# Theta-Bearing and Complement-Receptor Lymphocytes Are Distinct Populations of Cells

Abstract. *In the mouse, most (or all) complement-receptor lymphocytes and theta-bearing lymphocytes are part of nonoverlapping populations of cells. This finding validates the use of these membrane markers to characterize populations of lymphocytes. In addition, it is probable that, although receptors for complement and membrane-bound immunoglobulins coexist on the same lymphoid cells, these markers are located at separate sites on the membrane.*

Lymphocytes do not constitute a functionally homogeneous population of cells. The identification of membrane characteristics of lymphocytes (*1*) has allowed for their separation into distinct subpopulations. Some distinct markers have been described on the membrane of the mouse lymphocyte: the $\theta$ antigen (*2, 3*), the receptors for a modified complement component (C3) (*4, 5*) and the membrane-bound immunoglobulins (Ig) (*5–7*). [The last two markers have been found so far in all mammalian species tested (*5, 8*).] In examining the relation between the cell populations bearing these three markers, two questions were asked: (i) Are C3-receptor lymphocytes (CRL) and $\theta$-bearing lymphocytes different populations of cells and (ii) What is the relation between the C3 receptor found on the membrane of certain lymphocytes and the Ig molecules which they also display on their surface? We now report that most (or all) CRL and $\theta$-bearing lymphocytes are part of nonoverlapping populations of cells and also that the C3 receptor and membrane-bound Ig, though present on the same cells, are probably located at distinct membrane sites.

The fact that CRL and $\theta$-bearing lymphocytes are independent of each other was established by specifically depleting either cell population and measuring the resulting variation in the distribution of the markers among the remaining cells. For instance, if CRL and $\theta$-bearing lymphocytes were part of separate populations of cells, the depletion of CRL should be accompanied by a predictable increase in the proportion of $\theta$-bearing cells, and vice versa.

The method for specific depletion of CRL has been described (*5*) and is based on the ability of CRL to bind sheep erythrocytes (E) sensitized by rabbit antibody to Forssman antigen (A) and complement (C). The interaction between CRL and EAC leads to the formation of clusters. As these CRL-containing clusters have a higher density than free lymphocytes, they can be separated by differential flotation in a discontinuous bovine serum albumin (BSA) gradient (*9*). The average results of three experiments in which the percentage of $\theta$-bearing cells determined by cytotoxic tests (*10*) was measured among CRL-depleted and nondepleted cells isolated from the spleens of AKR mice is shown in Table 1. The percentage of $\theta$-bearing cells increased significantly in the depleted preparation, and the values obtained were quite close to those theoretically expected, assuming that no $\theta$-bearing cells were eliminated by the procedure. It is important to point out that the recovery of cells after depletion was very high (nonspecific cell losses about 10 percent).

The reverse experiment, that is, depletion of $\theta$-bearing cells, was performed as follows. A suspension of lymphocytes isolated from the peripheral lymph nodes of AKR mice (0.5 ml of $4 \times 10^7$ cell/ml) was incubated with an optimum amount of antiserum to $\theta$ (0.2 ml) and guinea pig complement (0.8 ml) for 45 minutes at 37°C. A sample was then counted and tested for trypan-blue exclusion. The remaining suspension was then layered on the top of a BSA discontinuous gradient and submitted to ultracentrifugation (*9*). After centrifugation, most of the nonviable cells were found in the pellet. The supernatant containing 98 percent or more viable cells was collected and diluted four times with culture medium RPMI 1640; the cells were further washed by centrifugation at 200*g* for 10 minutes. Nonspecific cell losses after ultracentrifugation were about 15 percent. The proportion of CRL was significantly higher in the depleted population, and the relative increase in their concentration was close to that expected if no CRL had been lysed by the antiserum to $\theta$ in the presence of complement (Table 2) (*11*).

Thus most or all $\theta$-bearing lymphocytes and CRL are part of independent cell populations. We had shown by a similar procedure that C3 receptors and Ig may coexist on the membrane of the same population of cells (*5*). This finding raised the possibility that the membrane-bound Ig might actually be the C3 receptor itself. However, the following experiments showing that antibodies to Ig do not interfere with the function of the C3 receptor on the membrane of

Table 1. Enrichment of $\theta$-bearing cells in populations of spleen cells from AKR mice after depletion of CRL. Mouse spleen cells were mixed with E (nondepleted) or EAC (depleted) and incubated as for the standard assay. Clusters formed only in the tube containing EAC. Both preparations were ultracentrifuged in separate BSA density gradients, after which only free lymphocytes remained in the supernatants. The data represent means of three experiments.

| Cells | Cell recovery after ultracentrifugation (% ± S.E.) | Remaining population | |
|---|---|---|---|
| | | CRL (% ± S.E.) | $\theta$-Bearing cells (% ± S.E.) |
| Nondepleted | 88.1 ± 4.1 | 33.8 ± 4.1 | 14.3 ± 1.0* |
| Depleted | 61.3 ± 5.0 | 4.3 ± 0.9 | 22.8 ± 0.8* (21.6)† |

* The difference between these results is significant, $P < .01$, Student's $t$-test. † Percentage expected if CRL and $\theta$-bearing lymphocytes are part of distinct and nonoverlapping cell populations.

Table 2. Enrichment of CRL in populations of lymph node cells of AKR mice after depletion of $\theta$-bearing cells.

| Treatment of lymphocytes | Cells lysed (% ± S.E.) | Cell recovery after elimination of dead cells (% ± S.E.) | CRL in remaining population (% ± S.E.) |
|---|---|---|---|
| Normal C3H serum and C (control) | 3.4 ± 0.4 | 84.3 ± 6.0 | 10.9 ± 5.6* |
| C3H antiserum to $\theta$ (AKR) and C | 42.9 ± 1.3 | 49.9 ± 3.3 | 24.7 ± 1.0* (22.3)† |

* The difference between these results is significant, $P < .05$, Student's $t$-test. † Percentage expected if CRL and $\theta$-bearing cells are part of distinct and nonoverlapping cell populations.

CRL render this hypothesis unlikely. The $F(ab')_2$ fragments of rabbit antiserum against mouse Ig (*5*) were prepared as indicated in (*12*). This antiserum contained antibodies directed against heavy chains and light chains of mouse Ig. The IgG fraction of this antiserum was capable of lysing a maximum of 20 percent of mouse spleen cells when incubated for 45 minutes at 37°C in the presence of complement (Fig. 1a). However, if these spleen cells were first treated with the $F(ab')_2$ fragments of the same IgG fraction and washed twice, they were no longer vulnerable to the lytic action of native IgG and guinea pig complement (Fig. 1b). This indicates that the $F(ab')_2$ fragments interacted with the membrane-bound Ig. A relatively high concentration of $F(ab')_2$ fragments, however, did not inhibit the binding of EAC to CRL (Fig. 1c). This last experiment was performed with EAC displaying relatively few C3 sites on their membranes to enable easier detection of an eventual inhibitory effect (*13*).

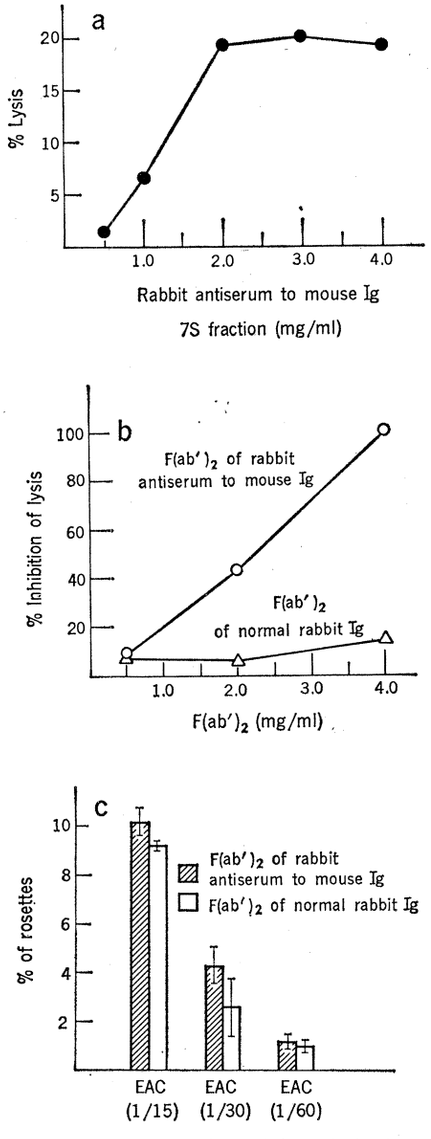


Fig. 1. (a) Cytotoxic activity of the IgG fraction of a rabbit antiserum to mouse Ig (fraction I) on mouse spleen cells. (b) Inhibition of the cytotoxic activity mediated by fraction I referred to above (2 mg/ml) by prior treatment of the spleen cells with $F(ab')_2$ fragments of the same fraction (control tubes contain fragments of normal IgG). (c) Lack of inhibitory activity of $F(ab')_2$ of rabbit antiserum to mouse Ig (4 mg/ml) on rosette formation between EAC and CRL. The test samples of EAC were prepared with three dilutions of fresh mouse serum (1/15, 1/30, and 1/60).

Our results along with those of others (*3, 5, 14*) validate the use of certain membrane markers for the characterization of lymphocyte populations and for studying cellular events of the immune response. The $\theta$ marker appears to be restricted to thymus or thymus-derived (T) lymphocytes, and membrane-bound Ig as well as the C3 receptor are found on bone marrow–derived, thymus-independent (B) lymphocytes. The membrane characteristics of CRL have been confirmed in our laboratory. A method has been devised to specifically isolate CRL from a mixed population of lymphoid cells, with good yields (15 to 30 percent) and a high degree of purity (above 90 percent). More than 90 percent of cells from this isolated population had Ig on their membranes, and none could be found to bear the $\theta$ marker, as indicated by the results of cytotoxic tests with specific antiserums (*15*).

The percentages of CRL (B) and $\theta$-bearing (T) lymphocytes found in peripheral lymph nodes of mice add up to only about 70 to 80 percent (*16*). In the spleen this total is somewhat lower, but in the mouse this organ contains large numbers of hemopoietic cells. At least two hypotheses can be proposed to explain these findings: (i) It is possible that a third population of lymphoid cells, distinct from B or T as defined by the markers discussed above, may be present in lymphoid organs of mice. (ii) Alternatively, subpopulations of B or T cells may have fewer C3 receptors or $\theta$ sites on their membranes, and for this reason would not be detected by the commonly used assays. It is known that the lymphocyte membrane may suffer quantitative and qualitative modifications during its differentiation. For example, T cells in peripheral lymphoid organs of the mouse do not display the TL antigen on their membranes [in TL+ strains of mice (*1*)] and have less $\theta$ antigen than the thymocytes from which they originated (*17*). Also, it appears that some plasma cells, although deriving from bone marrow precursors, have new membrane antigens (*18*) while lacking membrane-bound Ig (*19*) and C3 receptors (*5*).


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9. The BSA discontinuous gradient was prepared in 5-ml cellulose nitrate tubes. The bottom layer was made with 1.5 ml of BSA (specific gravity, 1.100), and the middle layer was made with 1.5 ml of a 1 : 2 dilution of the same BSA in medium RPMI 1640 (Associated Biomedic Systems, Inc.) The cell suspension was loaded on top of these layers. The tube was centrifuged in a Spinco ultracentrifuge at 4°C, 15,000*g*, for 30 minutes.
10. The antiserums to $\theta$ antigen were prepared by injection of thymocytes from AKR mice into C3HeB/Fe mice (*2*). Although both strains share the same H-2 specificities ($H\text{-}2^k$), they differ in their Ly specificities. AKR have the Ly-A.2/Ly-B.1/Ly-C.1 phenotype and C3H have the Ly-A.1/Ly-B.1/Ly-C.2 phenotype [E. A. Boyse, M. Miyazawa, T. Aoki, L. J. Old, *Proc. Roy. Soc. London Ser. B* **170**, 175 (1968); E. A. Boyse, K. Itakura, E. Stockert, C. Iritani, M. Miura, *Transplantation*, in press]. The Ly specificities of the variant C3HeB/Fe have not been determined. To check for the presence of antibody to Ly, the anti-$\theta$ AKR was tested against A/J ($H\text{-}2^a$, $\theta$C3H) and C57B1/6J ($H\text{-}2^b$, $\theta$C3H) thymocytes which carry the Ly-A.2/Ly-B.2/Ly-C.2 phenotype and against C58/J ($H\text{-}2^k$, $\theta$C3H) thymocytes which have the Ly-A.2/Ly-B.1/Ly-C.1 phenotype. No cytotoxic activity was detected. The cytotoxic tests were preformed as described by E. A. Boyse, L. J. Old, I. Chouroulinkov [*Methods Med. Res.* **10**, 39 (1964)] and in (*5*). The results were expressed as the percentage of cells taking up trypan blue in the experimental tubes, corrected for the percentage of lysis in control tubes, as follows: [(% lysis experimental—% lysis control) / (100—% lysis control)] × 100. The percentage of lysis in control tubes was consistently less than 6 percent.
11. These experiments also show that prior in-

cubation of CRL with antiserum to $\theta$ and guinea pig complement does not interfere with their subsequent capacity to bind EAC. Similar results were obtained when complement was omitted from the incubation medium; that is, we were unable to inhibit rosette formation between EAC and CRL (from AKR mice) by prior treatment of the lymphocytes with antiserum to $\theta$ (AKR) for 45 minutes at 37°C.

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13. The idea that membrane-bound Ig is the C3 receptor is unattractive for another reason. Distinct classes of Ig can be found on the membrane of mouse lymphocytes (*7*). This would probably restrict the site for the C3 receptors to the Fab portion of membrane-bound Ig. It has been observed, however, that normal serum Ig does not inhibit the interaction between EAC and CRL. This can only be reconciled with the aforementioned hypothesis, if it is further postulated that the properties of membrane-bound and free Ig are basically different.
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20. We thank Gertrude Fastaia for technical assistance and Dr. C. Merryman for the antiserums. Supported by NIH grants AI 08499, Health Research Council of the City of New York contract 1558, and a training fellowship from Fundação de Amparo à Pesquisa, São Paulo, Brazil.

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## Density Gradient Separation of Marrow Precursor Cells Restricted for Antibody Specificity

Abstract. *Potentially immunocompetent cells of* $(C57BL/6 \times DBA/2)F_1$ *mouse bone marrow are committed to antigenic determinants of sheep or burro erythrocytes prior to interaction with thymus-derived cells and participation in immune responses to administered antigens. At this stage of differentiation marrow cells of this particular mouse strain are not yet restricted for the immunoglobulin M or immunoglobulin G antibody class. By equilibrium centrifugation in discontinuous gradients of bovine serum albumin, precursors of cells that produce antibody to sheep erythrocytes migrate to denser regions, whereas the precursors of immunocytes that produce antibody to burro erythrocytes remain in the lower density regions.*

Recent evidence suggests that primitive precursors of antibody-forming cells, which are marrow derived in mice, are predetermined for the specificity of antibody to be synthesized by descendant immunocytes (*1*). This evidence was obtained by specifically paralyzing or sensitizing marrow-derived cells before interaction with thymus-derived and macrophage-like accessory cells. In addition, the population of marrow precursors undergoes long-term changes on exposure to antigens that specifically enhance immunological competence, while terminal differentiation and maturation remain dependent on accessory cells (*2*). Thus, the role of cellular interactions is not likely to be the transfer of information for antibody specificity from thymic cells to precursors of immunocytes, and marrow-derived cells could be the carriers of immunological memory.

In most mouse strains, marrow cells are predetermined also for the molecular class of descendant immunocytes, as demonstrated by limiting dilution analysis (*3*) and, more directly, by physical separation of unipotent cells (*4*). The only known exception occurs in $(C57BL/6 \times DBA/2)F_1$ mice whose marrow cells are pluripotent, according to limiting dilution experiments, for hemolytic antibodies to sheep erythrocytes (SRBC) which are of the IgM and IgG classes (*3*). We now present direct evidence confirming the lack of class restriction of $(C57BL/6 \times DBA/2)F_1$ marrow cells engaged in immune responses to SRBC (anti-sheep) and extending the finding to cells engaged in responses to burro erythrocytes (BRBC) (anti-burro). In addition, a second property of these cells is described, namely, the restriction for specificity of antibodies.

Bone marrow cells, harvested from the long bones of 12-week-old female mice, were suspended in Eagle's medium and subjected to equilibrium centrifugation in a discontinuous gradient of 17 to 35 percent bovine serum albumin (BSA), according to the method of Dicke *et al.* (*5*), as described earlier (*4*). Ten cell fractions were collected from the interphases beginning at the top between 17 and 19 percent BSA. The fractions were diluted in Ca- and Mg-free phosphate-buffered (*p*H 7.2) saline (PBS), washed once by centrifugation, and resuspended in PBS. Nucleated cells were counted with an electronic particle counter (*6*). Density distributions of nucleated cells have been described (*4*). The profiles were reproducible and displayed a major peak in the denser region of the gradient (29 to 35 percent BSA). Recovery of nucleated cells after centrifugation ranged from 65 percent to 73 percent of input in the three experiments described below.

To test for potentially immunocompetent cells, each marrow fraction was added to an equal volume of thymocyte suspensions, and the mixtures were transplanted by way of the tail vein into each mouse of several groups of syngeneic female mice that were heavily irradiated (*7*). Each group contained 10 to 20 animals, and each inoculum contained a limiting number of $2.5 \times 10^5$ bone marrow cells (fractionated or not) and an excess number of $4 \times 10^7$ thymocytes (*8*). Eighteen hours after transplantation, one-half of the recipients received an intravenous injection of $5 \times 10^8$ washed SRBC, and the other half received $5 \times 10^8$ washed BRBC. Direct (IgM) and indirect (IgG) plaque-forming cells (PFC) releasing hemolytic antibody were enumerated in cell suspensions of recipient spleens 9 days after transplantation by the agarose-slide technique (*9*). Spleen cells of recipients immunized against SRBC were assayed for anti-sheep PFC, and those of recipients immunized against BRBC for anti-burro PFC. Animals were classified as "positive" or "negative," depending on whether their spleens contained PFC in numbers greater than or equal to those of controls. Negative controls were irradiated mice inoculated with marrow cells and thymocytes but not with SRBC or BRBC, and mice inoculated with marrow cells (fractionated or not) and antigens but not with thymocytes. The numbers of PFC in spleens of these controls did not exceed 200 direct and 100 indirect. In positive spleens, the numbers of PFC ranged from 200 to