SPECIFICITY OF HUMAN LYMPHOCYTE
COMPLEMENT RECEPTORS*

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The first studies of complement (C) receptor specificity concerned the primate erythrocyte immune adherence receptor (1). The erythrocyte immune adherence receptor was at first thought to be specific for only the C3b fragment of immune complex-bound C3 (2, 3), but later Cooper demonstrated that immune complex-bound C4 (C4b) was also bound to erythrocytes (4). When bone marrow-derived lymphocytes (B cells) were demonstrated to have C3 receptors, these were at first thought to be also specific for the C3b fragment of C3 (5). Later however, B cells were found to have two different C3 receptors: one which was specific for C3b and antigenically similar to the erythrocyte immune adherence receptor, and another which was specific for C3b inactivator-cleaved C3b (C3d) and which was not found on erythrocytes (6). C3b inactivator is present in serum and has an enzyme-like activity which cleaves complex-bound C3b into the fragments C3c and C3d (7) such that the C3c fragment is released from the immune complex leaving the small C3d fragment complex bound (8). Recently, it has been shown that C3b inactivator also cleaves complex-bound C4. However, the presence of C2 apparently protects C4 from the action of this inactivator (N. R. Cooper, personal communication).

Studies of lymphocytes with C receptors (CRL)1 have indicated that many but not all possessed surface immunoglobulin (SIg), and that depending on the lymphoid source, some had either one or both types of C3 receptors (6, 9). Both types of C3 receptors were usually found on normal lymphocytes. However, peripheral lymphocytes from most patients with chronic lymphatic leukemia were found to have a large number of cells with C3d receptors but usually few cells with immune adherence (C3b) receptors. In 20% of the patients studied, lymphocyte SIg was found to be missing (9). Even though lymphocytes with C receptors and no SIg were rarely found in the peripheral blood of normal

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1Abbreviations used in this paper: BSA, bovine serum albumin; CLL, chronic lymphatic leukemia; CRL, complement receptor lymphocytes; DFP, diisopropylfluorophosphate; EA, sheep erythrocytes sensitized with γM rabbit antibody to sheep erythrocytes; EAC, sheep erythrocyte-γM rabbit antibody-complement complexes; EAγo, sheep erythrocytes sensitized with γG rabbit antibodies; GVB, gelatin veronal buffer; HBSS, Hanks' balanced salt solution; SIg, surface immunoglobulin; TRFC, thymus-derived rosette-forming cells (cells having nonimmune receptors for sheep erythrocytes).
individuals, 10–20% of spleen lymphocytes were of this type (9). However, since most of these spleen cells had both the immune adherence (C3b) receptor and the C3d receptor, it appeared that the type of cells observed in leukemic peripheral blood was different from any type of normal B cell.

In the present study, lymphocytes, granulocytes, and monocytes were shown to possess a receptor activity for C4 which was similar to that previously described to be present on erythrocytes (4). Specificity of the various lymphocyte C receptors was examined by assays for inhibition of sheep erythrocyte-γM rabbit antibody-C complex (EAC) rosette formation by highly purified C components and their fragments. By this technique the existence of only two different C receptors was demonstrated. Utilizing a double-label assay for these two receptors, it was demonstrated that each of the two types of C receptors could cap independently and thus were located on different molecules in the B-cell membrane.

Materials and Methods

Blood Cells, Tonsil Cells, and Lymphoblastoid Cell Lines. Blood from normal adults and patients with a confirmed diagnosis of chronic lymphatic leukemia or Waldenström's macroglobulinemia was collected into heparin. Cultured lymphoblastoid cell lines, except MOLT-4 and Raji, were initiated from normal buffy coat cells and were a gift from Dr. Thomas H. Hütteroth, Cornell University Medical College, New York (10). MOLT-4 lymphoblastoid cells were initiated from the lymphocytes of a patient with acute lymphoblastic leukemia (11) and were a gift from Dr. Mark Weksler, Cornell University Medical College, New York. Raji lymphoblastoid cells were initiated from a patient with Burkitt's lymphoma (12) and were a gift from Dr. Shu Man Fu, The Rockefeller University, New York. Tonsils were obtained from children not known to have any gross immunological defects who were undergoing routine tonsillectomy.

Separation of Lymphocytes. With normal blood, lymphocytes were isolated by treatment of the blood with dextran and carbonyl-iron followed by centrifugation on Ficoll-Hypaque at a density of 1.095 g/ml (6). Blood from patients with macroglobulinemia contained large numbers of granulocytes which poorly phagocytized carbonyl-iron. In order to remove granulocytes from these preparations, the dextran-carbonyl-iron step was followed by centrifugation on Ficoll-Hypaque at a density of 1.078 g/ml. Lymphocytes from patients with chronic lymphatic leukemia were isolated by Ficoll-Hypaque (d = 1.078 g/ml) centrifugation without carbonyl-iron pretreatment (6, 9). A similar method was utilized to remove erythrocytes and granulocytes from tonsil cell suspensions. Macrophage contamination in tonsil cell preparations, as assessed by measuring rosette formation and ingestion of sheep erythrocytes sensitized with γG rabbit antibody (EA,,) was found to be 5% or less.

Separation of Granulocytes and Monocytes. Granulocyte preparations were the adherent cell fraction isolated from dextran sedimented blood (6). Monocytes were isolated by adherence in a similar manner, except that the granulocytes were first removed from the dextran sedimented blood by bovine serum albumin (BSA) centrifugation (13). Removal of the monocyte-lymphocyte layer from the BSA with a Pasteur pipet was facilitated by overlayering the 28% BSA with Hanks' balanced salt solution (HBSS) before centrifugation. Monocyte preparations contained 85–90% monocytes, 6–10% lymphocytes, and 1–5% granulocytes.

Erythrocyte-antibody-C Complexes (EAC). Sheep erythrocytes sensitized with γM rabbit antibody to sheep erythrocytes (EA) were prepared as before (9). EAC1-3d, previously designated EA1-3b (preparation with purified human C components) are designated EAC1-3b and were prepared as previously described (9) except that the EAC1 used to prepare this complex was produced with only 3.5–7.5 μg C4/10⁶ EAC1. EAC1-3d, previously designated EA1-3bhu, were prepared by treating the EAC1-3d with C3b inactivator (6). The amount of C4 used in the preparation of EAC1-3d was found to be critical. If excess C4 was used, immune adherence activity due to cell-bound C4 remained after repeated treatments of the EAC1-3b intermediate with C3b inactivator. Since oxidized C2 (14) was used to form the EAC1-3b, much if not all of the C4 on the EAC was in stable complex with C2 and was
therefore resistant to the action of the C3b inactivator (N. R. Cooper, personal communication).

When native C2 was substituted for oxidized C2 in the preparation of the EAC1-3b complex, it was found that the native C2 offered bound C4 almost as much protection from the action of the C3b inactivator as did oxidized C2. The source of C3b inactivator used to prepare the EAC1-3d was either purified human C3b inactivator (15) or a reagent produced from modified whole human serum. In order to use the whole human serum source of C3b inactivator, it was found necessary to treat it with 1 M potassium thiocyanate (16), then to heat it 2 h at 56°C, and finally to absorb it with sheep erythrocytes. In addition, it was also found necessary to remove C1 from the EAC1-3b by incubation of the complex with 0.04 M EDTA in gelatin veronal buffer (GVB) for 90 min at 37°C. To permit inactivation of cell-bound C3b, 5 x 10^8 of the EAC4+23b in 1 ml of 0.04 M EDTA were mixed with 1 ml of the whole serum C3b inactivator reagent for 60 min at 37°C, followed by one wash step in 0.04 M EDTA and two wash steps in GVB. The EAC1-3d, prepared by treatment with purified C3b inactivator or with the whole serum reagent, were only used as a specific reagent for detection of C3d receptors when they had been tested and shown in tests for immune adherence with human erythrocytes to be more than 95% inactivated as compared to the EAC1-3b from which they were prepared (6).

Since granulocytes were more sensitive to C4 adherence than were lymphocytes or monocytes, only 2.5-5 μg C4/10^8 EAC1 were used during formation of the EAC1-3b when granulocyte receptors were being assayed. In each experiment, the EAC1 used to prepare the EAC1-3b were tested for lack of reactivity with the particular type of receptor-bearing cell being examined.

EAC14 were also prepared for detection of C4 receptor activity. These EAC14, in contrast to those used to prepare the EAC1-3b, were prepared with 100 μg C4/10^8 EAC1, an amount which was found to be optimal for the detection of all cells having C4 receptor activity.

Detection of CRL, SIg-Bearing Lymphocytes, and Lymphocytes Bearing a Receptor for Sheep Erythrocytes (TRFC). CRL, SIg, and thymus-derived rosette-forming cells (TRFC) were assayed as previously described (6, 9). CRL or TRFC were also assayed simultaneously with SIg-bearing cells by double-label assay (9).

In addition to the usual rosette assay, CRL were assayed by immunofluorescence. 5-10 x 10^4 lymphocytes were suspended in 0.2 ml of either C4, C3b, C3c, or C3d diluted to 5 x 10^-1 M in 0.03 M NaNO₃-HBSS. After 15 min incubation at 37°C, the cells were washed three times with ice-cold 0.03 M NaNO₃-HBSS and resuspended in either 0.2 ml of rabbit anti-C4 or 0.2 ml of rabbit anti-C3, each diluted in 0.03 M NaNO₃-HBSS. After 30 min at 4°C followed by three washes with ice-cold 0.03 M NaNO₃-HBSS, the cells were treated in a similar manner with goat antirabbit γ-globulin conjugated to fluorescein isothiocyanate and examined for fluorescence by the technique used for assay for SIg (9).

The anti-C4 and anti-C3 used had been prepared by immunization of rabbits with the highly purified components of C and were known to be monospecific. Before use in these experiments, they were absorbed with two different lines of cultured lymphoblastoid cells to remove natural antilymphocyte antibodies. The goat antiserum was purchased from Meloy Laboratories Inc., Springfield, Va., and before use was also absorbed with cultured lymphoblastoid cells. In each experiment, as a control, the lymphocytes without prior exposure to the fluid-phase C components were incubated with the antisera.

Immune Adherence. Adherence rosettes of EAC14 or EAC1-3b with human erythrocytes were quantitated by phase-contrast microscopy (6).

Detection of Granulocyte and Monocyte C Receptors. Monocytes and granulocytes were assayed for C receptors by an assay for rosette formation with EAC in a similar manner as that previously described for granulocytes (6).

Preparation of Purified C Components and their Fragments. C4 was purified in the usual manner (17) except that the pseudoglobulin was treated with diisopropylfluorophosphate (DFP) to destroy C1 esterase (C1s) activity (18). This pretreatment prevented the conversion of C4 to C4b during the isolation procedure. The purified C4 gave a single band in polyacrylamide gel electrophoresis and treatment of a sample of the C4 with purified C1s resulted in displacement of the entire band to positions characteristic for C4b, demonstrating that all of the untreated C4 was in the native uncleaved molecular form. C3 was purified in the usual manner (19) and was judged by polyacrylamide gels to be at least 95% pure and in its unconverted form. C4b was produced from C4 by treatment with C1s followed by addition of DFP to a final concentration of 2 x 10^-4 M to inactivate C1s and then dialysis against veronal-buffered saline. The fragments C3b, C3c, and C3d
were prepared by trypsin cleavage and Sephadex gel filtration according to the method of Bokisch et
al. (7). In calculations of molar concentrations the following mol wt were assumed: C4, 200,000
daltons (20); C3, 180,000 daltons; C3b, 173,000 daltons; C3c, 150,000 daltons; and C3d, 27,000 daltons
(7).

Purification of Bovine Conglutinin. Conglutinin was purified from adult bovine serum by a
combination of methods described by Lachmann (21). Briefly, conglutinin absorbed onto zymosan
from heat-inactivated serum was eluted with EDTA and precipitated from the eluate as a euglobulin.
The redissolved euglobulin was first treated with dithiothreitol to reduce contaminating IgM, and
then applied to a Sephadex G-200 column from which it eluted in the excluded volume.

Assay for Inhibition of C Receptors by Fluid-Phase C. C receptor target cells were resuspended
at 2 × 10^9/ml in a dilution of a particular C component in RPMI-1640 media (Microbiological
Associates, Inc., Bethesda, Md.). After 10 min at 37°C, the cells were assayed for EAC rosette
formation, either with or without first washing away unbound C with ice-cold RPMI-1640. Each of the
different types of EAC used in this assay was previously treated with 0.04 M EDTA-GVB at 37°C for
90 min to remove C1, thus preventing additional C4 uptake onto the EAC when inhibition was being
measured with fluid-phase native C4.

Since purity of the reagents was essential in this assay, the following control tests were included.
First, the EAC1-3b were tested for contaminating cell-bound C3d. Purified bovine conglutinin,
which specifically agglutinates complexes containing C3d, but not complexes containing only
uncleaved C3b (22), agglutinated EAC1-3d at a dilution of 1:6,000 and completely inhibited EAC1-3d
rosette formation. However, as expected it neither agglutinated EAC1-3b nor had any effect on
EAC1-3b rosette formation. Next, the C3d preparation was examined for contaminating C3b and
C3c. By both Ouchterlony and immunoelectrophoretic analysis the C3d preparation gave no reaction
with C3c-specific antiserum. Furthermore, the C3d preparation did not inhibit immune adherence
even at a dilution of 600 µg/ml, whereas either 60 µg/ml of the C3b preparation or 150 µg/ml of the C3c
preparation did inhibit immune adherence. Finally, by Ouchterlony analysis the C3b preparation did
not contain detectable C3d.

Assay for Inhibition of C Receptors by Antisera. The methods used for the preparation of
antierthrocyte and antilymphocyte sera and for the assay for inhibition of EAC rosette formation by
these antisera were the same as those previously described (6).

Depletion of TRFC from Peripheral Lymphocytes. TRFC rosettes were formed as in the TRFC
assay except that the sheep erythrocytes were treated with neuraminidase as described by Weiner et
al. (23) in order to stabilize the rosettes. Rosettes were separated from unrosetted lymphocytes by
centrifugation on Ficoll-Hypaque (d = 1.078 g/ml) by the same method used for isolation of
lymphocytes from peripheral blood (9). Unrosetted lymphocytes (non-TRFC) were removed from the
Ficoll-Hypaque interface and washed three times before testing for TRFC contamination. The
TRFC-depleted lymphocyte preparation was usually found to contain between 2 and 5% TRFC.

Results

C4 Receptor Activity on Lymphocytes, Erythrocytes, Granulocytes, and
Monocytes

Variation in the Amount of C4 Required. EAC14 formed rosettes with various
types of cells. The amount of C4 necessary to detect a maximum number of
rosettes varied considerably with the cell type (Fig. 1). Granulocytes were found
to be more reactive with small amounts of C4 than were erythrocytes. EAC14
prepared with 10 µg C4/10^9 EAC1 (an amount of C4 calculated to produce
approximately 2,000 molecules of bound C4/EAC14) rosetted only 1–2% of
erthrocytes but 60% of granulocytes (Fig. 1). The reactivity of both monocytes
and lymphocytes with complex-bound C4 increased over a broad range of C4
input.

Specificity of Adherence for C4 To ensure that EAC14 did not contain
contaminating C3, the complex was tested with anti-C3. This antibody did not agglutinate the EAC14 nor did its monovalent fragments (6) inhibit EAC14 rosette formation. The same antibody preparation agglutinated EAC1-3b at a dilution of 1:25,000, while a 1:100 dilution of its monovalent fragments completely inhibited both EAC1-3b and EAC1-3d rosette formation.

Lymphocytes with C4 Receptor Activity are B Cells

NORMAL LYMPHOCYTES FROM THE BLOOD AND TONSILS. When EAC14 rosette formation was assayed with normal lymphocytes from the blood or tonsils which had been previously labeled with fluorescein-conjugated anti-Ig, most of the lymphocytes which formed EAC14 rosettes were fluorescent. However, the peripheral blood C receptor-bearing cells appeared to be a subpopulation of the SIg-bearing cells, since more than half of the cells which were fluorescence labeled with anti-Ig did not form rosettes with any type of EAC (Table I). Occasionally, though, some normal individuals were found who had 1-4% CRL in the peripheral blood which lacked SIg as detected by immunofluorescence. To ascertain whether C4 receptor activity was also present on TRFC, experiments were performed in which a peripheral blood cell population was depleted of TRFC and the effect of this manipulation on the EAC14 rosette-forming cells was determined. It was found that depletion of the proportion of peripheral blood TRFC from 75% to 5% increased the proportion of non-TRFC from 25% to 95% (a 3.8-fold increase). By this manipulation, the proportion of lymphocytes forming EAC14 rosettes was increased from 8% to 30% (a 3.75-fold increase). To determine whether C4 receptors were on the same lymphocytes as the receptors for C3, EAC14 was mixed with EAC1-3b or with EAC1-3d. In each case it was found that only slightly more rosettes formed than when EAC14 was used individually, indicating that most lymphocytes with C4 receptor activity also had C3b and C3d receptor activity.

Lymphocyte preparations from tonsils contained more cells with C4 receptor activity than did peripheral blood lymphocytes and most SIg-bearing tonsil cells
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were found to have C receptors (Table I). All tonsil lymphocytes which formed rosettes with EAC14 also formed rosettes with EAC1-3b and EAC1-3d. However, tonsil lymphocytes were unlike peripheral blood lymphocytes in that each type of EAC detected a number of CRL on which SIg was not detectable by immunofluorescence. EAC14 detected 2-5% SIg-negative CRL in tonsils, while EAC1-3b detected 3-9% and EAC1-3d detected 9-22%.

LYMPHOCYTES FROM PATIENTS WITH LYMPHOPROLIFERATIVE DISEASES. In four patients with chronic lymphatic leukemia, most CRL had C3d receptor activity, but only 10-67% of the CRL with C3d receptor activity had C3b receptor activity and even fewer CRL had C4 receptor activity (Table II). In peripheral lymphocytes from three patients with Waldenström's macroglobulinemia a higher percentage of cells had C3b and C4 receptor activity than C3d receptor activity. Futher, two of the patients with macroglobulinemia had some CRL which did not contain detectable SIg.

LYMPHOBLASTOID CELLS FROM B- AND T-CELL-LIKE LINES. Lymphoblastoid cells from only four out of seven B-cell like lines formed rosettes with EAC14, even

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**Table I**

| Source of lymphocytes | No. of samples tested | CRL EAC14 | EAC1-3b | EAC1-3d | SIg % | TRFC % |
|-----------------------|-----------------------|-----------|---------|---------|-------|-------|
| Normal blood          | 11                    | 9 (4-14)  | 11 (8-16) | 9 (4-14) | 26 (19-33) | 71 (64-79) |
| Range                 | 8 (25-75)             | 53 (42-81) | 71 (58-84) | 62 (39-78) | 30 (15-52) |
| Tonsils               | 8                     | 53 (25-75) | 67 (42-81) | 71 (58-84) | 62 (39-78) | 30 (15-52) |

**Table II**

| Disease               | CRL EAC14 | EAC1-3b | EAC1-3d | SIg % | TRFC % |
|-----------------------|-----------|---------|---------|-------|-------|
| CLL                   | 4         | 21      | 31      | 8     | 13    |
| F. H.                 | 1         | 2       | 25      | 68    | 30    |
| L. E.                 | 17        | 47      | 85      | 81    | 9     |
| H. R.                 | <1        | 10      | 67      | 74    | 7     |
| Macroglobulinemia     | 10        | 27      | 8       | 22    | 66    |
| B. G.                 | 9         | 4       | 1       | 12    | 76    |
| S. S.                 | 41        | 32      | 9       | 23    | 40    |
though all seven lines formed rosettes with both EAC1-3b and EAC1-3d (Table III). C4 receptor activity was also found on a small number of cells in the MOLT-4 line, a line which resembles T cells since it is totally devoid of SIg and contains TRFC.

**Specificity of Lymphocyte C Receptors**

**INHIBITION OF ROSETTE FORMATION BY FLUID-PHASE C COMPONENTS.** EAC rosette formation was assayed with C4, C3, and their fragments present in the reaction mixture. Since a preliminary investigation indicated that there was no significant difference in the degree of inhibition obtained with native C4 as compared to that obtained with C4b, in subsequent inhibition studies only native C4 and not C4b was utilized. On the other hand, native C3 was not used extensively in the various inhibition assays because preliminary studies with this component indicated that the inhibition by native C3 was not dose dependent over a broad range of concentrations that were tested (Figs. 2-4).

**INHIBITION OF NORMAL LYMPHOCYTE C RECEPTORS.** Tonsil lymphocytes were selected as normal lymphocyte target cells because preparations of these cells contained more CRL than did peripheral blood lymphocytes. In the tonsil preparations, more than 50% of the cells were found to have receptor activity for C4, C3b, and C3d. The inhibitory effect of C4, C3, and its fragments on rosette formation with tonsil lymphocytes and EAC was first assayed with the C present at the time of rosette formation (Figs. 2-4). In order to determine if the C components or fragments from the fluid-phase became firmly bound to the specific receptors, a sample was removed from each complement-lymphocyte suspension and washed from one to three times before addition of EAC (Table IV).

EAC14 rosette formation was strongly inhibited by fluid-phase C3, C3b, and C3c, as well as by fluid-phase C4 (Fig. 2 and Table IV). Rosette formation with EAC1-3b was inhibited by the same fluid-phase components that inhibited EAC14, except that partial inhibition was consistently observed with fluid-phase C3d, and fluid-phase C4 and C3c each produced much less inhibition than did

### Table III

**CRL, SIg, and TRFC in Lymphoblastoid Cells**

| Cell line | CRL | SIg | TRFC |
|-----------|-----|-----|------|
|           | EAC14 EAC1-3b EAC1-3d | percentage | percentage |
| H. H.-O   | 30  93 91 | 18  | 0  |
| H. H.-1   | 2   89 95 | 10  | 0  |
| T. M.     | 0   77 98 | 63  | 0  |
| W. O.     | 0   29 52 | 80  | 0  |
| C. S.     | 74  79 77 | 70  | 0  |
| M. W.     | 0   70 93 | 46  | 0  |
| Wil       | 3   87 96 | 35  | 0  |
| MOLT-4    | 4   9  83 | 0   | 31 |

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| H. H.-1   | 2   89 95 | 10  | 0  |
| T. M.     | 0   77 98 | 63  | 0  |
| W. O.     | 0   29 52 | 80  | 0  |
| C. S.     | 74  79 77 | 70  | 0  |
| M. W.     | 0   70 93 | 46  | 0  |
| Wil       | 3   87 96 | 35  | 0  |
| MOLT-4    | 4   9  83 | 0   | 31 |
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Fig. 2. Inhibition of EAC14 rosette formation in the presence of various C components and their fragments. $10 \times 10^{-7}$ M inhibitor corresponds to 200 µg/ml of C4, 180 µg/ml of C3, 173 µg/ml of C3b, and 27 µg/ml of C3d.

Fig. 3. Inhibition of EAC4X23b rosette formation in the presence of various C components and their fragments. See legend of Fig. 2.

Fig. 4. Inhibition of EAC4X23d rosette formation in the presence of various C components and their fragments. See legend of Fig. 2.

Inhibition of C receptors on erythrocytes and lymphoblastoid cells. Lines C. S. and H. H.-1 were selected as target cells because they had been previously shown (Table III) to differ in that whereas both lines formed rosettes with EAC1-3b and EAC1-3d, only C. S. formed a significant number of rosettes with EAC14. Erythrocytes were studied because they contained an immune adherence (C3b) receptor which was antigenically shared with that of lymphocytes, but did not have a C3d receptor (6).

With erythrocytes (Table V), EAC1-3b rosette formation (immune adherence) was inhibited by fluid-phase C3b, C4, and C3c, but not by fluid-phase C3d. The
TABLE IV
Effect of Washing Procedures on the Inhibition of Tonsil Lymphocyte C Receptors by Fluid-Phase C Components and C Fragments

| Rosette type | Fluid-phase* component | Inhibition of rosette formation |
|--------------|-------------------------|--------------------------------|
|              | Before washing | After 1 wash | After 2 washes | After 3 washes |
|              | % | % | % | % |
| EAC14        | C4 | 99 | 94 | 90 | 83 |
|              | C3 | 75 | 60 | 52 | 40 |
|              | C3b | 97 | 94 | 93 | 88 |
|              | C3c | 49 | 0  | -  | -  |
|              | C3d | 0  | -  | -  | -  |
| EAC1-3b      | C4 | 0† | -  | -  | -  |
|              | C3 | 14 | 0  | -  | -  |
|              | C3b | 81 | 54 | 42 | 37 |
|              | C3c | 9  | 0  | -  | -  |
|              | C3d | 21 | 0  | -  | -  |
| EAC1-3b      | C4 | 0  | -  | -  | -  |
|              | C3 | 24 | 22 | 14 | -  |
|              | C3d | 40 | 36 | 15 | 0  |
|              | C3c | 0  | -  | -  | -  |
|              | C3d | 77 | 38 | 24 | 21 |

* Initial concentrations: C4, 70 μg/ml; C3, 87 μg/ml; C3b, 220 μg/ml; C3c, 182 μg/ml; and C3d, 40 μg/ml.
† Inhibition of EAC1-3b not obtained with less than 100 μg/ml of C4.

TABLE V
Inhibition of EAC1-3b Rosette Formation by Fluid-Phase C Components

| Target cell            | Inhibition by fluid-phase components* |
|------------------------|----------------------------------------|
|                        | C3b | C4 | C3c | C3d |
| Human erythrocytes     | 56  | 43 | 33  | 0   |
| Lymphoblastoid cells   |     |    |     |     |
| C. S. line             | 68  | 55 | 28  | 5   |
| H. H.-1 line           | 33  | 10 | 4   | 47  |

* Concentrations: C3b, 180 μg/ml; C4, 200 μg/ml; C3c, 150 μg/ml; and C3d, 36 μg/ml with lymphoblastoid cells and 75 μg/ml with human erythrocytes

lymphoblastoid cell lines differed from each other in that the C. S. line was inhibited more by fluid-phase C3c than it was by C3d, while the H. H.-1 line was inhibited more by fluid-phase C3d than it was by C3c. Further, C4 was found to produce greater inhibition with C. S. than with H. H.-1.

INHIBITION OF NORMAL LYMPHOCYTE RECEPTORS FOR THE EAC1-3b. Normal tonsil
lymphocytes were examined for inhibition of EAC1-3b rosette formation by C components and their fragments and by critical combinations of two of these. The results (Table VI) demonstrated that mixtures of C3c plus C3d and of C4 plus C3d inhibited EAC1-3b rosette formation more strongly than did fluid-phase C4, C3c, or C3d alone. The inhibition produced with these mixtures was as much or more than that inhibition produced by an equimolar concentration of fluid-phase C3b. However, the inhibition produced by a mixture of C4 plus C3c was not greater than that produced by C3c alone.

INHIBITION OF C RECEPTORS BY ANTISERA. Previously (6) it had been demonstrated that certain antisera had the ability to inhibit EAC rosette formation. Antierthrocyte serum inhibited normal lymphocyte rosettes with EAC1-3b but not with EAC1-3d. On the other hand, antichronic lymphatic leukemia lymphocyte (anti-CLL) serum inhibited normal lymphocyte rosette formation with EAC1-3d but not with EAC1-3b. Utilizing these same two antisera in the present study, it was found that the antierthrocyte but not the anti-CLL serum inhibited EAC14 rosette formation. Next, the antisera were used to study the C receptors on H. H.-1 cells and Raji cells. Raji cells resembled H. H.-1 cells in that they formed rosettes with EAC1-3b and EAC1-3d, but not with EAC14. Significantly, both of the lines differed from normal lymphocytes in that the anti-CLL serum inhibited both EAC1-3b (77% and 78%) and EAC1-3d (60% and 69%) rosette formation. Furthermore, the antierthrocyte serum did not inhibit EAC1-3b rosette formation with either line.

**Table VI**

| Inhibitor | Inhibition of EAC1-3b rosette formation* |
|-----------|-----------------------------------------|
|           | 10 x 10^-4 M‡ | 2.5 x 10^-4 M |
| C3b       | %            | %            |
| C4        | 24           | 7            |
| C3c       | 32           | 15           |
| C3d       | 9            | 5            |
| C3c + C3d | 46           | 40           |
| C4 + C3d  | 47           | 33           |
| C3c + C4  | 30           | 10           |
| C3b + C4  | 45           | 24           |
| C3b + C3d | 52           | 32           |

* In the absence of fluid-phase complement, EAC1-3b rosetted 59% of the tonsil lymphocytes.
† Final concentration of each inhibitor in the mixture.

Independent Capping of Different C Receptors

**Fluorescence Labeling of C Receptors.** After washing away unbound fluid-phase C, the bound components and thus indirectly the C receptors themselves,
were detected on tonsil lymphocytes by immunofluorescence (Table VII). Cells labeled at 4°C in the presence of sodium azide showed an even distribution of fluorescent patches. When the labeled cells were washed free of sodium azide and incubated at 37°C for 30 min, up to 50% of the fluorescence-labeled cells exhibited a redistribution of fluorescence into a single bright polar cap. The specificity of the fluorescence for CRL was confirmed by assaying fluorescence-labeled cells for rosette formation with each of the respective EAC complexes. By sequential observation of the same microscopic field by incident light fluorescence followed by visible light-phase contrast microscopy, no fluorescence-labeled cells were found that were not rosetted by EAC.

**Double-Label Assay for Capping of Two Receptors.** By combining the fluorescence assay for CRL with the rosette assay for CRL, two different C receptors could be labeled simultaneously on the same lymphocyte.

C3b on tonsil lymphocytes was fluorescence labeled and then induced to form caps by incubation of the cells at 37°C for 10 min. EAC1-3b were then incubated

| EAC type | Rosette formation | FITC*-labeled component | Fluorescent cells |
|----------|------------------|-------------------------|------------------|
| EAC14    | 48               | C4                      | 47               |
| EAC1-3b  | 57               | C3b                     | 52               |
| —        | —                | C3c                     | 46               |
| EAC1-3d  | 67               | C3d                     | 57               |

*FITC, fluorescein isothiocyanate-conjugated antibody label.

with the C3b fluorescence-capped lymphocytes for 15 min at 37°C to form C3b-dependent rosettes. 76% of the C3b fluorescence-capped cells contained an EAC1-3b rosette which was capped at the same place on the cell membrane as the fluorescence cap ("cocapping", Fig. 5a). Furthermore, the distribution of individual EAC1-3b on lymphocytes was the same as the fluorescence, even on cells on which the fluorescence was not located in a single cap. For example, when the fluorescence was distributed into three large patches, the EAC1-3b were attached at these same three points. On the other hand, only 25% of the C3b fluorescence-capped cells contained a rosette cap when EAC1-3d was used instead of EAC1-3b. In the other 75% of these C3b fluorescence-capped cells, the EAC1-3d rosettes either completely surrounded the lymphocytes (independent capping, Fig. 5 b) or capped at another location on the cell membrane. On most cells the distribution of the individual attached EAC1-3d seemed to bear no relationship to the distribution of the fluorescence. However, most of the EAC1-3d rosettes which were capped were located at a position on the cell membrane which was rotated 90° or less around the cell from the C3b fluorescence cap, and only on rare occasions was the EAC1-3d rosette cap observed to be 180° from the C3b fluorescence cap.
SPECIFICITY OF COMPLEMENT RECEPTORS

FIG. 5. Demonstration of independent capping of immune adherence receptors and C3d receptors. (a) Cocapping: Immune adherence receptors were first labeled by sequential addition of C3b, rabbit anti-C3, and goat antirabbit γ-globulin conjugated to fluorescein isothiocyanate. Next, the cells were incubated for 10 min at 37°C to cap the immune adherence receptors. Then EAC14 was mixed with the lymphocytes for 15 min at 37°C. The EAC14 rosette is cocapped with the C3b fluorescence cap (arrow) showing that complete capping of immune adherence receptors had occurred at the site of the fluorescence cap. (b) Independent capping: The same procedure as above was followed except that EAC1-3d was used instead of EAC14. In this case the fluorescence indicated the distribution of immune adherence receptors (indicated by arrow) while the rosette indicated the distribution of C3d receptors.

The tonsil lymphocyte receptors for EAC14 also cocapped (61%) with fluorescence-labeled C3b. In addition, when bound C4 was fluorescence-labeled instead of C3b, 80% of C4 fluorescence-capped cells were cocapped with EAC14, but only 13% were cocapped with EAC1-3d. The remaining 87% of these C4 fluorescence-capped cells contained an EAC1-3d rosette whose orientation was unrelated to the fluorescence cap. Finally, with caps of fluorescence-labeled C3d there was 64% cocapping with EAC1-3d rosettes, but only 34% cocapping with EAC1-3b rosettes and 23% cocapping with EAC14 rosettes.

Discussion

In order to ascertain the number of different types of C receptors on human lymphocytes and their specificity, experiments were performed in which highly purified C components or their fragments were tested for their ability to inhibit rosette formation with EAC. These inhibition studies demonstrated only two different types of C receptors and these were shown to be the same two which had been previously distinguished with antilymphocyte sera (6). These two receptors however, were responsible for binding the C contained in three different types of immune complexes: EAC14, EAC1-3b, and EAC1-3d.

First with normal lymphocytes, it was found that EAC1-3b rosette formation was inhibited by fluid-phase C4, C3b, C3c, and C3d. However, the degree of inhibition produced by each inhibitor was different. C4, C3c, and C3d each produced only partial inhibition of normal lymphocyte EAC1-3b rosette formation compared to that amount produced by C3b. Since both C3c and C3d inhibited EAC1-3b rosette formation, it appeared possible that EAC1-3b were
bound to two different types of C receptors simultaneously: one specific for the C3c region of uncleaved C3b, and another specific for the C3d region of uncleaved C3b. That this was indeed the case was demonstrated by utilizing a mixture of C3c plus C3d. This mixture produced as much inhibition of EAC1-3b rosette formation as did an equal protein concentration of C3b, and more inhibition than did either C3c or C3d alone. Furthermore, fluid-phase C4 could be substituted for fluid-phase C3c but not for fluid-phase C3d in this EAC1-3b inhibition mixture, so that it appeared that C4 was bound to the same C3c-region-specific receptor which bound C3b. Inhibition studies with EAC14 and EAC1-3d rosette formation confirmed the specificities of these two normal lymphocyte receptors. EAC14 rosette formation was strongly inhibited by fluid-phase C4, C3b, or C3c, but C3d had no effect on EAC14 rosette formation. Thus it appeared that EAC14 were only bound to the C3c-region-specific receptors and not to the C3d-region-specific receptors. On the other hand, EAC1-3d rosette formation was inhibited by C3b or C3d, but not by C3c or C4. These data indicated that EAC1-3d were only bound to the C3d-region-specific receptors and not to the C3c-region-specific receptors.

Similar inhibition assays were also performed to study the specificity of C receptors on human erythrocytes and two different lines of cultured lymphoblastoid cells. Erythrocytes formed rosettes with EAC14 and EAC1-3b, but not with EAC1-3d. The two lymphoblastoid lines differed from each other in that only one of the two lines formed significant numbers of rosettes with EAC14, even though both lines formed rosettes with EAC1-3b and EAC1-3d. With human erythrocytes, both EAC1-3b and EAC14 rosette formation were inhibited by fluid-phase C4, C3b, or C3c, but not by C3d. Thus it appeared that the same erythrocyte immune adherence receptor was responsible for binding both EAC14 and EAC1-3b. The inhibition studies however, indicated that the two lymphoblastoid lines differed qualitatively in their EAC1-3b rosette formation. With the EAC14 rosette-forming line (C. S., Table III), EAC1-3b rosette formation was primarily dependent on C3c-region-specific receptors, while in the other line (H. H.-1) EAC1-3b rosette formation was mainly dependent on C3d-region-specific receptors. These results indicated that EAC1-3b were primarily bound to immune adherence receptors in the case of the cell line which formed EAC14 rosettes. Even though this line also contained C3d receptors (as indicated by EAC1-3d rosette formation), EAC1-3b were mainly bound to immune adherence receptors, so that fluid-phase C3d had almost no effect on EAC1-3b rosette formation. On the other hand, in the other cell line, EAC1-3b were mainly bound to C3d receptors and not to C3c-region-specific (immune adherence) receptors. Since C3d receptors were not able to bind C4, this latter cell line was not able to form EAC14 rosettes.

In addition to the specificity studies with fluid-phase C, C receptors were also examined for inhibition by antisera. The antisera were the same ones which had been previously used to selectively inhibit either C3b or C3d receptors (6). The antierythrocyte serum, which inhibited normal lymphocyte rosette formation with EAC1-3b but not with EAC1-3d (6), also inhibited lymphocyte rosette formation with EAC14. The anti-CLL serum, which inhibited normal lymphocyte rosette formation with EAC1-3d but not with EAC1-3b (6), did not have any
effect on EAC14 rosette formation. The antisera were also used to study the C receptors on two lymphoblastoid lines which formed rosettes with EAC1-3b and EAC1-3d but not with EAC14. These two lines (H. H.-1 and Raji) differed significantly from normal lymphocytes in that the anti-CLL serum inhibited both EAC1-3b and EAC1-3d rosette formation. Furthermore, with these two cell lines, the anti-CLL serum produced more inhibition of EAC1-3b rosette formation than of EAC1-3d rosette formation. This further confirmed the finding with fluid-phase C3d that the C3d receptor on certain cell lines was responsible for rosette formation with both EAC1-3b and EAC1-3d.

From these studies in was concluded that lymphocytes contained only two different C receptors: the immune adherence receptor and the C3d receptor. The immune adherence receptor was specific for both C4 and the C3c region of C3b. The C3d receptor was specific for the C3d produced by C3b inactivator cleavage of C3b, but would also react to a lesser extent with the C3d region of uncleaved C3b. With normal lymphocytes which contained both receptors, EAC1-3b were bound preferentially to immune adherence receptors, though some simultaneous binding to C3d receptors also occurred. Due to the presence of some interaction between the C3d receptor and the uncleaved C3b molecule, certain lines of cultured lymphoblastoid cells which primarily contained C3d receptors, were able to form rosettes with EAC1-3b as well as with EAC1-3d. However, many other types of cells which contained only C3d receptors were able to form rosettes only with EAC1-3d and not with EAC1-3b. These latter C3d receptor-bearing cells were observed in normal tonsils, in certain other lymphoblastoid lines, and in the peripheral blood of patients with chronic lymphatic leukemia (6). Since EAC14 did not react with C3d receptors, EAC14 rosette formation was thus a more reliable indicator for immune adherence receptors in the presence of C3d receptors than was EAC1-3b rosette formation.

Recently, Bokisch and Sobel (24) have also described lymphocyte receptors for C4 and compared the specificity of these receptors to the previously described C3b receptors. Their data on the inhibition of normal lymphocyte rosette formation by fluid-phase C was similar to ours in that it demonstrated that C4 was probably bound to the same receptors which bound C3b. However, they did not conclude that C4 was bound to C3b receptors because the Raji cells which they also investigated formed rosettes with EAC1-3b but not with EAC14. This characteristic of Raji and other similar cell lines appeared to indicate that the C4 receptor might not be the same as the C3b receptor. In the study described herein, several cell lines similar to Raji were found, and one of them, H. H.-1, was studied extensively. The explanation for the difference in the binding of C4 and C3b to lymphocytes was that C3b could be bound to cells containing either the immune adherence receptor or the C3d receptor, while C4 could only be bound to lymphocytes containing the immune adherence receptor. Cell lines such as H. H.-1 and Raji, which contained only C3d receptors, bound C3b, but since they did not have immune adherence receptors, did not bind C4. With both H. H.-1 and Raji cells, the involvement of C3d receptors in EAC1-3b rosette formation was demonstrated by inhibition of EAC1-3b rosette formation with antileukemia lymphocyte serum. Furthermore, with these C3d receptor-bearing cell lines, rosette formation with EAC1-3b was shown to be much weaker than with
EAC1-3d. EAC1-3b rosette formation was inhibited by a lower concentration of either fluid-phase C3d or antileukemic lymphocyte serum than was EAC1-3d rosette formation. Indeed, EAC1-3d rosette formation with H. H.-1 cells was so strong that inhibition required at least $10 \times 10^{-7}$M C3d, and inhibition by fluid-phase C3b was difficult to demonstrate at all. This probably explains why Theofilopoulos et al. (25) found that with Raji cells, C3b inhibited EAC1-3b rosette formation but not EAC1-3d rosette formation. With normal lymphocytes and cell lines which formed weaker EAC1-3d rosettes, as little as $5 \times 10^{-8}$M C3b inhibited EAC1-3d rosette formation.

Previously it was shown that patients with CLL had lymphocytes with C3d receptors but had diminished numbers of lymphocytes with immune adherence receptors (6). Data obtained in the present study which utilized EAC14 to assay immune adherence receptors agree well with that previous finding in that few lymphocytes from these patients formed rosettes with EAC14. Furthermore, antiserum to leukemic lymphocytes did not inhibit EAC14. Lymphocytes from three patients with Waldenström’s macroglobulinemia differed from CLL lymphocytes in that they appeared to contain immune adherence receptors but diminished amounts of C3d receptors. Lymphocytes from patients with CLL also occasionally lack Slg (9). Such lymphocytes, having C3d receptors but lacking Slg and immune adherence receptors, have not been observed in normal blood, spleen, or thoracic duct (9), but in this study were observed to make up a significant proportion of tonsil lymphocytes.

Purified C components and their fragments were bound sufficiently firmly to C receptors on normal lymphocytes to allow their detection by indirect immunofluorescence. Similar fluorescence staining of C receptors on cells from the Raji lymphoblastoid line had been previously reported (26). The distribution of fluorescence-labeled C receptors was found to depend on labeling conditions in a manner similar to that reported for Slg (27). In our study, simultaneous detection of the two different C receptors on individual cells was accomplished by utilizing fluorescence to distinguish one type of receptor and EAC rosette formation to distinguish the other type. This technique permitted a study of the physical interrelationships of the two different receptors. It was found that 61-80% of cells containing caps of fluorescence-labeled C3b or C4 also contained immediately adjacent rosette caps of either EAC1-3b or EAC14 (cocapping). In contrast to this finding, only 13-25% of the cells with C3b or C4 fluorescence caps were cocapped with EAC1-3d rosettes. In the remaining 75-87% of these C3b or C4 fluorescence-capped cells, the EAC1-3d rosette either completely surrounded the cell or was capped at a location on the cell membrane that was rotated 90° or less around the cell membrane from the fluorescence cap. The ability of the immune adherence receptor and the C3d receptor to each cap independently of the other indicated that each was located on different molecules, each of which could move through the fluid membrane matrix independently of the other. Furthermore, it was of interest that both types of receptors were able to bind EAC after they had been fluorescence-labeled and capped. Much of the C bound from the fluid phase was removed by the washing steps during the fluorescence-labeling procedure and this permitted EAC rosette formation with the fluorescence-labeled cells. However, even though the remaining fluorescence-labeled C was insufficient to
inhibit rosette formation, it was able to cap almost all of the C receptors detectable by rosette formation. That the two types of fluorescence-capped receptor molecules were able to bind EAC, and almost always at the location of the fluorescence cap, indicates that both types of receptor molecules are probably multivalent.

The identification of C4 receptor activity on lymphoid cells and phagocytic cells demonstrates an activity for complex-bound C4 that was not previously recognized. This activity appears to be the same as the C4-dependent erythrocyte immune adherence originally described by Cooper (4). The C4 receptor activity of the immune adherence receptor may have more significance in vivo than the C3b activity of the receptor, since complex-bound C4, but not C3b, is protected from the action of C3b inactivator by the presence of C2 (N. R. Cooper, personal communication). In experiments performed with granulocytes, it was found that small amounts of bound C4 were sufficient for rosette formation and would greatly enhance the phagocytosis of EAγδ (28). Since monocytes required much more bound C4 than did granulocytes for rosette formation, it may be significant that monocytes, but not granulocytes (6), were found to have receptors for complex-bound C3d. Furthermore, in other studies the C3d receptors on monocytes were found to enhance the phagocytosis of erythrocytes in a similar manner as did the C4 receptors on granulocytes.

Summary

Erythrocytes, bone marrow-derived lymphocytes, monocytes, and granulocytes were shown to have a receptor activity for C4. This C4 receptor activity was studied in relation to the previously identified C3b and C3d receptors. By assay for inhibition of rosette formation by fluid-phase complement (C), only two different lymphocyte C receptors were demonstrated. The immune adherence receptor, the only one of the two shared in common with erythrocytes, was specific for C4 or the C3c region of C3b, but was unreactive with C3d. The other lymphocyte receptor, the C3d receptor, was specific for C3d fragments, but would also react to a lesser extent with the C3d region of uncleaved C3b. The C3d receptor did not react with either C3c or C4. This specificity of the C3d receptor allowed certain cells which contained only C3d receptors to form rosettes with EAC1-3b and EAC1-3d, but not with EAC14. However, because C3d receptors bound EAC1-3d or C3d fragments more firmly than they did EAC1-3b or C3b fragments, many other types of cells containing only C3d receptors, formed rosettes with EAC1-3d but not with EAC1-3b. Erythrocytes and those lymphocytes which contained only immune adherence receptors, formed rosettes with EAC14 and EAC1-3b but not with EAC1-3d. A double-label assay was devised for the simultaneous detection of both types of C receptors on individual lymphocytes. This assay involved fluorescence labeling of one of the two C receptors with soluble C fragments in combination with the usual rosette method for labeling the other type of C receptor. With this double-label assay, it was observed that the two different lymphocyte C receptors capped independently and thus were located on different molecules which could each move through the fluid membrane matrix independently of the other.

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