RECEPTOR FOR THE FOURTH COMPONENT OF COMPLEMENT ON HUMAN B LYMPHOCYTES AND CULTURED HUMAN LYMPHOBLASTOID CELLS*

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In this paper we describe a receptor for C4b on human peripheral B lymphocytes and cultured human lymphoblastoid cells. The C4b receptor was demonstrated by rosette formation with EAC14. We also found that this receptor cooperates with the C3b receptor in the binding of antigen-antibody complement complexes to lymphocytes. A preliminary report of this work was presented in the form of an abstract (1). Independently, Ross and Polley (2) recently presented evidence for the presence of such a receptor on polymorphonuclear leukocytes and B lymphocytes. The occurrence of a C4b receptor on human erythrocytes was originally described by Cooper (3) who observed C4b-dependent immune adherence.

Human peripheral B lymphocytes have receptors for IgG Fc (4–6), receptors for C3b (7, 8) and C3d (9), and membrane-bound IgG, which presumably functions as receptor for antigens. Although these receptors are useful for the characterization of cells, more important is their biologic role. IgG Fc, C3b, and C3d receptors mediate contact between antigen-antibody complement complexes and cells bearing these receptors. After contact, monocytes (10), macrophages (7), and polymorphonuclear leukocytes (11) engulf and digest such complexes. It was shown that IgG Fc and C3b receptors on monocytes (10) and polymorphonuclear leukocytes (2) cooperate in the phagocytosis of immune complexes. Another function of C3b receptors was demonstrated in an earlier report from this laboratory. C3b bound to C3b receptors of Raji cells, which are cultured human lymphoblastoid cells derived from a patient with Burkitt's lymphoma, lysed these cells through activation of the alternate complement pathway (12, 13).

Materials and Methods

Peripheral Blood Lymphocytes. Peripheral blood lymphocytes were prepared from defibrinated blood of healthy donors by sedimentation in 1% Dextran and centrifugation on Isopaque Ficoll as described earlier (12). Macrophages were removed by either incubation of the cells in plastic dishes and separation of the nonadhering cells or by carbonyl iron ingestion and separation in a magnetic field. The lymphocyte preparations obtained with these procedures contained 94–97% lymphocytes.

Lymphoblastoid Cell Lines. The cell lines used in this study were kindly provided by Dr. F. Jensen and Dr. A. N. Theofilopoulos, Scripps Clinic. Raji cells were derived from a Burkitt's lymphoma patient (14, 15), and Wil$_2$WT cells from the spleen of a patient with hereditary spherocytic anemia (16). SCRF 5001 are cultured from peripheral blood lymphocytes of SLE patients, 1454-9 cells are embryonic lymphocytes derived from the placenta of a patient with SLE. Sommer B 8432 cells are cultured from the peripheral blood of a patient with acute lymphocytic leukemia.

EAC Intermediate Complexes. EAC complexes were prepared with isolated human complement components. C1, C2, C3, and C4 were isolated from human serum according to published methods (17). For the preparation of EAC14, 1×10^9 sheep erythrocytes sensitized with rabbit anti-erythrocyte antibody were incubated with purified C1 for 20 min at 37°C, then washed at 37°C with GVB[1] (Veronal-buffered saline containing 0.1% gelatin, 1.5×10^{-4} M calcium and 5×10^{-4} M magnesium). EAC1 were then incubated with varying amounts of C4, ranging from 1 μg to 100 μg, for 30 min at 37°C. EAC14 were washed in cold GVB and stored at 4°C at a concentration of 1×10^9 cells per ml. In order to produce EAC1423, EAC14 cells were again incubated with C1 for 15 min at 37°C, washed, and reacted with an excess amount of C2 for 30 min at 32°C. After washing, EAC142 cells were incubated with varying amounts of C3, ranging from 1 μg to 100 μg for 20 min at 30°C. EAC1423 were washed in cold GVB and stored at 4°C at a concentration of 1×10^9 cells per ml. For the quantitation of molecules per cell, ^{125}I-labeled C3 or C4 were used.

Radioiodination of C3 and C4. C3 and C4 were labeled with ^{125}I according to the method of McConahey and Dixon (18).

Fragments of C3 and C4. C3b, a fragment of C3, was produced by cleaving C3 either with the complement enzyme $\overline{C42}$ or trypsin (19). C4b, a fragment of C4, was produced by treatment of C4 with C1s or trypsin (20).

Rosette Assay. 5×10^5 peripheral lymphocytes were incubated with 1×10^7 EAC in a total vol of 0.4 ml minimal essential medium (MEM) containing 5% fetal calf serum and 0.01 M EDTA. The cell mixture was incubated for 30 min at 37°C in a CO_2 incubator. The percent of rosette-forming cells (three or more bound EAC per lymphocyte) was determined by viewing at least 300 lymphocytes in a hemocytometer using a Leitz phase microscope (E. Leitz, Inc., Rockleigh, N. J.). In inhibition experiments various amounts of inhibitor were added to the cell mixture at the start of the incubation period.

Immune Adherence. Immune adherence experiments were performed in microtiter plates (Cook Engineering Co., Alexandria, Va.). Each well was filled with 25 μl of Veronal-buffered saline containing 0.1% human serum albumin, pH 7.5 (SAVB). Then 25 μl of 1×10^9 EAC was placed in the first well and diluted serially. Thereafter, 25 μl SAVB and 25 μl of 6×10^7 human blood group O erythrocytes were added to all wells. The cells were incubated for 30 min at 37°C, after which time the sedimentation pattern was evaluated. For inhibition experiments the highest dilution of EAC giving an agglutination pattern was chosen. In a typical inhibition experiment, all wells received 25 μl SAVB, then 25 μl of inhibitor was added to the first well and diluted serially. Thereafter, 25 μl of EAC suspension and 25 μl of 6×10^7 human blood group O erythrocytes were added to all wells and the plates were incubated for 30 min at 37°C and evaluated.

Removal of Rosette-Forming Lymphocytes by Albumin Gradient Centrifugation. Albumin gradient centrifugation was performed according to the method described by Bianco et al. (8).

KAF Treatment of EAC14 and EAC1423. KAF (C3b inactivator) was kindly provided by Dr. Cooper, Scripps Clinic. 1×10^9 EAC14 and EAC1423 were incubated with KAF for 60 min at 37°C. Thereafter, the treated cells were examined for immune adherence reactivity.

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[1] *Abbreviations used in this paper:* GVB, Veronal-buffered saline containing 0.1% gelatin, 1.5×10^{-4} M calcium and 5×10^{-4} magnesium; SAVB, Veronal-buffered saline containing 0.1% human serum albumin, pH 7.5.

Results

Demonstration of a C4b Receptor on Human Peripheral B Lymphocytes.
While studying the C3b receptor on human peripheral B lymphocytes, it was noticed that certain batches of EAC14 used as controls formed rosettes with virtually the same number of lymphocytes as did EAC1423. This observation suggested the presence of a receptor for C4b on lymphocytes. Table I compares the percentage of lymphocytes of several healthy blood donors which formed rosettes with EA, EAC14, and EAC1423. Virtually the same percentage of lymphocytes formed rosettes with EAC14 (14.4%) and EAC1423 (13.5%). Only 2.8% lymphocytes formed rosettes with EA. Approximately the same number of lymphocytes forming rosettes with EAC14 also interacted with EAC142 or EAC14oxy2.

The observation that a comparable number of lymphocytes in each preparation

TABLE I
Receptors for C4b on Human Peripheral Lymphocytes

Lymphocyte Donors	Rosette-forming lymphocytes		
	EA	EAC14	EAC1423
	%	%	%
H. S.	1	16	14
M. G.	6	15	17
E. S.	0	16	15
P. U.	3	14	13
A. S.	4	12	7
M. Y.	1	9	7
P. B.	5	19	22
Average	2.8	14.4	13.5

formed rosettes with EAC14 and EAC1423 suggested that it was the same cell population which interacted with both intermediate complexes. This was demonstrated by two different methods: (a) 1×10^7 intact EAC14 and 1×10^7 lysed EAC1423 were incubated together with 5×10^5 lymphocytes. Rosettes consisting of lymphocytes and both intermediate complexes were observed under phase microscopy. (b) Reduction of the number of EAC1423 rosette-forming lymphocytes in a lymphocyte preparation also diminished the number of EAC14 rosette-forming lymphocytes. In these experiments, one batch of 1×10^7 lymphocytes was first allowed to form rosettes with 2×10^8 EAC1423. EAC1423 were prepared from EAC14 containing a low number of C4b sites per cell which per se did not induce rosette formation. As controls, one batch of 1×10^7 lymphocytes was incubated with 2×10^8 EAC1 and another batch in medium alone. Subsequently, the three batches of lymphocytes were subjected to centrifugation in a 23–33% bovine serum albumin gradient, in which rosettes and erythrocytes are pelleted and the free lymphocytes remain in the upper portion of

the gradient. These lymphocytes were subsequently analyzed for their ability to form rosettes with either EAC14 or EAC1423. The results of a representative experiment are graphed in Fig. 1. In the batch of lymphocytes which had been incubated with EAC1423, the number of both EAC1423 lymphocyte rosettes and EAC14 lymphocyte rosettes was significantly reduced. The results obtained show that the receptors for C4b and C3b are present on the same cell. Furthermore, staining of lymphocytes with fluorescein-conjugated antihuman IgG showed that EAC14 as well as EAC1423 rosette-forming cells have membrane-bound Ig, characterizing these cells as B-type lymphocytes.

Exclusion of Traces of C3b on EAC14.   Since it was suggested that only 60–100 C3b molecules per EAC1423 are required for immune adherence (3), it had to be excluded that EAC14-forming rosettes with lymphocytes contained trace amounts of C3b. To assess whether EAC14 had taken up traces of C3b, EAC14 was prepared with C4 which was purposely contaminated with [125I]-labeled C3, in
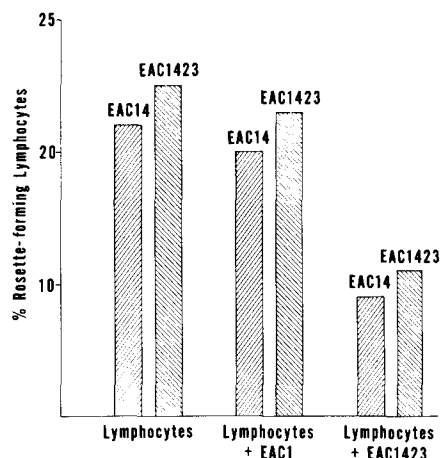

FIG. 1.   EAC14 and EAC1423 rosettes after removal of EAC1423 lymphocyte rosettes from the lymphocyte preparation.

two separate experiments. In the first experiment, batch (a) was incubated with 15 μg of C4 alone, batch (b) with 15 μg C4 and 15 μg [125I]C3, and batch (c) with 15 μg [125I]C3 alone. The second experiment followed the same protocol with the exception that, instead of 15 μg, 30 μg of each component was used. All complement-cell complexes were analyzed for the presence of C3b by hemagglutination with rabbit antihuman C3 and by measuring uptake of radiolabeled C3. The results of these experiments are summarized in Table II. None of the intermediate complexes was agglutinated with anti-C3. EAC1 which was incubated with either C4 alone or with C4 and [125I]C3 agglutinated strongly with antihuman C4, demonstrating the presence of C4 on those cells. In both experiments, EAC1 incubated with C4 and [125I]C3 and EAC1 incubated with [125I]C3 alone bound the same number of C3 molecules suggesting that C3 was bound nonspecifically. Furthermore, there was no difference in [125I]C3 uptake between the two experiments which differed in the amounts of C4 and [125I]C3

TABLE II

Analysis of EAC14 for the Presence of C3b

Treatment of EAC1 cells*	Rosette formation	Immune adherence	Hemagglutination with:		No. of C3 or C3b molecules per cell
			Anti-C3	Anti-C4	
15 μg C4 + /	0	0	0	+	0
15 μg C4 + 15 μg [125I] C3	0	0	0	+	46
/ + 15 μg [125I] C3	0	0	0	0	46
30 μg C4 + /	+	+	0	+	0
30 μg C4 + 30 μg [125I] C3	+	+	0	+	50
/ + 30 μg [125I] C3	0	0	0	0	45

* 1 × 10^9 EAC1 cells were incubated with C4 and/or C3 in a total vol of 0.5 ml.

used. The results demonstrate that binding of EAC14 to lymphocytes or erythrocytes is mediated by C4b. When tested in the rosette assay or immune adherence, intermediate complexes prepared with 15 μg C4 were negative, whereas the intermediate complexes prepared with 30 μg of C4 were positive. The observation that only EAC14 cells prepared with 30 μg of C4 were positive in immune adherence and rosette formation suggested a dose dependency of this phenomenon.

Dose Response of C4b and C3b Rosette Formation and Immune Adherence. To measure the C4 dependence of rosette formation and immune adherence, EAC14 with a defined number of [125I]C4b molecules per cell was used. For comparison, EAC1423 with a comparable number of [125I]C3b molecules per cell was also tested in both assays. The results of this experiment which are depicted in Fig. 2 show that more than 4,000 C4b molecules per cell are required to produce a maximal number of rosettes. EAC1423 containing less than 1,000 C4b molecules per cell needed at least 2,000 C3b molecules per cell in order to produce maximal rosette formation. The immune adherence reactivity of the two different types of intermediate complexes roughly paralleled their rosette-forming ability.

To investigate whether C3b and C4b cooperate in the rosette-forming function, the following experiment was performed. Two batches of EAC14 were prepared, one containing 350 molecules of C4b per cell and the other 5,500 C4b molecules per cell. Samples of these preparations were then reacted with C2 and C3, such that the number of bound C3b molecules varied in different samples between 0 and 2,100 per cell. Fig. 3 shows the percentage of rosette formation as a function of cell-bound C3b.

The apparent rosette-forming ability of cells with a small number of bound C4b molecules is entirely dependent on the presence of a relatively large number of C3b molecules. Rosette formation by cells with a large number of C4b molecules is independent of, however, increases by cell-bound C3b. In this particular experiment the same percentage of rosette formation was observed with either 5,500 C4b molecules per cell or 350 C4b plus 1,500 C3b molecules per
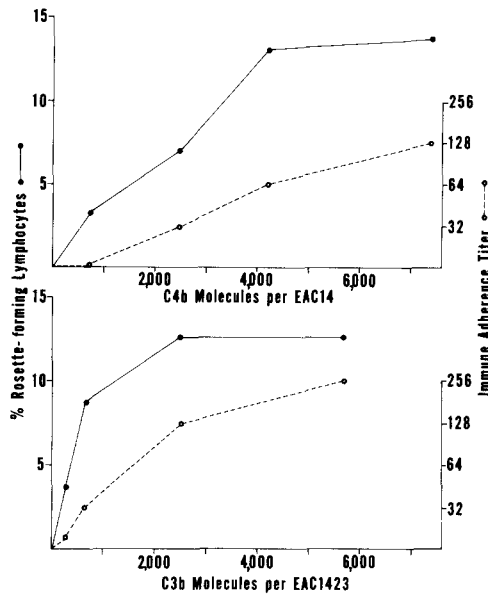
FIG. 2. Dose response of C4 and C3 in lymphocyte rosette formation and immune adherence with EAC14 and EAC1423.
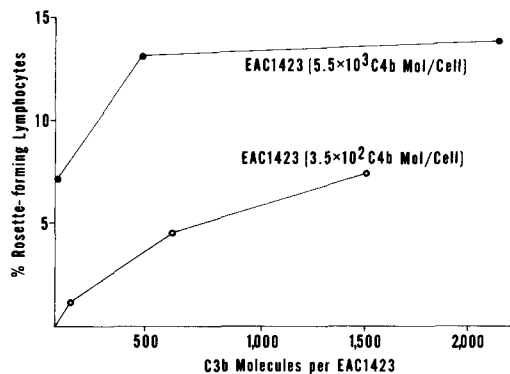

FIG. 3. Cooperation of C3 and C4 in lymphocyte rosette formation.

cell. These results suggest that C3b is more efficient than C4b or that both fragments act cooperatively in the binding of EAC1423 to human lymphocytes.

Characterization of C3b and C4b Receptors as Identical or Distinct Entities. Since C3b and C4b bind to the same cells, the question arose as to whether these cells have one receptor which binds both ligands or, alternatively, two different receptors, one specific for C3b and the other for C4b. To distinguish between the two possibilities, we tried to inhibit EAC14 lymphocyte rosettes with soluble C3b or C4b. The results depicted in Fig. 4 show that the same degree of inhibition was obtained with equal amounts of C3b or C4b. EAC1423 lymphocyte rosettes are also inhibited by equal amounts of either protein. These results suggest that human B lymphocytes have a single receptor capable of binding C3b and C4b.
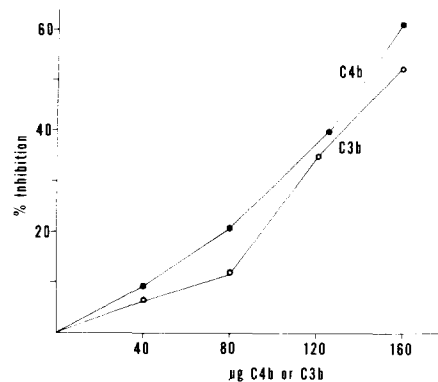
Fig. 4. Inhibition of EAC14 lymphocyte rosettes by C3b or C4b.

To inhibit immune adherence of EAC14 or EAC1423 to human erythrocytes an approximately 300-fold excess of free over bound C3b or C4b was needed. Considerably more free overbound molecules were required to inhibit lymphocyte rosette formation with either intermediate complex. As shown in Fig. 4, 160 μg of C3b or C4b resulted in 60% inhibition of rosette formation. Since EAC14 intermediates used in this experiment contained 5,500 C4b molecules per cell, the molar ratio of bound C4b to free C4b or C3b was approximately 1 to 10,000. In spite of the large amount of inhibitor required, the inhibition was specific since the same amount of albumin or IgG was not inhibitory.

In contrast to the inhibition experiment which suggested a single receptor for C3b and C4b, evidence for a C3b receptor distinct from the C4b receptor was found by testing several human lymphoblastoid cell lines for their ability to form rosettes with EAC14 or with EAC1423. Table III shows that, of all cell lines tested, fewer cells formed rosettes with EAC14 than with EAC1423. Raji cells formed rosettes only with EAC1423 and not with EAC14. As shown in Fig. 5, no rosettes were observed even when EAC14 was used containing 14,000 C4b molecules per cell, whereas approximately 65% of Raji cells formed rosettes with EAC1423 containing the same number of C3b molecules per cell.

Lack of Receptors for KAF-Treated C4b on Human Peripheral Lymphocytes and Erythrocytes.   Cooper[2] demonstrated that KAF (C3b inactivator) cleaves cell-bound C4b in a manner similar to cell-bound C3b. Following KAF treatment of cell-bound C4b, a portion of the molecule (C4d) analogous to C3d remains cell bound, whereas the remainder of the C4b molecule is dissociated from the cells. Because C3d reacts with human lymphocytes and certain cultured lymphoblastoid cells,[3] (9, 21) we tested whether human B lymphocytes have receptors for C4d. EAC14 and EAC1423 were incubated with purified KAF for 60 min at 37°C, washed, and then used in the rosette test with human peripheral lymphocytes. As control, the same intermediate complexes were incubated with NaCl. As shown

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[2] Cooper, N. R. Manuscript in preparation.

[3] Theofilopoulos, A. N., C. B. Wilson, F. J. Dixon, and V. A. Bokisch. Binding of soluble immune complexes to human lymphoblastoid cells. II. Use of Raji cells to detect circulating immune complexes in animal and human sera. *J. Exp. Med.* **140**:1230.

TABLE III

C4b and C3b Receptors on Human Lymphoblastoid Cells

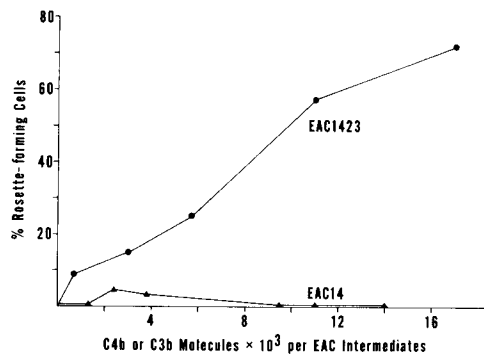| Cell lines	Rosette-forming cells		
	EA	EAC14	EAC1423
	%	%	%
Wil₂ Wt	5	1	7
SCRF 5001	6	8	38
1454-9	0	15	45
Sommer B 8432	5	39	70
Raji	2	1	82

FIG. 5.   Dose response of C4 or C3 in rosette formation of Raji cells with EAC14 or EAC1423.

in Fig. 6, KAF treatment of the intermediate complexes significantly reduced the number of EAC14 lymphocyte rosettes, while only a slight reduction of EAC1423 lymphocyte rosettes was observed. These results indicate that KAF treatment abolishes the binding site of cell-bound C4b and, that human lymphocytes lack a receptor for C4d.

Discussion

This report describes a receptor for C4b on peripheral human B lymphocytes. The receptor reacts with red cell-bound C4b (EAC14) as well as with soluble C4b. The presence of a C4b receptor on erythrocytes has previously been shown by Cooper (3) who demonstrated that EAC14 cells produced the immune adherence phenomenon with human erythrocytes. Recently, Ross et al. described a similar receptor on human polymorphonuclear leukocytes (2). Since it was previously shown that only 60-100 C3b molecules per EAC1423 cell were needed for a positive immune adherence reaction with human erythrocytes (3), the presence of minute amounts of C3b on EAC14 cells had to be excluded. For this purpose, known amounts of [¹²⁵I]C3 were added to C4 preparations and C3 uptake on EAC14 was measured. In several experiments using different amounts of C4,
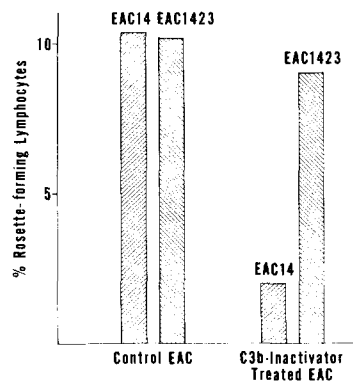
FIG. 6. Effect of KAF (C3b inactivator) on EAC14 and EAC1423 lymphocyte rosette formation.

purposely contaminated with C3, we found no uptake of specifically bound C3 by EAC14 which could account for their interaction with human erythrocytes or B lymphocytes.

The question whether the C4b receptors are distinct from the C3b receptors on human erythrocytes and B lymphocytes remains unresolved. The observation that both C3b and C4b inhibit EAC14 and EAC1423 lymphocyte rosettes and the immune adherence reaction of both intermediate complexes with human erythrocytes suggested that the C4b and C3b receptors are either identical or are different entities, exhibiting cross specificity for the other ligand. Receptors which bind more than one type of ligand have been described. For example, C3b receptors on Raji cells (12) and other human lymphoblastoid cells bind, in addition to human C3b, native C3, as well as C3b and C3 of other species[3] (21).

Furthermore, recent studies revealed a striking similarity between C3[4] and C4[5] (19, 20). In the course of complement activation, C3 and C4 are cleaved by the complement enzymes C3 convertase and C1 esterase, respectively, into a low molecular weight a fragment and a large b fragment. Upon cleavage, C3b and C4b acquire two binding sites, a labile binding site which allows the molecules to attach themselves to cell membrane surfaces, immune complexes and other particles, and a stable binding site which enables C3b and C4b to react with C3b or C4b receptors on the surface of various cells. Both molecules are also susceptible to the action of KAF, or C3b inactivator.[2]

In contrast to human peripheral lymphocytes, which formed the same number of rosettes with EAC14 and EAC1423, cultured human lymphoblastoid cells gave fewer rosettes with EAC14 than with EAC1423. More importantly, Raji cells formed rosettes only with EAC1423, demonstrating that these cells lack receptors for C4b and that the C3b receptors on these cells do not cross-react with C4b. These results may suggest that the receptors for C3b and C4b are distinct entities. However, since cells in culture alter some of their properties, it is

conceivable that receptors of cultured cells may have changed their specificity, or may have been lost.

Since KAF cleaves C4b[2] as well as C3b (22, 23), rendering EAC14 and EAC1423 unreactive in immune adherence with human erythrocytes, we investigated whether such treatment exposed a new binding site on the C4b molecule analogous to the C3d binding site which appears after KAF treatment of EAC1423. Peripheral human lymphocytes (9) and lymphoblastoid cells[3] (21) have a receptor for C3d. The fact that KAF-treated EAC14 did not form rosettes with peripheral human lymphocytes indicates that lymphocytes lack a receptor for KAF-treated C4b (C4d).

Cell-bound C4b and C3b cooperate in the binding of EAC1423 to C3b and C4b receptor bearing cells. This was demonstrated by lymphocyte-rosette formation with various EAC1423, prepared from two batches of EAC14 cells differing in the amount of cell-bound C4b. In these experiments, the number of cell-bound C3b molecules required to give a maximal number of rosettes was significantly lower for EAC1423 containing a large number of C4b molecules as compared to EAC1423 with few C4b molecules per cell, indicating a cooperative effect of C3b and C4b. Because of the cooperation of C3b and C4b in EAC1423-lymphocyte rosette formation or immune adherence reactions the number of cell-bound C4b has to be taken into account in estimating the number of cell-bound C3b required for the binding of EAC1423 to receptor-bearing cells. Indeed, the discrepancy in the numbers of C3b on EAC1423 needed for immune adherence between our results and those of others may be due to different amounts of C4b per EAC1423.

It has been shown that the receptor for C3b is essential in phagocytosis of antigen-antibody complement complexes. The C3b receptor on phagocytic cells such as monocytes or polymorphonuclear leukocytes and macrophages mediates contact between these complexes and the cells. Huber et al. (10) have shown that binding of antigen-antibody complexes without complement to phagocytic cells is inhibited by physiologic amounts of IgG. Binding of complexes, however, can be mediated by C3b even in the presence of IgG. In a model system, using Raji cells which have receptors for IgG Fc and C3b, we could show that human aggregated IgG which has interacted with complement bound to these cells only through the complement receptors for C3b and C3d.[3] In the present report we show that C4b like C3b mediated the binding of antigen-antibody complement complexes to human B lymphocytes and human erythrocytes via C4b receptors on these cells. Similar receptors are present on polymorphonuclear leukocytes (2). We have preliminary evidence for the presence of C4b receptors on human monocytes. Since attachment of immune complexes to phagocytic cells is mostly followed by phagocytosis of these complexes, it is evident that the C4b receptor plays a role in immune clearance of such complexes. The reduced immune clearance of immune complexes in C4-deficient guinea pigs as compared to normal animals may exemplify the contribution of C4b to this mechanism (24).

Summary

This report describes receptors for C4b on human peripheral B lymphocytes. The simultaneous presence of C3b and C4b receptors on the same lymphocytes was

demonstrated by the formation of mixed rosettes consisting of the lymphocytes, EAC14 and EAC1423. Furthermore, reduction of the number of EAC1423 rosette-forming lymphocytes in a lymphocyte population by albumin gradient centrifugation concomitantly reduced EAC14 rosette-forming lymphocytes. Binding of EAC14 intermediates to receptors on human lymphocytes and erythrocytes could be inhibited by equal amounts of soluble C3b or C4b, suggesting the presence of a single receptor for both ligands on those cells. In contrast, the results of the rosette assay with Raji cells, cultured human lymphoblastoid cells, EAC14 and EAC1423 suggested that the receptors for C4b and C3b are distinct entities, since Raji cells formed rosettes with EAC1423, but not with EAC14. Moreover, this report demonstrates a cooperation of erythrocyte-bound C4b and C3b in the binding of EAC1423 to B lymphocytes. In contrast to KAF-treated C3b, KAF-treated C4b did not bind to B lymphocytes, indicating that these cells lack a receptor for C4d.

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