ONTOGENY OF B LYMPHOCYTES

II. RELATIVE RATES OF APPEARANCE OF LYMPHOCYTES BEARING SURFACE IMMUNOGLOBULIN AND COMPLEMENT RECEPTORS

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The class of mammalian lymphocytes equivalent to the bursa-derived (B) lymphocytes of birds may be identified by the presence of large amounts of immunoglobulin (Ig) on the surface of most of these cells (1, 2). In addition, many of these B lymphocytes, but not thymus-derived (T) lymphocytes, bear a surface receptor for an activated component of C3 allowing them to bind antigen-antibody-complement complexes (3). These cells are referred to as complement receptor lymphocytes (CRL).

Although no functional role for the C receptor of B lymphocytes has yet been established, at least two major possibilities have been suggested. Firstly, the C receptor by binding antigen-antibody-complement complexes may serve as an antigen concentrating mechanism and thus increase the likelihood that antigens would interact with specific receptors on the surface of immunocompetent B lymphocytes and then activate these cells (4). Alternatively, it has been proposed that B-lymphocyte activation requires the interaction of that cell both with specific antigen and with another ligand leading to the generation of two signals within the cell. A major candidate for the ligand generating the so-called “second signal” would be activated C3 binding to the C receptor (5). In such a model the binding of activated C3 would be crucial to B-cell stimulation.

In view of the potential importance of the C receptor in B-lymphocyte function, we have initiated studies on the development of this receptor in the neonatal period. Our results demonstrate that Ig-bearing lymphocytes lacking C receptors appear in large numbers in the spleens of mice during their first few days of life. Lymphocytes with surface Ig and with C receptors are not found until considerably later, although the pace of appearance of cells

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1 Abbreviations used in this paper: AAC, antigen-antibody-complement; B, bursa derived; BSA, bovine serum albumin; CRL, complement receptor lymphocytes; EA, sheep erythrocytes sensitized with antibody; EAC, sheep erythrocytes sensitized with antibody and mouse complement, LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; poly I-C, polyinosinic-cytidilic acid.
with the C receptor varies among mouse strains. In particular, some strains (AKR, A, and SJL) have substantial numbers of CRL at 2 wk of age, whereas other strains (DBA/2, BALB/c, and C57BL/6) have very few CRL at this age. The rate of appearance of CRL appears to be under precise genetic control. Analysis of CRL frequency in backcross progeny of (AKR × DBA/2)F₁ and DBA/2 mice suggests that two independent genes control the rate of appearance of CRL. Furthermore, the relative pace of appearance of Ig-bearing lymphocytes and CRL in irradiated mice reconstituted with syngeneic bone marrow is similar to their rate of appearance during normal development, suggesting that these genes play an important role in B-cell development throughout life.

**Materials and Methods**

**Animals.**—A/HeN, AL/N, AKR/N, BALB/CanN, C3H/HeN, C57BL/6N, DBA/2N, and (BALB/c × DBA/2)F₁ [CDF₁] mice were obtained from the NIH Animal Production Section, Bethesda, Md. A/HeJ, AKR/J, DBA/2J, SJL/J, and (AKR × DBA/2)F₁ [AKD2] mice were obtained from Jackson Laboratories, Bar Harbor, Maine. (AKR × C57BL/6)F₁ [AKB6] and F₂ mice, and progeny of AKR × AKD2 and of DBA/2 × AKD2 crosses were bred in our own animal quarters.

**Spleen Cell Suspensions.**—Mice were sacrificed by cervical dislocation and spleens immediately removed. Single spleen cell suspensions were prepared by teasing with fine forceps and passing cells through a fine mesh screen. For immunofluorescence studies, erythrocytes were lysed with isotonic Tris-buffered NH₄Cl (6).

**Preparation of Sheep Erythrocytes (E) Sensitized with Antibody (A) and Mouse Complement (C).**—Sheep erythrocytes sensitized with antibody and mouse complement (EAC) were prepared according to the method of Bianco et al. (3). Sheep erythrocytes were washed in 0.15 M NaCl solution and then incubated for 30 min at 37°C with the 19S fraction of rabbit antibody to boiled erythrocyte stromata as previously described (7). Such EA were washed and made up to a 5% suspension in veronal-buffered saline. Equal volumes of this 5% EA suspension were incubated for 30 min at 37°C with a 1:20 dilution of fresh serum from CDF₁ mice as a source of complement.

The EAC were washed twice in veronal buffer, once in EDTA, and brought to a concentration of 1 × 10⁶ cells/ml in EDTA buffer. The spleen cells to be evaluated for C receptors were brought to a final concentration of 5 × 10⁶/ml in EDTA buffer.

This procedure, the formation of rosettes in EDTA buffer using EAC prepared with 19S antibody, precludes the formation of rosettes with macrophages through either the C receptor or the Fc receptor of such cells because the macrophage C receptor is Mg²⁺ dependent and the Fc receptor does not interact with Ig of the γM class (8). Moreover, the antigen-antibody complex receptor of B lymphocytes does not cause rosette formation with the EAC (or EA) used here (9). Thus, rosette formation occurs exclusively with lymphocytes through interaction of EAC with C receptors.

**Rosette Formation.**—Equal volumes (0.3–0.5 ml) of EAC and spleen cell suspension were mixed and placed on a rotating wheel for 30 min at 37°C. A drop of the suspension was placed on a slide, covered with a petrolatum-sealed cover slip, and observed at 400X magnification in a standard light microscope. The number of rosetted cells (lymphocytes with three or more surrounding EAC’s) and the total number of cells counted were recorded. At least 300 cells/sample were evaluated.

**Preparation of EAC Rosettes with Spleen Cells Preincubated in Plasma.**—Spleen cells from 2-wk old and adult BALB/c mice were incubated at 50 × 10⁶/ml for 1 h at 37°C in undiluted plasma from 2-wk old or adult BALB/c mice. The plasma was separated from blood...
collected in the presence of $10^{-2}$ M EDTA. After incubation, the cells were washed three times and brought to a concentration of $5 \times 10^6$ ml in EDTA buffer. Rosettes were then formed as described above.

Detection of Ig-Bearing Lymphocytes.—Ig-bearing spleen cells were detected using a fluoresceinated rabbit antimouse k-chain antibody. The serum, which was the gift of Dr. R. Mage (NAID, NIH), was prepared by immunizing a panel of rabbits with a mixture of several k-type Bence Jones proteins. The antiserum was purified by absorption to and elution from agarose affinity columns to which a mouse k-type myeloma protein of the $\gamma_1$-class had been coupled. The purified antibody was fluoresceinated by the method of Wood et al. (10), and absorbed with an equal volume of mouse spleen cells. Fluoresceinated anti-k antibody (50 $\mu$l) was added to $5 \times 10^6$ spleen cells. The cells were incubated at 4°C for 30 min and then washed three times through 5% bovine serum albumin (BSA). After the third wash, the pelleted cells were suspended in two drops of medium and examined in the live state with a Leitz Ortholux microscope equipped with an incident light fluorescence illuminator (E. Leitz, Inc., Rockleigh, N. J.). Cells were first located by bright-field illumination with a tungsten light source, and the presence of fluorescence then noted after switching to the ultraviolet illuminator. The number of fluorescent small round cells and the total number of small round cells counted were recorded.

Detection of Ig on CRL.—To detect lymphoid cells bearing both Ig and the receptor for C3, spleen cells were stained with fluorescent anti-$\xi$ in the usual manner and then incubated with EAC to form rosettes. Small round cells were located by bright-field illumination. These cells were classified as having formed or not formed rosettes and then, by switching to the ultraviolet light source, the presence or absence of surface fluorescence was noted. In control experiments, the frequency of Ig-bearing cells and of CRL as determined by single- and double-labeling procedures was compared and found to be similar.

Preparation of BSA-Anti-BSA-Complement (Antigen-Antibody-Complement [AAC]) Complexes and Detection of AAC-Binding Cells.—AAC complexes were prepared according to the method of Eden et al. (11). Mouse anti-BSA serum (the kind gift of Dr. V. Nussenzweig) was diluted 1:2 and 0.48 ml incubated with 2.4 ml of ultracentrifuged $^{125}$I-labeled BSA (5 $\mu$g/ml; 0.7 $\mu$Ci/$\mu$g) for 30 min at 37°C. As a source of C, 0.17 ml of fresh CDF1 serum was added and the mixture incubated for 30 min at 37°C. $5 \times 10^6$ lymphoid cells in 0.1 ml were added to 0.05 ml of AAC. The mixture was then incubated at room temperature for 40 min with frequent agitation. After four washes with phosphate-buffered saline, the cells were placed on a gelatin-coated slide, air dried, dipped in nuclear track emulsion (NTB-2, Eastman Kodak Co., Rochester, N. Y.), and exposed for 4–5 days. Lymphoid cells with five or more surrounding grains detected by microscopic evaluation of slides stained with methyl green-pyronin were considered to have bound AAC.

Preparation of Irradiated Mice and Reconstitution with Syngeneic Bone Marrow Cells.—8- to 12-wk old mice received 750 R of total body irradiation from a Westinghouse 250 KV (Peak) X-ray machine (Westinghouse Electric Corp., Pittsburgh, Pa.). 1 day later these animals were given intravenous injections of $20 \times 10^6$ syngeneic bone marrow cells. 1, 2, and 3 wk after reconstitution, the mice were sacrificed and the frequencies of splenic Ig-bearing lymphocytes and CRL determined.

RESULTS

The frequency of Ig-bearing lymphocytes in spleens of BALB/c mice increases rapidly in the neonatal period. Immediately after birth, such cells constitute approximately 4% of the splenic lymphocytes. By 3 days of age,

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2 Gelfand, M. C., R. Asofsky, and W. E. Paul. 1974. Ontogeny of B lymphocytes. I. In vitro appearance of Ig-bearing lymphocytes. Manuscript submitted for publication.
approximately 24% of spleen lymphocytes bear surface Ig and adult percentages of Ig-bearing lymphocytes are attained by 2 wk of age (Table I). On the other hand, lymphocytes capable of forming rosettes with EAC are not detected (<1%) in the spleens of 3-day or 1 wk old BALB/c mice and only 2.6% of splenic lymphocytes of 2-wk old BALB/c form rosettes with EAC. Thereafter, the frequency of CRL increases, reaching adult levels at 6 wk of age (Table I). Thus, in the development of the B-lymphocyte population of BALB/c mice, there is a substantial interval between the appearance of surface Ig and the appearance of C receptors as judged by the capacity to form rosettes with EAC.

The low frequency of CRL, in contrast to that of Ig-bearing lymphocytes, in the spleens of 2-wk old BALB/c mice is also observed in a series of other inbred strains (Fig. 1). Thus, in the spleens of the 2-week old C57BL/6, DBA/2, and CDF1 mice, fewer than 6% of the lymphocytes are CRL. We will refer to such strains as “low CRL” strains. In sharp contrast, 27.9% of the splenic lymphocytes of 2-wk old AKR mice bear C receptors. Intermediate levels of CRL are observed in A/He, SJL, (AKR X C57BL/6)F1, and (AKR X DBA/2)F1 mice.

Because of the striking difference in the frequency of CRL in the spleens of 2-week-old BALB/c and AKR mice, we examined the ontogeny of Ig-bearing lymphocytes and CRL in AKR mice more completely. These mice display a lag between the appearance of surface Ig and C receptors on lymphocytes as did the BALB/c mice. This is shown by the frequencies of Ig-bearing lymphocytes (25.0) and CRL (1.5%) at 3 days of age (Table I). However, by 1 wk of age, AKR mice have a high frequency of CRL in their spleens (16.5%) while in BALB/c mice of this age fewer than 1% of spleen lymphocytes bear C receptors. As noted previously (Fig. 1), the frequency of CRL in 2-week old AKR is 27.9% while that of 2-week-old BALB/c is 2.6%.

### TABLE I

Appearance of CRL and Ig-Bearing Lymphocytes in Spleens of BALB/c and AKR Mice

| Age   | BALB/c |       | AKR |       |
|-------|--------|-------|-----|-------|
|       | CRL    | CRL   |     | CRL   |
|       | %      | %     | %   | %     |
| 3 days| 23.8   | <1    | 25.0| 1.5 ± 1.0 |
| 1 wk  | 35.8   | <1    | 38.3| 16.5 ± 2.5 |
| 2 wk  | 46.5   | 2.6 ± 0.4 | 34.0| 27.9 ± 2.5 |
| 4 wk  | 38.8   | 13.5 ± 1.0 | 42.2| 36.8 ± 3.5 |
| 6-12 wk | 37.3 | 25.2 ± 4.8 | 47.3| 42.0 ± 3.8 |

At the indicated ages, mice were sacrificed and spleens evaluated for the frequencies of CRL and Ig-bearing lymphocytes. CRL data are presented as mean ± standard error of the mean.
In order to verify both the low levels of lymphocytes with C receptors in the spleen of young low CRL mice such as BALB/c and DBA/2, and the differences between such young mice and young AKR mice, a series of additional experiments were performed. The possibility was considered that cells from 2-wk old low CRL mice failed to form rosettes with EAC not because they lacked C receptors but because of an inability to form rosettes. To study this point, the relative capacity of lymphocytes from "high" and low CRL strains to bind soluble AAC complexes was determined. Soluble AAC complexes were prepared using $[^{125}\text{I}]$BSA as antigen, mouse anti-BSA antiserum as antibody, and mouse serum as a source of complement. These complexes were mixed with spleen cell suspensions at room temperature for 40 min. The cells were extensively washed, autoradiographs were prepared, and the frequency of labeled cells determined. On the same cell preparations, the frequency of cells forming rosettes with EAC was also measured. As shown in Table II, quite similar results were obtained with the two procedures for detecting lymphocytes with C receptors. Specifically, only 3% of spleen cells from 2-wk old DBA/2 mice were labeled by AAC and only 1% formed rosettes with EAC, while 26% of 2-wk old AKR spleen cells bound AAC and 18% formed EAC rosettes. This experiment indicates that young DBA/2 mice fail to form rosettes with EAC because they lack sufficient C receptors to do so, and not because of any physical difficulty in forming rosettes.

A second possible explanation for the difference between young and adult mice and between low and high CRL strains in the frequency of CRL is that the density of C on EAC which is required for rosette formation might differ in these cases. To study this point, we prepared EAC with a 1:2 dilution of mouse serum and also with a 1:20 dilution. The latter is the serum concen-
Lymphoid cells binding BSA-anti-BSA-complement complexes were detected as follows: Mouse anti-BSA serum diluted 1:2 was incubated with $^{125}$I-BSA for 30 min at 37°C after which fresh mouse serum (as a source of complement) was added and the mixture reincubated for 30 min at 37°C. $5 \times 10^6$ cells in 0.1 ml were incubated at room temperature for 40 min with 0.05 ml of the BSA-anti-BSA-complement mixture, washed four times, spread on gelatin-coated slides, and prepared as autoradiographs. Lymphoid cells with five or more grains were considered to have bound AAC. Lymphoid cells forming rosettes with EAC were detected on samples of the same spleen cell preparation used to determine the frequency of AAC-binding cells.

Table II

| Strain    | Age | Binding of soluble ACC complexes | Rosette formation |
|-----------|-----|----------------------------------|-------------------|
|           |     | Pos./Total (%)                   | Pos./Total (%)    |
| DBA/2     | 2   | 20/600                           | 3/300             |
| AKR       | 2   | 154/600                          | 108/600           |
| AKD2(F1)  | 2   | 76/400                           | 44/250            |
| AKB6(F1)  | 2   | 50/400                           | 54/250            |
| C57BL/6   | 12  | 166/600                          | 157/500           |

The low frequency of CRL in the spleens of 2-wk old BALB/c mice might have resulted from the presence of a plasma factor which prevents rosette formation. Such a plasma factor might be lacking in young AKR mice and adult mice of all strains. To investigate this possibility, spleen cells from 2-wk old and adult BALB/c mice were incubated in plasma from 2-wk old or adult BALB/c mice before rosette formation. Preincubation of adult spleen cells in plasma from 2-wk old mice did not affect the frequency of rosette-forming cells (Table IV) indicating that an inhibitory plasma factor is not likely to account for the low levels of CRL in young BALB/c mice.

It has been recently reported (12) that preincubation of human lymphoid cells with 2-mercaptoethanol (2-ME) increases the frequency of cells which form rosettes with EAC. To rule out the possibility that mercaptoethanol pretreatment of spleen cells from 2-wk old low CRL mice might increase the frequency of CRL to a level similar to that of 2-wk old AKR or even adult mice, we incubated spleen cells from 2-wk old and adult DBA/2 and AKR mice with $10^{-5}$ M 2-ME. Such pretreatment did not increase the frequency of rosette-forming lymphocytes in any of the populations tested (Table V).
TABLE III

Frequency of CRL using EAC prepared with serum at different concentrations and from different sources

| Cell donor (age) | Serum donor | Serum dilution | CRL |
|------------------|-------------|----------------|-----|
|                  | Strain      | C5             |     |
| C3H (2 wk)       | CDF<sub>1</sub> | 1:20           | 4.4 |
| C3H (2 wk)       | CDF<sub>1</sub> | 1:20           | 3.2 |
| DBA/2 (2 wk)     | CDF<sub>1</sub> | 1:20           | 5.6 ± 0.6 |
| DBA/2 (2 wk)     | AKR         | 1:20           | 6.2 ± 0.2 |
| AKR (2 wk)       | CDF<sub>1</sub> | 1:20           | 23.5 ± 1.6 |
| AKR (2 wk)       | AKR         | 1:20           | 24.4 ± 2.4 |
| BALB/c (14 wk)   | CDF<sub>1</sub> | 1:20           | 40.8 |
| BALB/c (14 wk)   | AKR         | 1:20           | 41.1 |

The frequency of spleen cells forming rosettes with EAC prepared using the dilution and source of serum noted was determined by counting 300 cells/sample. In the comparison of CDF<sub>1</sub> and AKR serum for rosette formation by 2-wk old DBA/2 and AKR spleen cells, four individual animals of each strain were tested. The results are presented as mean ± standard error.

TABLE IV

CRL Frequency in BALB/c Mice After Incubation with Plasma

| Age of cell donor | Source of plasma | None | 2 wk old BALB/c | Adult BALB/c |
|-------------------|------------------|------|-----------------|--------------|
| 2 wk              |                  | 16/301 (5.3) | 18/301 (6.0) | 15/300 (5.0) |
| Adult             |                  | 105/300 (35.0) | 138/333 (41.4) | 116/300 (38.7) |

Spleen cells from BALB/c mice were incubated for 1 h at 37°C in undiluted plasma from 2-wk old or adult mice, washed three times, and then the capacity to form rosettes with EAC determined. The number of CRL and the total number of lymphocytes counted are shown with the per cent of CRL in parenthesis.

TABLE V

Effect of 2-Mercaptoethanol Pretreatment on CRL Frequency in Two-wk Old DBA/2 and AKR Mice

| Cell Donor     | Pretreatment | None | 2-ME |
|----------------|--------------|------|------|
| 2-wk old DBA/2 |              | 10/400 (2.5) | 11/420 (2.5) |
| Adult DBA/2    |              | 153/400 (38.2) | 226/800 (28.3) |
| 2-wk old AKR   |              | 90/400 (22.5) | 105/400 (25.1) |
| Adult AKR      |              | 235/600 (39.1) | 90/400 (22.5) |

Spleen cells from 2-wk old and adult DBA/2 and AKR mice were incubated with 10⁻⁵ M 2-ME for 45 min and washed three times. Frequency of CRL was determined in the usual manner.
The difference between AKR mice and low CRL strains in the rate of appearance of C receptors might reflect either a genetic or environmental difference between these animals. Before an examination of the genetics of CRL appearance, we attempted to rule out the possibility that the differences were due to environmental influences. Thus, we obtained AKR mice from Jackson Laboratories and from the Animal Production Center, NIH at 2 wk of age. In addition, a group of pregnant AKR mice delivered and reared their young in our animal quarters. The frequency of splenic lymphocytes bearing C receptors in 2-wk-old AKR mice from these three sources were identical.

Similarly, we evaluated the effect of environmental influence on the appearance of CRL by determining the frequency of CRL and of Ig-bearing lymphocytes in the spleens of germ free BALB/c mice at 2, 3, and 4 wk of age (Table VI). These mice were kindly provided by Dr. Richard Asofsky (NAID, NIH).

### Table VI

| Age   | k⁺ CRL | CRL |
|-------|--------|-----|
|       | Pos./total (%) | Pos./total (%) |
| 2 weeks | 70/152 (46) | 22/550 (4) |
| 3 weeks | 84/200 (42) | 39/300 (13) |
| 4 weeks | 51/150 (34) | 69/333 (21) |

Germ-free BALB/c were sacrificed at the indicated ages, and the frequencies of splenic CRL and k⁺-bearing lymphocytes determined.

The results obtained were not appreciably different from our results with conventional BALB/c mice (Table I), suggesting that environmental influences have little effect on the rate of appearance of CRL.

The genetic control of the age of CRL appearance was studied by determining the proportion of lymphocytes with C receptors in the spleens of 2-wk old progeny of crosses between (AKR X DBA/2)F₁ and AKR mice and between (AKR X DBA/2)F₁ and DBA/2 mice, and comparing the CRL frequencies to those of AKR (high), DBA/2 (low) and AKR X DBA/2 (“intermediate”) mice. In addition, the frequency of CRL in spleens of 2-wk old F₂ mice from (AKR X C57BL/6)F₁ matings was determined.

As noted previously, and as shown in Fig. 2, 2-wk old (AKR X DBA/2)F₁ mice have a CRL frequency in their spleen (14.8%) which is intermediate between that of the AKR (27.9%) and DBA/2 (4.8%) parents. Progeny of parental X F₁ crosses have CRL frequencies consistent with a relatively simple genetic control of this phenotype. Thus, 15 of 51 offspring of DBA/2 X F₁ matings have CRL frequencies similar to those of the DBA/2 parent while the frequency of CRL in the remaining mice fall within the F₁ range. The
Fig. 2. Percent of lymphocytes bearing C receptors in spleens of individual 2-wk old AKR, (AKR × AKD2), (AKR × DBA/2)F₁ [AKR2], (DBA/2 × AKD2), and DBA/2 mice. Each point is the frequency of CRL in an individual mouse.

Assignment of the 15 mice to the DBA/2 category depended on their being included within the range of values observed for DBA/2 mice and falling more than two standard deviations below the mean value for F₁ mice. No relation between sex and CRL frequency was noticed. The frequency of “DBA/2-type” mice among the progeny of DBA/2 × F₁ crosses (29.4%) is more similar to the frequency anticipated for two independent genes (25%) than to the frequencies expected for either one (50%) or three (12.5%) genes.

An analysis of the progeny of AKR × AKD₂ crosses is consistent with this conclusion. In this case, the range of CRL frequency in the parental types (AKR and AKD₂) overlaps. If the progeny with CRL frequency greater than two standard deviations above the AKD₂ mean are taken as “AKR type,” then 2 of 16 progeny would be in this class. On the other hand, if all progeny within the range of AKR are taken as “AKR” type, then 7 of 16 would be included. Because of overlap of the ranges, the former value is likely
to be an underestimate and the latter an overestimate of the frequency of "AKR" type in the offspring. Nonetheless, neither frequency is significantly different from 25% (P > 0.05).

24 F$_2$ progeny of (high X low)F$_1$ matings, (AKR X C57BL/6)F$_1$ X (AKR X C57BL/6)F$_1$, were analyzed at 2 wk of age for CRL frequency. Of these, two had frequencies of CRL clearly within the low range (3.3 and 4.5%) while the remainder had frequencies within the intermediate or high range (9.3% or more) (Table VII). This frequency of low CRL type (2/24; 8.3%) is similar to the frequency anticipated for two independently segregating genes (6.25%). However, the frequency is not significantly different from that anticipated for either one (25%) or three (1.56%) genes, presumably because of the relatively small numbers of animals studied.

The clear genetic control of the rate of appearance of CRL in young mice might be operative only in the perinatal period. Alternatively, these genes may regulate the basic pattern of B-lymphocyte differentiation throughout life. In order to evaluate these possibilities, we compared the capacity of bone marrow cells from C57BL/6 and CDF$_1$ mice (low CRL strains) and from AKR mice (a high CRL strain) to reconstitute Ig-bearing lymphocyte populations and CRL populations in the spleens of irradiated syngeneic animals. C57BL/6, CDF$_1$, and AKR mice received 750 R of X irradiation. 1 day later, 20 X 10$^6$ syngeneic bone marrow cells were infused and after 1, 2, and 3 wk the mice were sacrificed and the frequencies of lymphocytes bearing C receptors and surface Ig were determined.

The pace of reconstitution of CRL in AKR mice is clearly faster than in either C57BL/6 or CDF$_1$ animals (Table VIII). 2 wk after reconstitution, 25.8% of AKR spleens formed EAC rosettes whereas 2.1% of C57BL/6 and 6.5% of CDF$_1$ spleens formed such rosettes. The frequency of Ig-bearing lymphocytes in CDF$_1$ and AKR mice was similar at this time. Thus, the pattern of B-lymphocyte reconstitution of irradiated animals resembles the ontogenetic pattern in neonatal animals and suggests that the genes governing rate of appearance of CRL have a continuing role in B-lymphocyte development.

| TABLE VII |
| Frequency of CRL in 2-wk Old F$_2$ Progeny of (AKR X C57BL/6) F$_1$ Mice |
|-----------------|------------------|
| **Individual Values** | **Frequency** |
| Low CRL type (<6.6%) | 3.3; 4.5 | 2/24 (8.3%) |
| Intermediate and high CRL type | 9.3; 11.3; 11.4; 12.0; 12.3; 13.2; 14.0; 14.7; 14.7; 15.0; 15.3; 15.3; 15.7; 17.1; 18.0; 18.7; 21.3; 23.0; 30.0 | 22/24 (91.7%) |
TABLE VIII
Appearance of CRL and Ig-Bearing Lymphocytes in Irradiated Mice Reconstituted with Syngeneic Bone Marrow

| Strain      | 1 wk | 2 wk | 3 wk |
|-------------|------|------|------|
|             | κλ  | CRL  | κλ  | CRL  | κλ  | CRL  |
|             | %   | %    | %   | %    | %   | %    |
| C57BL/6     | 6   | 0.6  | 36  | 3.3  | 80  | 25   |
|             | 12  | 0.3  | 7   | 1.0  | 59  | 36   |
|             | 5   | ND*  | ND  | 1.6  | 46  | 34   |
|             | ND  | 2.4  | ND  | 44   | ND  | 44   |
| Mean        | 7.7 | 0.5  | 2.1 | 61.6 | 34.8|
| CDF1        |     |      |     |      |     |      |
|             | 42  | 5.4  |     |      |     |      |
|             | 44+ | 8.0+ |     |      |     |      |
|             | 44+ | 6.0+ |     |      |     |      |
| Mean        | 43  | 6.5  |     |      |     |      |
| AKR         |     |      |     |      |     |      |
|             | 14  | 6    | 43  | 31   | 64  | 37   |
|             | 26  | 23   | 53  | 14   | 65  | 43   |
|             | 16  | 3    | 41  | 20   |     |      |
| Mean        | 18.7| 10.7 | ND  | 38   | 64.5| 40   |

On the day after 750 R whole body irradiation, mice were given 20 × 10⁶ syngeneic bone marrow cells intravenously. At the indicated times after reconstitution, mice were sacrificed and the spleens evaluated separately for the frequencies of CRL and κ-bearing lymphocytes.

* ND, not done.

† Frequency of κ-bearing cells in pooled sample from two donors; CRL frequency was measured on individual samples.

The control exerted by these genes may involve the regulation of the proportion of distinct B-lymphocyte lines with characteristic phenotypes (e.g. surface Ig⁺, C receptor⁺; surface Ig⁺, C receptor⁻; surface Ig⁻, C receptor⁻). Alternatively, B lymphocytes may be of only a single lineage but acquire various surface markers according to a genetically determined schedule. As an initial step in studies on this issue, we determined whether the CRL of young AKR mice had surface Ig. That is, we asked whether the distinction between young mice of high and low CRL strains was that high CRL strain mice possessed surface Ig⁻, C receptor⁺ lymphocytes.

Splenic lymphocytes from 14- to 17-day old, 4-wk old, and 6- to 12-wk old AKR mice were simultaneously evaluated for surface Ig and C receptors by staining with fluorescent-labeled anti-κ followed by rosette formation. Rosettes were located under tungsten bright-field illumination and fluorescence determined by switching to ultraviolet epillumination. In each group evaluated, the great majority of CRL also had detectable surface Ig (Table IX). Indeed, of 349 CRL evaluated, all but 4 were found to fluoresce with anti-κ. In one
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TABLE IX

Presence of Surface Immunoglobulin on CRL in AKR Mice

| Age          | No. of CRL Counted | No. of Ig+ CRL | No. of Ig- CRL |
|--------------|--------------------|----------------|---------------|
| 14-17 days   | 140                | 138            | 2             |
| 4 wk         | 76                 | 74             | 2             |
| 6-12 wk      | 133                | 133            | 0             |

At the indicated ages, mice were sacrificed and the spleens evaluated simultaneously for EAC rosette formation and the presence of Ig on the surface of rosette-forming lymphocytes using a fluorescein-labeled antibody to mouse k-chain. In one experiment, fluoresceinated anti-k was ultracentrifuged (100,000 g) for 30 min immediately before use. In this experiment, all of the 45 CRL from 2-wk old AKR mice and 47 CRL from 8-wk old AKR mice were labeled by the fluorescent antibody.

In this paper, we have examined the timing of appearance of lymphocytes bearing C receptors (or the appearance of C receptors on existing lymphocytes) since the C receptor appears to be one of the major surface markers of B lymphocytes, or a subline thereof. Indeed, as indicated in the introduction, the C receptors may have importance in the activation of B lymphocytes by antigens and/or mitogens.

Our results indicate that there is substantial lag between the appearance of lymphocytes with surface Ig and those bearing C receptors. Thus BALB/c,
DBA/2, and C57BL/6 mice have virtually no detectable (<1%) splenic CRL before 2 wk of age and have very few CRL at 2 wk, although a substantial number of cells with surface Ig appear during the 1st day after birth. Moreover, studies using soluble AAC complexes and EAC prepared in a variety of ways suggest that the results are not due to some artifact in methodology, but in fact represent either the complete absence of C receptors or the presence of very small numbers of such receptors on surface of B cells of young mice of these strains. It should be obvious that B lymphocytes from such young mice will be useful in attempting to assess the importance of C receptors in B-lymphocyte activation and tolerance induction.

Studies to be published separately\(^a\) show that spleen cells from 3-day old BALB/c mice respond vigorously to the B-cell mitogens bacterial lipopolysaccharide (LPS) and poly inosinic-cytodilic acid (poly I-C). This suggests that the response of B cells to LPS and poly I-C is not due to binding to the lymphocyte C receptor of C3 activated by these agents.

Although a lag between appearance of surface Ig and C receptors seems general to mouse strains, a clear difference in the time of appearance of CRL exists between AKR mice and the low CRL strains noted above. Moreover, this ontogenetic pattern is also noted in studies of repopulation of lymphoid tissue of adult-irradiated mice who have received syngeneic bone marrow. AKR mice repopulate the CRL content of their spleens much more rapidly than do either C57BL/6 or CDF1 mice.

It appears then that a continuing and relatively precise control of the pattern of B-lymphocyte differentiation exists. Breeding studies suggest that there are at least two independent genetic loci involved in determining the time of appearance of CRL. More precisely, AKR and DBA/2 mice appear to differ at two independent loci important in CRL appearance as shown by the fact that 15 of 51 progeny of DBA/2 × AKD2 matings have CRL frequencies which resemble that of the DBA/2. Possession of either of the two postulated CRL genes confers an intermediate or high CRL frequency on 2-wk old mice. Recent studies show a substantial difference in the frequency of CRL in 2-wk old mice in two strains congenic for the \(H-2\) region (C57BL/10 and B10.A (19)). As these mice differ from each other, genetically, in the \(H-2\) region but are believed to be identical at most other loci, this implies that one locus important in controlling ontogeny of CRL is linked to the \(H-2\) region. In view of the control of other aspects of lymphocyte function and structure by genes in this region (20–22), this result strongly suggests that the \(H-2\) region has a special relation to the immune system and to lymphocyte activation.

Although it is clear that C receptors appear after surface Ig, our studies do not yet allow a decision to be made as to whether the control observed is con-

\(^a\) Gelfand, M. C., G. I. Elfenbein, and W. E. Paul. Ontogeny of B lymphocytes. IV. Development of responsiveness to B-cell mitogens. Manuscript in preparation.
cerned with differentiation along a single line of B-lymphocyte development or, alternatively, the regulation of independent subpopulations of two (or more) types of B lymphocytes with distinct markers (Table X).

Data concerning the relative frequency of several phenotypic forms of B lymphocytes exist which may aid in clarifying this issue. Although there is no definitive evidence establishing that a lymphocyte (as distinct from a multipotent) stem cell exists, a thymus-independent lymphocyte which is both Ig- and lacks C receptors (CR-) has been described (23). The B-cell progenitors indicated in Table X may be members of this group of cells. There is ample evidence in mice that a substantial portion (up to 40%) of Ig-bearing lymphocytes in spleen and lymph node lack detectable C receptors (24, 25) and, as shown here, these cells appear before cells which bear C receptors. In human peripheral blood, it has also been reported that a substantial fraction of Ig+ cells lack the C receptor (26), although the frequency of CRL does depend on the source of complement used (27). Thus, an Ig+ CR- lymphocyte is well established, as of course is the Ig+ CR+ lymphocyte. The existence of an Ig- CR+ B lymphocyte is less certain. If such a cell could be documented, it would tend to support the existence of multiple B-cell lines, although this cell could also represent a further differentiation step beyond the Ig+ CR+ step in a single line. Ross et al. (26) have reported that although Ig- CR+ lymphocytes are rare in peripheral blood, in spleens of two out of three humans tested, 20-30% of the CRL observed lacked surface Ig. On the basis of this finding, we made a search for Ig- CR+ lymphocytes in the spleens of AKR mice. Our goal was to determine whether the developmental difference between AKR mice and mice of low CRL strains was the more rapid appearance of an Ig- CR+ cell in the former. Our data indicate that this is not the case as more than 95% of the CRL evaluated possessed surface Ig.

Finally, it is obvious that the precise mechanism by which the "CRL genes" function is unknown. We think it unlikely, however, that the rate of appearance of CRL is determined by genes which control T-lymphocyte functions directly and B-lymphocyte (and CRL) frequency only indirectly. In experiments not shown here, the frequency of CRL was the same in intact and

### Table X

**Possible Schema of B-Cell Differentiation**

| A. One line | B. Two or more lines |
|-------------|---------------------|
| I\(\text{g}^-\text{CR}^-\) \(\rightarrow\) I\(\text{g}^+\text{CR}^-\) \(\rightarrow\) I\(\text{g}^+\text{CR}^+\) \(\rightarrow\) I\(\text{g}^-\text{CR}^+\) | I\(\text{g}^-\text{CR}^-\) \(\rightarrow\) I\(\text{g}^+\text{CR}^+\) |
| I\(\text{g}^-\text{CR}^-\) \(\rightarrow\) I\(\text{g}^+\text{CR}^-\) | I\(\text{g}^-\text{CR}^-\) \(\rightarrow\) I\(\text{g}^+\text{CR}^-\) |
| I\(\text{g}^-\text{CR}^-\) \(\rightarrow\) I\(\text{g}^-\text{CR}^+\) | I\(\text{g}^-\text{CR}^-\) \(\rightarrow\) I\(\text{g}^-\text{CR}^+\) |
thymectomized irradiated mice at 2 wk after reconstitution with syngeneic bone marrow.

The demonstration of a timetable of B-lymphocyte differentiation, under strict genetic control, is likely to be of considerable importance in understanding the acquisition of immunocompetence in the B-lymphocyte line and in assessing the importance of the C receptor to the function of B lymphocytes.

SUMMARY

Many bursa-equivalent (B) lymphocytes of adult mice bear surface Ig and receptors for C3. The frequency of Ig-bearing cells increases rapidly immediately after birth, but these cells lack complement (C) receptors. Lymphocytes bearing C receptors are not found in the spleens of BALB/c, DBA/2, and C57BL/6 mice until 2 wk of age and do not attain substantial numbers until 3–4 wk of age. In AKR mice, a lag between appearance of Ig-bearing and complement receptor lymphocytes (CRL) is also observed but it is of much shorter duration. AKR mice have a frequency of CRL at 2 wk of age of 28% in comparison to a frequency of 4.8% for DBA/2 mice. The difference in frequency between young and adult mice and between “low” and “high CRL” strains cannot be explained by a nonspecific inability to form rosettes as similar results are obtained with soluble antigen-antibody-complement complexes. Analysis of CRL frequency in (AKR X DBA/2)F1 mice and F1 X parental backcross progeny suggests that two independent genes control the rate of appearance of CRL. Furthermore, the genetic difference in the ontogeny of CRL is recapitulated in the repopulation of the B-lymphocyte line in adult-irradiated mice restored with syngeneic bone marrow. Thus, the “CRL genes” described here appear to control B-cell differentiation throughout life.

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REFERENCES

1. Raft, M. C. 1970. Two distinct populations of peripheral lymphocytes distinguishable by immunofluorescence. *Immunology. 29*:367.
2. Rabellino, E., S. Colon, H. M. Grey, and E. R. Unanue. 1971. Immunoglobulin on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.* 133:150.
3. Bianco, C., R. Patrick, and V. Nussenzweig. 1970. A population of lymphocytes bearing a membrane receptor for antigen-antibody-complement complexes. I. Separation and characterization. *J. Exp. Med.* 132:702.
4. Bianco, C., P. Dukor, and V. Nussenzweig. 1971. In Morphological and Functional Aspects of Immunity. K. Linkdahl-Kiessling, G. Alan, and M. G. Hanna, Jr., editors. Plenum Publishing Corporation, New York. 251.
5. Dukor, P., and K. V. Hartmann. 1973. Bound C3 as the second signal in B-cell activation. *Cell. Immunol.* 7:349.
6. Boyle, W. 1968. An extension of the ³¹Cr-release assay for the estimation of mouse cytotoxins. *Transplantation.* 6:761.
7. Frank, M. M., and T. Gaither. 1970. The effect of temperature on the reactivity of guinea pig complement with γG and γM hemolytic antibodies. *Immunology.* 19:967.
8. Lay, W. A., and V. Nussenzweig. 1968. Receptors for complement on leukocytes. *J. Exp. Med.* 128:991.
9. Shevach, E., R. Herberman, R. Lieberman, M. M. Frank, and I. Green. 1972. Receptors for immunoglobulin and complement on mouse leukemias and lymphomas. *J. Immunol.* 108:325.
10. Wood, B. T., S. H. Thompson, and G. Goldstein. 1965. Fluorescent antibody staining. III. Preparation of fluorescein-isothiocyanate-labeled antibodies. *J. Immunol.* 95:225.
11. Eden, A., C. Bianco, and V. Nussenzweig. 1973. Mechanism of binding of soluble immune complexes to lymphocytes. *Cell. Immunol.* 7:459.
12. Daniele, R. P., and D. T. Rowlands, Jr. 1973. "Unmasking" of complement receptors on normal and CLL lymphocytes after *in vitro* treatment with mercaptoethanol. 16th Annual Meeting of the American Society of Hematology. 108. (Abstr.).
13. Basten, A., J. F. A. P. Miller, J. Sprent, and J. Pye. 1972. A receptor for antibody on B lymphocytes. I. Method of detection and functional significance. *J. Exp. Med.* 135:610.
14. Dickler, H. B., and H. C. Kunkel. 1972. Interaction of aggregated γ-globulin with B lymphocytes. *J. Exp. Med.* 136:191.
15. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen-reactive cells. *Transplant. Rev.* 1:3.
16. Claman, H. N., and E. A. Chaperon. 1969. Immunologic complementation between thymus and marrow cells. A model for the two-cell theory of immunocompetence. *Transplant. Rev.* 1:92.
17. Davies, A. J. S. 1969. The thymus and the cellular basis of immunity. *Transplant. Rev.* 1:43.
18. Cooper, M. D., A. R. Lawton, and P. W. Kincade. 1972. A developmental approach to the biological basis for antibody diversity. *In* Contemporary Topics in Immunobiology. M. G. Hanna, editor. Plenum Publishing Corporation, New York. 33.
19. Gelfand, M. C., D. Sachs, R. Lieberman, and W. E. Paul. 1974. III. H-2 linkage of a gene controlling the rate of appearance of complement receptor lymphocytes. *J. Exp. Med.* 139:800.
20. Benacerraf, B., and H. O. McDevitt. 1972. Histocompatibility-linked immune response genes. *Science (Wash. D. C.)* 187:273.
21. Bach, F. 1973. The major histocompatibility complex in transplantation immunology. *Transplant. Proc.* 5:23.
22. Boyse, E. A., L. J. Old, and E. Stockert. 1965. The TL (thymus leukemia) antigen, a review. *In* IV International Symposium on Immunopathology. P. Grabar and P. A. Miescher, editors. Grune & Stratton, Inc. New York. 23.
23. Stobo, J. D., A. S. Rosenthal, and W. E. Paul. 1973. Functional heterogeneity of murine lymphoid cells. V. Lymphocytes lacking detectable surface θ or immunoglobulin determinants. *J. Exp. Med.* 138:71.
24. Bianco, C., and V. Nussenzweig. 1971. Theta-bearing and complement-receptor lymphocytes are distinct populations of cells. *Science (Wash. D. C.)* 173:154.

25. van Boxel, J. A., W. E. Paul, M. M. Frank, and I. Green. 1973. Antibody-dependent lymphoid cell-mediated cytotoxicity. Role of lymphocytes bearing a receptor for complement. *J. Immunol.* 110:1027.

26. Ross, G. D., E. M. Rabellino, M. J. Polley, and H. M. Grey. 1973. Combined studies of complement receptor and surface immunoglobulin-bearing cells and sheep erythrocyte rosette-forming cells in normal and leukemic human lymphocytes. *J. Clin. Invest.* 52:377.

27. Ross, G. D., M. J. Polley, E. M. Rabellino, and H. M. Grey. 1973. Two different complement receptors on human lymphocytes. One specific for C3b and one for C3b inactivator-cleaved C3b. *J. Exp. Med.* 138:798.