrocyte-antibody complement (EAC) (human) using a previously described method (18). Briefly, sheep erythrocytes were sensitized with a 1:3,000 dilution of rabbit anti-sheep cell serum (Wellcome Reagents, Beckenham, England), washed, and incubated with a subhemolytic dilution of human serum, the titer of which was determined extempore. After three washes 5×10^7 EAC were incubated with 10^6 rabbit lymphocytes in serum-free HBSS at 37°C for 15 min. After brief centrifugation the cell pellet was resuspended vigorously and the percentage of rosettes counted in a hemocytometer chamber. Under these conditions rabbit lymphocytes did not make rosettes with erythrocyte or erythrocyte-antibody alone. When lymphocytes prestained for sIgM were tested for EAC-binding, the rosettes were identified by phase contrast microscopy and the central cell examined for fluorescence.

Results

Identification of Rabbit Pre-B Cells. The cytoplasmic IgM of human and mouse pre-B cells is more difficult to detect by immunofluorescence than the sIg of B lymphocytes; the fluorescence intensity of these cells is very much weaker than that of plasmablasts and plasmacytes. We therefore paid special attention to optimizing conditions for detection of weak fluorescence, and to the identification of pre-B cells in tissues when more mature B cells were absent. In rabbit fetuses of 19 or 21 days gestational age, we could find no liver cells that stained for IgM, IgG, or IgA at either surface or cytoplasmic levels. We found large and small lymphoid cells in fetal liver at 23 days gestation and bone marrow at 25 days gestation which stained weakly but distinctly for cIgM. These preparations contained no cells having detectable sIgM, nor were cells bearing other sIg isotypes found at these ages in the corresponding tissues (see below). Detection of pre-B cells was optimal when live cells were treated first with fluoresceinated (FITC) anti- μ to detect sIgM and then, after fixation had made the cytoplasmic Ig accessible, restained with the same antibody labeled with RITC.

The frequency of pre-B cells in liver preparations reached a peak of 2% 2 days after birth, and then fell to very low levels (Table I). In a single experiment liver cells from a fetus of 23 days gestation, which contained pre-B cells but no sIg⁺ cells, generated

TABLE I
*Percentage of Pre-B Cells in Rabbit Marrow, Liver, and Spleen Preparations at Different Ages**

| Tissue | Age in days | | | | | | | | | | | |
|--------|-------------|----|-----|-----|-----|-------------|-----|-----|-----|-----|-----|-----|
| | Fetal | | | | | After birth | | | | | | |
| | 19 | 21 | 23 | 25 | 28 | 1 | 2 | 3 | 5 | 6 | 10 | >21 |
| Marrow | 0 | 0 | 0 | 0.2 | —‡ | 9 | 2.4 | 3.2 | 2.5 | 0.5 | 1.5 | 1 |
| Liver | 0 | 0 | 0.6 | 0.4 | 0.3 | 0.7 | 2 | 0.5 | 0.1 | 0.1 | — | 0 |
| Spleen | — | — | — | — | — | 0.1§ | 0 | 0 | 0 | — | — | — |

* Mononuclear cells were prepared from cell suspensions by centrifugation on Ficoll-Hypaque. Pre-B cells were identified as cIgM⁺·sIgM⁻ cells by immunofluorescence as described in the text. Fetal results are from pooled single litters; postnatal results are the means of two or three animals. At least 500 cells were counted in each preparation. Where the frequency of positives was <2% the entire preparation was scanned.

‡ Not done.

§ 2 pre-B cells were seen of 2,000 cells scanned.

a low frequency (<0.1%) of sIgM⁺ cells after incubation for 3 days (but not before) in tissue culture.

Bone marrow cells were not obtained in sufficient numbers for examination before 25 days gestation, when 0.2% of the mononuclear cells were pre-B cells. By the day of birth the frequency of pre-B cells had risen to a mean of 9%, followed by a fall to adult levels of less than 1% (Table I). An extensive search for pre-B cells in the appendix or mesenteric lymph nodes of newborn or adult rabbits failed to reveal any (data not shown). Moreover, beyond the 1st day of life, no pre-B cells were found in the spleen. Other fetal preparations were stained for sIgM and then for cytoplasmic IgG or IgA. No cIg⁺ cells of these isotypes were found before the appearance of plasma cells at 3 days of age.

Expression of Kappa Chain Allotypes in Pre-B Cells. Marrow cells from b⁴b⁵ heterozygous rabbits were stained with FITC anti-μ for sIgM, then fixed and stained with RITC anti-allotype antibodies to detect cIg. Cells negative for sIgM were found which stained faintly but unequivocally for cytoplasmic b allotype. The morphology of these cells resembled those identified by their cIgM⁺·sIgM⁻ phenotype as pre-B cells. The cIg-allotype⁺·sIgM⁻ cells were present in neonatal marrow samples before the appearance of B cells bearing sIgG or sIgA (compare Table II and Table V). Although it was not technically possible to simultaneously detect sIgM, cIgM, and cytoplasmic allotype, we conclude that the population of sIgM⁻·cIg-allotype⁺ cells found in fetal tissues must be among the population of sIg⁻·cIgM⁺ pre-B cells.

In b⁴b⁵ heterozygotes, b⁴ pre-B cells outnumbered b⁵ pre-B cells but their combined percentages were generally less than the number of pre-B cells stained by anti-μ antibodies (Table II). Marrow cells of homozygous b⁴b⁴ and b⁵b⁵ rabbits had pre-B cells of the expected allotype only and no positive cells were seen in b⁶b⁶ or b⁶b⁹ preparations stained with our anti-b⁴ or anti-b⁵ antibodies. This confirms the specificity of the alloantibodies and indicates that binding of any residual IgG in the affinity purified F(ab)₂ fragments to Fc receptor bearing cells was not detectable.

Allotype Exclusion in Pre-B Cells. Cytocentrifuge preparations of marrow cells from one 2-day-old and two 3-day old b⁴b⁵ heterozygous rabbits were fixed and then stained sequentially with RITC anti-b⁴ and FITC anti-b⁵ antibodies. Cells positive

TABLE II
Percentages of Pre-B Cells Stained With Anti- μ and Anti-Allotype Antibodies
in Marrow Preparations from b^4b^5 Rabbits*

| Percent positive for: | Donor age in days‡ | | |
|-----------------------|--------------------|----------|--------------------|
| | 2 | 3 | >21 |
| μ | 1.8, 2.8 | 2.4, 2.5 | 0.95 (\pm 0.28) |
| b4 | 1.0, 2.0 | 1.0, 0.8 | 0.63 (\pm 0.11) |
| b5 | 0.3, 1.0 | 0.8, 0.5 | 0.45 (\pm 0.1) |

* Mononuclear cells were prepared from marrow and stained as described in the text.

‡ Results are from single animals except ages >21 days for which mean observations (\pm standard error) on eight animals are given.

TABLE III
Allotype Exclusion by Mononuclear Cells from Marrow of b^4b^5 Heterozygous
Rabbits 2-3 Days Old*

| Age | Percent of cells stained for: | | |
|-------------|-------------------------------|-----|---------|
| | b4 | b5 | b4 + b5 |
| <i>days</i> | | | |
| 2 | 1.6 | 1.0 | 0.1‡ |
| 3 | 3.5 | 2.9 | 0 |
| 3 | 2.9 | 1.0 | 0 |

* Cytocentrifuged fixed marrow mononuclear cell preparations were sequentially stained for the two allotypes with purified antibodies labeled with contrasting fluorochromes without prior staining for surface immunoglobulin.

‡ 2 cells were seen that stained for both allotypes of 2,000 cells examined.

for one allotype were identified on the slides and examined for staining by the alternative fluorochrome. Of over 6,000 marrow mononuclear cells scanned, only two of 100 pre-B cells in one preparation were stained for both allotypes (Table III). In samples from the two other animals, the pre-B cells stained exclusively for one allotype. The percentage of pre-B cells identified with anti- μ was generally greater than the sum of the pre-B cells identified with the anti-b4 and anti-b5 (Table II). Part of this difference is probably due to the presence of pre-B cells expressing lambda light chains. Additional possibilities include expression of one or both b allotypes in amounts below the level of detection by our techniques, or lack of production of light chains of any type.

In these experiments we used both fluorochromes to stain fixed cells so we were unable to stain for sIg. On this account we selected animals 2-3 days old for study since pre-B cells are relatively abundant and slightly outnumber sIg⁺ B lymphocytes in the marrow at this age. It is nevertheless likely that some of the positive cells we observed were B lymphocytes rather than pre-B cells. Since we found that both marrow and spleen B lymphocytes (Table IV) exhibited allotype exclusion it is unlikely that the inclusion of some B lymphocytes in our counts of pre-B cells affects the validity of our results. Furthermore, pre-B cells from liver of heterozygous rabbits at 23-25 days of gestation exhibited allelic exclusion.

ALLELIC EXCLUSION IN PRE-B CELLS

TABLE IV
Allotype Exclusion by B Lymphocytes from the Bone Marrow and Spleens of b⁴b⁵ Heterozygous Rabbits, Shown by Immunofluorescence

| Tissue | Age days | Percent of cells stained for: | | | |
|-------------|-------------|-------------------------------|------------|------------|-----------|
| | | μ | $\mu + b4$ | $\mu + b5$ | $b4 + b5$ |
| Bone marrow | 14 | 6 | 3 | 2 | 0* |
| Spleen | 3 | 44 | 31 | 4 | 0 |
| | 21 | 28 | 19 | 10 | 0 |
| | 21 | 38 | 19 | 16 | 0 |

* No cells positive for both allotypes seen among 200 positive for sIg.

TABLE V
*Postnatal Development of B Lymphocytes Bearing Different Isotypes in Rabbits: Percentage of Cells Positive by Immunofluorescence**

| Source | Isotype | Donor age in days | | | | | |
|----------|---------|-------------------|-----|-----|-----|------|-----------|
| | | 1 | 2 | 3 | 4-6 | 7-10 | Adult |
| Blood | IgG | 0.5 | 0.5 | —‡ | 7 | 9 | 6 ± 1 |
| | IgA | 0 | 0 | — | 0 | 0.1 | 3 ± 1 |
| | IgM | 15 | 29 | 42 | 51 | 34 | 56 ± 10 |
| Spleen | IgG | 0 | 1 | 3 | 7 | 11 | 6 ± 2 |
| | IgA | 0 | 0 | 0 | 0.5 | 0.5 | 1.5 ± 0.5 |
| | IgM | 25 | 48 | 49 | 39 | 38 | 60 ± 7 |
| Appendix | IgG | 0 | 0 | 0 | 1 | 3.5 | 2 ± 0.8 |
| | IgA | 0 | 0 | 0 | 1 | 0.2 | 3 ± 1 |
| | IgM | 0 | 0 | 0 | 33 | 27 | 36 ± 7 |
| Marrow | IgG | 0 | 0 | 0 | 0.3 | 0.3 | 0.3 ± 0.2 |
| | IgA | 0 | 0 | 0 | 0 | 0 | 0.1 ± 0.1 |
| | IgM | 1.3 | 2 | 2 | 2 | 3 | 5 ± 2 |
| Liver | IgG | 0 | 0 | 0 | 0.3 | 1.5 | 2 ± 0.5 |
| | IgA | 0 | 0 | 0 | 0.1 | 1 | 3 ± 1.5 |
| | IgM | 2 | 2 | 0.5 | 7 | 5 | 7.5 ± 2 |

* Results on neonates are the means of observations on two or three animals at each age. Five adults were studied, and means ± standard error are presented.

‡ Not done.

Development of sIgM⁺ B Lymphocytes. A single B lymphocyte was found among over 1,000 mononuclear cells scanned from the liver at 25 days gestation, giving a frequency of <0.1%. This is close to the time at which B cells appeared in 23 days gestation liver cultured in vitro for 3 days (23 + 3 = 26 days equivalent). By 28 days gestation sufficient blood lymphocytes were obtained for counting, and 26% of these had sIgM. The postnatal development of sIg⁺ lymphocytes is summarized in Table V. Although the percentages of sIg⁺ cells in the spleen and appendix declined slightly between days 4 and 14 the weights of each tissue increased four to fivefold in the same period, so the absolute number of B cells they contained would not have fallen. Less than 2% of marrow lymphocytes had sIgM in the 1st wk of life; the proportion in adults (mean 5%) was higher.

Development of B Cells With sIgG and sIgA. The first sIgG⁺ cells were found in the blood on the day of birth; later they appeared in other tissues (Table V). All the

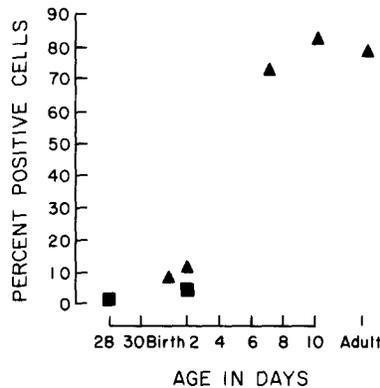


FIG. 1. Percentage of sIgM⁺ B lymphocytes in liver (▲) and spleen (■) at different ages which rosetted with EAC. Cells were prepared and tested as described in the Materials and Methods section.

sIgG⁺ cells in the blood and spleen of 6 and 10-day-old rabbits were found by double staining with FITC-anti- μ and RITC-anti- γ to be positive for sIgM. By 28 days only 14% of spleen sIgG⁺ cells had sIgM, and this fell to between 0 and 10% in the blood of adult rabbits, and 3% in the spleen.

A single sIgA⁺ lymphocyte was seen among 500 mononuclear cells counted from the liver of a 5-day-old rabbit. On day 6 sIgA⁺ cells were present at low frequency (<1%, Table V) in the appendix, spleen, and liver. They were not found in the blood until 10 days of age. In double-staining experiments the spleen and liver sIgA⁺ lymphocytes of 8-day-old rabbits were negative for sIgG and sIgM.

Development of Complement Receptors on B Cells. Although mature B cells, among other cell types, have receptors for C3, these have been found to be lacking on the B cells of immature mice (19). We therefore examined the sIgM⁺ cells of developing rabbits for complement receptors. 10% of sIgM⁺ spleen cells at 2 days of age bound EAC, and this combination rose to 80–90% after the 1st wk of life (Fig. 1). Complement receptor-positive cells accounted for 29% of mononuclear cells in the fetal liver preparations at 28 days gestation but these cells did not have sIgM.

Discussion

Our observations indicate that primary B-cell development in rabbits resembles that of mouse and man. This analogy is strongly supported by similarities in the ontogenetic development and tissue distribution of pre-B cells among the three species.

Mouse pre-B cells appear in the fetal liver at 12–13 days of a 20-day gestation, about 4 days before the first B cells (1, 2). Human pre-B cells are present in the fetal liver before the marrow becomes hemopoietic, and before B lymphocytes appear (3). During fetal life, pre-B cells are found in low frequency in various lymphoid tissues, while in adults they are almost exclusively confined to the bone marrow. In both species pre-B cells lack sIg by fluorescence staining and the intensity of their cytoplasmic staining is much less than that of plasma cells. The distinction from plasma cells is facilitated in ontogeny since the latter first appear some time after B lymphocytes. Furthermore, mouse B lymphocytes and plasma cells are eliminated by *in vivo* injections of anti- μ antibodies while pre-B cells survive (20).

Rabbit pre-B cells appeared at 23 days gestation in the liver and at 25 days in the

marrow. B lymphocytes were found first at 25 days or 26 days equivalent in tissue culture of 23 days fetal liver. These observations are compatible with previous results in mouse and human fetuses. Pre-B cells were not found in the appendix or mesenteric lymph nodes and only very rarely in the spleen, so it is unlikely that these lymphoid tissues are sites of primary B-cell generation. No rabbit pre-B cells contained cytoplasmic IgG or IgA but, because the frequency of pre-B cells is low, we cannot exclude the possibility that rare cells might contain two or more isotypes.

In common with mice and men, the first rabbit B lymphocytes had sIgM. sIgG⁺ or sIgA⁺ lymphocytes appeared later, at 1 and 5-6 days of life, respectively. The appearance of sIgM⁺ lymphocytes before sIgG⁺ cells has previously been reported by Nowygrod et al. (21), and in this respect our observations confirm these authors' work. The proportion of sIg⁺ cells we found in the blood and tissues of newborn and young rabbit are also compatible with those found by Harrison and Mage (22). The sIgG⁺ lymphocytes of young rabbits were also sIgM⁺, while in older animals the majority of sIgG⁺ cells were singles. Similar observations have been made in both man (3) and mouse (23). In contrast, sIgA⁺ cells were negative for sIgG and sIgM, although such combinations occur in man (3). Possibly the amounts of sIgA on young rabbit B cells bearing multiple isotypes would be below the threshold for detection. The delayed and simultaneous appearance of sIgA⁺ cells in spleen and appendix of rabbits we studied may indicate that we detected IgA memory cells, which might be expected to have proportionally more IgA on their surface.

The delay in appearance of complement receptors on developing rabbit B cells resembles results obtained in mice (19). Since the earliest B cells in both species lack C3 receptors there is no direct evidence that the C3 receptor-positive cells which outnumber sIg⁺ cells in the spleen of newborn rabbits are B-cell precursors. We found rather fewer C3 receptor-positive cells in the spleens of 2-day-old rabbits than did Elfenbein and co-workers (24). This difference may have arisen from our use of Ficoll-Hypaque gradients to prepare lymphocytes from the tissues or from other technical differences. The present data on EAC-rosetting cells were obtained using human complement whereas Elfenbein et al. used mouse complement.

In b⁴b⁵ rabbits we did not find pre-B or B lymphocytes which expressed both allotypes in any significant number. Cells from homozygous animals had only the allotypes expected from the genotype. These results confirm the specificity of the reagents, but they provide no support for the generality of the observation that one rabbit synthesized three different b allotypes during hyperimmunization (25). However, low levels of unexpected b allotypes which may be detected by sensitive radioimmunoassay methods in healthy rabbits (Francis and Mandy, unpublished observations; and cited by Strosberg in reference 26) might be undetectable by fluorescence techniques. The striking differences in the amino acid sequences of constant regions of different kappa chain allotypes leave open the possibility that allotypic behavior in breeding studies results from the action of regulatory rather than structural genes. The finding of allotype exclusion among pre-B cells suggests that preferential activation or selective repression of the kappa chain allotype genes has taken place at this or an earlier stage in cell development. In the b⁴b⁵ animals described here, there were more b⁴ than b⁵ pre-B cells. This trend has been established for B lymphocytes and serum immunoglobulins. Since pre-B cells lack detectable receptors for antigen (sIg), the ratio of b⁴ to b⁵ is likely to be genetically regulated.

Drastic alterations in serum b4 and b5 allotype levels result from administration of antibody to the paternal haplotype to neonatal b⁴b⁵ rabbits (allotype suppression, 27). We are currently investigating the effect of suppression of pre-B cells. In our studies to date, pre-B cells which stained for the suppressed allotype were found in marrow preparations of all the suppressed animals even when there were no B cells of the suppressed allotype detectable (28).²

Summary

Pre-B cells in developing rabbits were identified by immunofluorescence as cells containing small amounts of cytoplasmic IgM (cIgM) but lacking surface immunoglobulin (sIg). During ontogeny the first pre-B cells appeared in fetal liver at 23 days gestation, 2 days before the appearance of sIgM⁺ B lymphocytes. Pre-B cells were relatively frequent in fetal and adult bone marrow, but were not found in other tissues except rarely in fetal spleen. Allelic exclusion is apparently established at this early stage of development, because individual pre-B cells and B lymphocytes from heterozygous rabbits expressed only one of the alternative alleles in amounts sufficient for detection.

Development of isotype diversity among rabbit B lymphocytes followed the general pattern seen in mouse and man. sIgM⁺ cells were detected before birth. Expression of sIgG was detected in neonatal rabbits on cells which were also sIgM⁺ but in older animals most sIgG⁺ cells lacked sIgM. Cells bearing sIgA were not found until 5–6 days of age, and had no other isotype on their surface.

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