

RECEPTOR FOR SOLUBLE C3 AND C3b ON HUMAN LYMPHOBLASTOID (RAJI) CELLS

PROPERTIES AND BIOLOGICAL SIGNIFICANCE*

BY ARGYRIOS N. THEOFILOPOULOS,† VIKTOR A. BOKISCH,§ AND
FRANK J. DIXON

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

(Received for publication 6 December 1973)

Membrane receptors for C3b are present on a variety of cells, including erythrocytes, polymorphonuclear leukocytes, macrophages, and bone marrow-derived (B) lymphocytes (1). Upon enzymatic cleavage of C3 into the fragments C3a and C3b, two binding sites become exposed on the C3b molecule. One binding site is short-lived and allows attachment of C3b to a receptor site on the cell membrane other than the C3b receptor (2). The other binding site is stable and interacts with the C3b receptor (immune adherence receptor) (3, 4). C3b's property of binding in two different ways on cell membranes enables the molecule to mediate cell to cell contact, which can be demonstrated by techniques such as immune adherence and rosette formation between EAC1423 and C3b receptor-bearing cells.

Occupation of the C3b receptor by C3b may in many instances induce changes in the activity of the receptor bearing cells. An example of this phenomenon is the phagocytosis of EAC1423 by human monocytes after contact through C3b (5). Similarly, in other experiments it was shown that the same monocytes exerted a cytolytic effect on chicken EAC1423 (6). These effects appeared to be triggered solely by C3b since the phagocytosis or cytolysis did not require additional complement components.

C3b is a factor in the alternate pathway of complement. Together with C3PA convertase (C3PAse)¹ it cleaves C3 proactivator (C3PA) which in turn triggers cleavage

* This is Publication No. 777 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation. This work was supported in part by National Institutes of Health grant AI-07007, and grant IC-73 from the American Cancer Society. Presented in part at the 5th International Complement Workshop, 1973, Coronado Island, California and at the 57th Annual Meeting of the Federation of American Societies for Experimental Biology, 1973, Atlantic City.

† Supported by grant DADA 17-73-C-3137 from the Department of the U.S. Army.

§ Recipient of the National Institute of Health Career Research Development Award 5KO4 AI 70263-02.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; C3bina, C3b inactivator; C3PA, C3 proactivator; C3PAse, C3PA convertase; CoF, cobra venom factor; EA, antibody-sensitized sheep erythrocytes; FITC, fluorescein isothiocyanate; GVB, Veronal-buffered (5 mM) saline containing 1.0% gelatin, 1.5×10^{-4} M CaCl₂, 5×10^{-4} M MgCl₂; MEM, minimum essential medium; NHS, normal human serum; NRS, normal rabbit serum; PBS, phosphate-buffered saline; RBC, red blood cell.

of C3 into the fragments C3a and C3b (7, 8). It is conceivable that receptor-bound C3b may initiate complement activation at the membrane. Attachment of additional components may result in changes of cell activity or even cell death.

The present study concerns itself with the characterization of a C3 receptor on the Raji line of lymphocytes derived from a Burkitt lymphoma. The receptor binds soluble C3 as well as C3b. Lysis of cultured Raji cells occurs when C3b is attached to the receptor in the presence of fresh serum. The lytic effect is abolished by inactivating serum C3PA and requires C6.

Materials and Methods

Lymphoblastoid Cell Lines.—The four cell lines used in this study were originally provided by Dr. R. Lerner. The Raji and Daudi cell lines were derived from patients with Burkitt lymphoma (9, 10). 8866 cells were derived from the peripheral blood of a patient with acute myelocytic leukemia (11), and WiL₂WT cells were cultured from the spleen of a patient with hereditary spherocytic anemia (12). Suspension cultures of these cell lines were propagated in Eagle's minimal essential medium (MEM; Autopow, Flow Laboratories, Inc., Rockville, Md.) supplemented with glutamine, nonessential amino acids, pyruvate, 10% fetal bovine serum, penicillin, streptomycin, and Fungizone as described by Lerner et al. (13). Cell viability was determined by trypan blue exclusion.

Peripheral Blood Lymphocytes.—Peripheral blood lymphocytes were obtained from normal healthy adults. Venous blood was defibrinated by agitation with sterile glass beads. Erythrocytes were sedimented by incubating two volumes of blood with one volume of 3% dextran (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) in MEM for 30 min at 37°C. The leukocyte-rich supernatant was layered on an Isopaque-Ficoll gradient and centrifuged for 10 min at 1,500 *g* (14). The layer aspirated from the interface was washed three times in MEM, and the macrophages were removed by using columns packed with nylon fibers (15). The remaining preparation contained 90–95% lymphocytes.

C3 and C3 Fragments.—C3 was prepared from human serum according to the method described by Nilsson and Müller-Eberhard (16). The fragments C3b and C3c were prepared by enzymatic cleavage of C3 with trypsin (17). They were isolated by column chromatography and pevikon electrophoresis. C3 and the fragments were freed of aggregates and possible contamination with aggregated endotoxin by centrifugation for 1 h at 100,000 *g*. The upper two-thirds of the contents of the centrifuge tubes were filtered through a 0.65 μ Millipore filter and stored in PBS at 4°C.

The addition of C3b to whole serum resulted in cleavage of C3PA. The C3PA cleaving activity of C3b was determined by immunoelectrophoresis. In a typical experiment 10–25 μ g of C3b were added to 100 μ l of fresh human serum and incubated for 30 min at 37°C. The reaction mixture was subjected to immunoelectrophoresis and developed with a rabbit anti-human C3PA serum (7).

Radioidination.—C3, C3b, and C3c were labeled with ¹²⁵I according to the method of McConahey and Dixon (18). After labeling, each preparation was dialyzed against phosphate-buffered saline (PBS) and then centrifuged for 90 min at 100,000 *g* in order to remove any aggregates. No conversion of C3 was detected after labeling. The specific activity of the preparations used was approximately 2×10^5 cpm/ μ g protein.

Cobra Venom Factor.—Cobra venom factor (CoF), a gift from Dr. O. Götze, was purified from the venom of *Naja naja* (Miami Serpentarium, Miami, Flor.) according to the method of Müller-Eberhard and Fjellström (19). The isolated material was centrifuged for 1 h at 100,000 *g* to free it from possible contamination by aggregated endotoxin, the upper two-

thirds of the contents of the centrifuge tubes was filtered through a 0.65 μ Millipore filter and stored in PBS at -70°C .

Antisera.—Antihuman C3 sera were prepared by immunizing rabbits with isolated C3 (17). The anti-C3 sera used in this study reacted with C3 and the fragments C3b, C3c, and C3d when tested by double diffusion in agar. The anti-C3 serum gave a single precipitin line when tested against normal human serum (NHS) or isolated C3 on Ouchterlony analysis and immunoelectrophoresis. The anti-C3 serum agglutinated EAC1423 up to a 1:256 dilution. Anti-C3a was produced by immunizing rabbits with isolated C3a (17). The anti-C3a serum formed a precipitin line with C3a as well as C3 in agar double diffusion. No reaction was observed with C3b. Antirabbit IgG sera were produced by immunization of goats with isolated rabbit IgG.

Immunofluorescent Staining.—Antisera were conjugated according to described methods (20). C3 and C3b bound to cell surface were detected by immunofluorescent staining with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-C3. In a typical experiment 5×10^6 cells in 25 μl of MEM were incubated under gentle shaking with the various amounts of C3, C3b, aged human serum, or NHS at 37°C for 30 min. Aged human serum used in this study contained the fragments C3c and C3d but not C3 and C3b as demonstrated by immunoelectrophoretic analysis using a potent anti-C3 serum (17, 21). Subsequently, cells were washed three times with MEM and 25 μl of FITC anti-C3 was added. The mixture was incubated with gentle shaking for 30 min at 4°C . The cells were then washed three times with MEM and resuspended in 10 μl of 10% bovine serum albumin (BSA) in PBS. Cell smears were air dried, fixed in 95% cold ethanol, and mounted under glycerol. Wet preparations were made by sealing a drop of the cell solution with paramount under a cover slip. Smears were observed by using a Zeiss RA microscope (Carl Zeiss, Inc., New York) equipped with the FITC interference filter system (Optisk Laboratorium, Lyngly, Denmark) and illuminated from a HBO 200 watt mercury light source.

To demonstrate native C3 on cell surfaces, an indirect staining technique was used. 5×10^6 C3 or C3b bearing cells were allowed to react with rabbit anti C3a. The washed cells were subsequently stained with fluorescein-conjugated goat antirabbit IgG.

Erythrocyte-Antibody-Complement Complexes (EAC).—EAC1423 cells were prepared with purified human complement components. Purified macromolecular C1 was prepared from human serum according to Nelson et al. (22). C2 was purified as described previously (23) and was used exclusively in its oxidized form (24) unless otherwise stated. C4 (25) and C3 (16) were obtained according to published methods. 5×10^9 sheep erythrocytes (E) sensitized with rabbit antierythrocyte antibody (A) were incubated with purified C1 for 20 min at 37°C , washed with GVB (5mM veronal buffered saline with 1.0% gelatin, 1.5×10^{-4} M calcium, and 5×10^{-4} M magnesium) and then reacted with 100 μg of purified C4 for 30 min at 37°C . Subsequently, the EAC14 cells were washed with GVB and reacted again with C1 for 15 min at 37°C . After additional washing with GVB an excess amount of oxyC2 was added and the cells were incubated for 30 min at 32°C . After three washes in 0.01 M EDTA containing GVB, 1×10^9 EAC14oxy2 cells were incubated with 100 μg purified C3 for 20 min at 30°C . Thereafter, the EAC14oxy23 cells were washed three times with GVB and stored at 4°C at a final concentration of 1×10^9 cells/ml. EAC1423 were made as with EAC14oxy23 but C2 was used in its nonoxidized form. One batch of cells could be used over a period of seven days.

Rosette Formation of C3 Receptor-Bearing Cells with EAC14oxy23.— 2×10^6 lymphoid cells were incubated with 5×10^7 EAC14oxy23 in a total volume of 200 μl of MEM at 37°C for 30 min during which time the mixture was shaken twice by hand. Thereafter, the cells were suspended in 1 ml MEM and placed on ice for 10 min. Rosetted (four or more bound EAC14oxy23) and unrosetted lymphocytes were counted in a hemocytometer using a Zeiss phase contrast microscope (Carl Zeiss, Inc.). 500 cells were counted and the per cent of rosette-forming cells was calculated.

Immune Adherence.—Immune adherence experiments were performed in microtiter plates (Cook Engineering Co., Alexandria, Va.). In a typical experiment each well was filled with 25 μ l of veronal buffer pH 7.5, T/2 0.1 containing 0.1% human serum albumin. 25 μ l of 1×10^9 Raji cells, which had been preincubated with C3b, were placed into the first well and dilutions were made using a 25 μ l microtiter loop. After addition of 25 μ l of a human O group red cell suspension containing 2×10^8 red cells per ml to all wells, the plates were agitated for 5 min and then incubated for 30 min at 37°C after which time the sedimentation pattern was evaluated. Dilutions of EAC14oxy23 were used as positive controls.

RESULTS

Binding of C3 and C3b in Free Solution to Receptors on Raji Cells.—When 5×10^6 Raji cells were incubated for 30 min at 37°C in MEM containing 10 μ l of fresh human serum nearly 100% of the cells were positive when subsequently stained with FITC anti C3 (Fig. 1). In contrast, incubation with even ten times as much aged human serum gave no positive cells. Human serum treated with CoF, resulting in cleavage of C3 into C3a and C3b, (26) gave a staining (not shown) similar to that of fresh human serum. These results indicate that C3 and C3b bind to receptor sites on Raji cells. In contrast, it was shown that C3c and C3d which are present in aged human serum did not bind to the cells.

A possible mediation of the binding of C3 and C3b to Raji cell receptors by other complement or serum factors was excluded, since incubation with isolated C3 and C3b at concentrations equivalent to that of serum resulted in nearly 100% staining of the cells (Fig. 1). Binding of C3 and C3b proceeds in the presence of 0.01 M EDTA.

Binding of C3 and C3b to Raji cells is to some extent temperature dependent. When incubation with C3 and C3b was carried out at 4°C, only 40% were fluorescence positive as compared to nearly 100% after incubation at 37°C. The pattern of staining also varied with temperature (Fig. 2). Incubation of

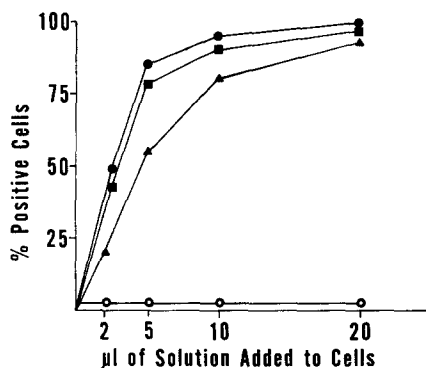


FIG. 1. Demonstration of Raji cell-bound C3 and C3b by immunofluorescence. 5×10^6 Raji cells were incubated with various amounts of NHS (●—●), C3 (■—■), C3b (▲—▲) and aged human serum containing C3c and C3d (○—○). C3 and C3b were at concentrations of 1.5 mg/ml. Cells were stained with FITC antihuman C3.

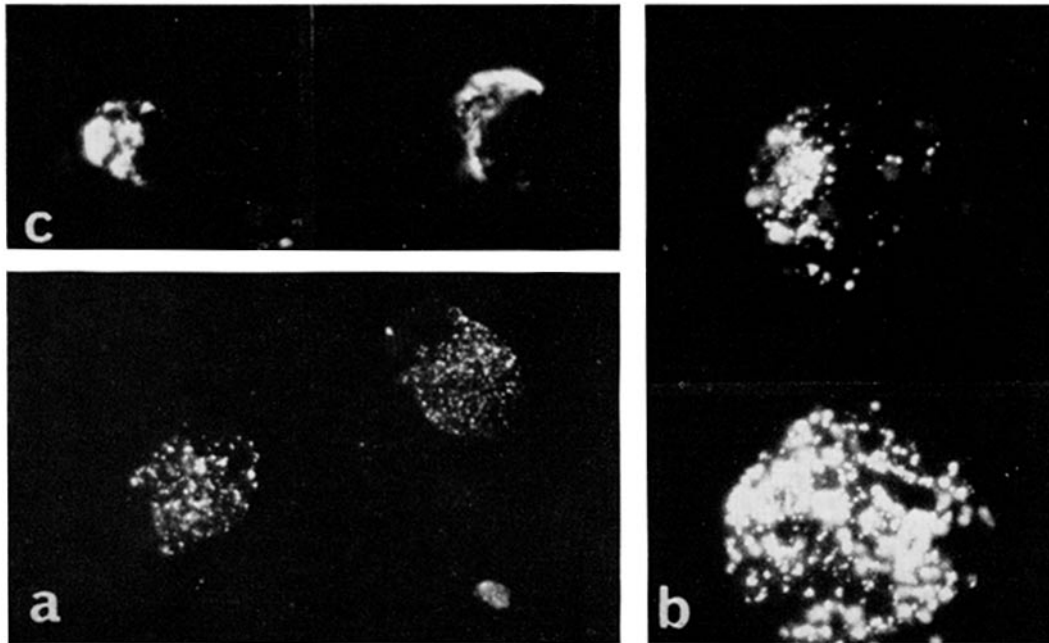


FIG. 2. Immunofluorescent staining patterns of C3-bearing Raji cells. (a) Incubation of cells with C3 and staining with FITC anti-C3 were carried out at 4°C. (b) Incubation at 37°C and staining at 4°C. (c) Incubation and staining at 37°C.

Raji cells with C3 or C3b at 4°C resulted in fine granular staining (Fig. 2 *a*) whereas incubation at 37°C produced coarse spots in often polar distribution (Fig. 2 *b*). In these experiments fluorescent staining was carried out at 4°C. When incubating with C3 or C3b and staining was carried out at 37°C, cap formation was observed in some cells (Fig. 2 *c*).

Physicochemical State of Bound C3.—In the preceding section it was shown that binding of C3 and C3b was not mediated by additional complement or serum factors. It is conceivable that cell membrane-bound enzymes might cleave C3 before attachment, in this case only the C3b portion of the molecule would bind to the receptor. Antiserum to the C3a fragment and the fluorescent sandwich technique were employed to study the physicochemical state of receptor-bound C3. The results of these experiments are summarized in Table I. 100% of the Raji cells incubated with 50 μ g C3 reacted with anti C3a antiserum, whereas only 5% of cells incubated with 50 μ g C3b reacted with this antiserum, indicating that C3 was bound to Raji cells in its native form.

C3 and C3b-bearing Raji cells gave no immune adherence pattern when tested at various concentrations with human erythrocytes. This suggests that binding of C3b occurs through the immune adherence region of the molecule and not the C3d portion.

TABLE I
Demonstration of Native C3 on Raji Cell Surface

Incubation	Staining procedure		Positive cells
			%
Raji + medium	Rab anti-C3a	FITC sheep anti-Rab IgG	0
Raji + C3	Rab anti-C3a	FITC sheep anti-Rab IgG	100
Raji + C3b	Rab anti-C3a	FITC sheep anti-Rab IgG	5
Raji + C3	NRS	FITC sheep anti-Rab IgG	0

Comparison of the Binding of Soluble C3 and C3b and of RBC Bound C3b to Lymphocyte Membrane C3 Receptors.—The ability of four different cell lines and of peripheral lymphocytes to bind C3 and C3b in free solution was compared to their ability to form rosettes with EAC14oxy23 cells. Binding of soluble C3 and C3b was determined by immunofluorescence as described above. The results of these experiments are summarized in Table II. Within each cell type, roughly the same percentage of cells bound soluble C3b and formed rosettes with EAC14oxy23 100% of Raji cells were positive in both tests, whereas only 5% of the other three lines were positive. In contrast to peripheral blood lymphocytes, all four cell lines also bind soluble C3 to almost the same extent as C3b.

Specificity of the C3 Receptor on Raji Cells.—While it was not possible to show directly that both C3 and C3b bound to the same receptor site, it was possible indirectly to demonstrate that they bound to the same or closely associated sites by inhibition of Raji cells-EAC14oxy23 rosette formation. Fig. 3 shows that incubation of Raji cells with increasing amounts of C3 or C3b and subsequent suspension in MEM before addition of EAC14oxy23 inhibited rosette formation. However, it had to be considered that red cell-bound C4oxy2 enzyme with a half life at 37°C of 150–200 min (27) could cleave Raji cell-bound C3 into C3a and C3b. Thus, inhibition of Raji-EAC14oxy23 rosette formation could be due to C3b rather than C3. To exclude this possibility EAC1423 were prepared with nonoxidized C2, which forms a C42 enzyme with a half life at 37°C of only 10 min (27). When EAC1423 previously decayed to EAC143 were used, C3 and C3b inhibited rosettes to the same extent as with EAC14oxy23 cells. Rosettes could also be completely inhibited by preincubation of Raji cells with fresh NHS. In contrast, aged human serum which contained the fragments C3c and C3d did not significantly inhibit rosettes (Fig. 3). Rosettes between Raji cells and EAC1423 could also be dissociated by C3 or C3b. In both inhibition and dissociation of rosettes, C3 was more effective than C3b when used at equal concentrations.

Quantitation of C3 and C3 Fragments Bound to Raji Cell Membrane.—The number of molecules of C3, C3b, and C3c which were bound per Raji cell was determined by uptake experiments. 2.5×10^6 cells were incubated with increasing amounts of radiolabeled C3, C3b, or C3c in a total volume of 250 μ l of

TABLE II
Comparison of the Binding of Soluble C3 and C3b and of RBC-Bound C3b to Lymphocyte Membrane C3 Receptors

Cell type	Percent cells binding*		Cells forming rosettes with EAC1423
	C3	C3b	
		%	%
Raji	100	100	95-100
Wil ₂ WT	5	2-3	2-3
8866	5	5	5
Daudi	20	5	2-3
Peripheral lymphocytes†	0	20	20

* The binding of soluble C3 and C3b was assessed by staining with FITC antihuman C3.

† Average of ten experiments.

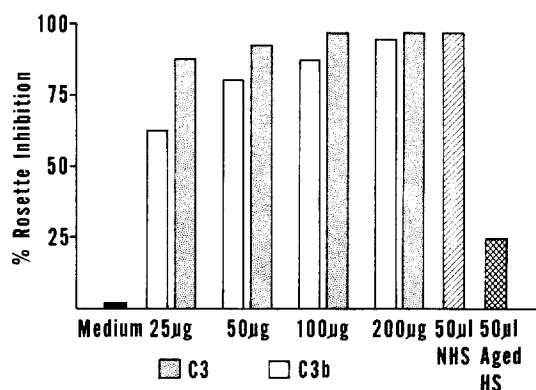


FIG. 3. Inhibition of Raji cell-EAC1423 rosette formation by NHS, C3, C3b, and aged human serum (C3c and C3d).

0.1% human serum albumin in MEM. The binding curves of the different proteins are depicted in Fig. 4. Addition to Raji cells of 250 μ l of MEM containing 20 μ g C3, an amount corresponding approximately to one-twentieth of the serum C3 concentration, occupied 80% of the available C3 receptors (Fig. 4). At saturation, approximately 4×10^5 molecules of C3 were bound per Raji cell. The receptor on Raji cells has a greater binding affinity for C3 than C3b prepared with trypsin; almost two times more C3b than C3 had to be offered to the cells in order to bind the same number of molecules. Uptake of C3c molecules was approximately one-seventh that of C3 and C3b.

Effect of C3b Inactivator on Raji Cell-EAC14oxy23 Rosette Formation.—Binding of soluble C3c and C3d present in an aged human serum on Raji cells could not be detected by the immunofluorescence method. However, since a receptor for red cell-bound C3d has been recently reported to be present on human lymphocytes (28), experiments were performed in order to explore the possibility of red cell-bound C3d binding to Raji cells. Since C3b inactivator (C3bina)

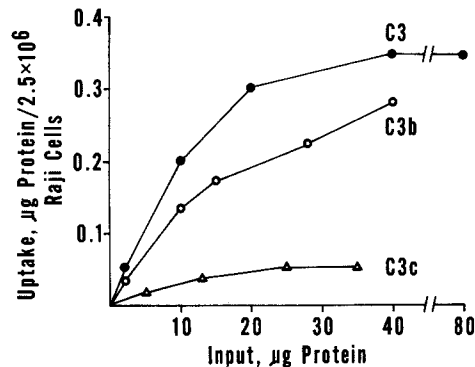


FIG. 4. Uptake of ^{125}I -labeled C3, C3b, and C3c by Raji cells.

can cleave C3b to C3c and C3d (29) and treatment of EAC14oxy23 with C3bina results in release of C3c and retention of C3d on the red cell surface (29), the following experiments were performed: (a) 5×10^7 EAC14oxy32 , preincubated for 90 min at 37°C with a sufficient amount of C3bina² to render them immune adherence negative, were incubated with Raji cells and the number of rosettes was counted. It was found that Raji cells continue to form rosettes with such erythrocytes in a percentage similar to that obtained with EAC14oxy23 not treated with C3bina. (b) A similar amount of C3bina was added to preformed Raji cell- EAC14oxy23 rosettes; after incubation for 90 min at 37°C no dissociation of rosettes was seen. (c) C3bina mixed with EAC14oxy23 cells was added to Raji cells; after incubation for 90 min at 37°C no inhibition of rosette formation was seen.

Effect of Trypsin on the C3 Receptor.—Raji cells incubated in a 0.01% trypsin solution in MEM for 15 min at 37°C were no longer capable of binding soluble C3 and C3b or of forming rosettes with EAC14oxy23 .

Effect of Complement Activation by CoF on Cell Viability.—Since C3b activates the alternate pathway of complement (7), receptor-bound C3b might cause lysis of C3 receptor-bearing cells through complement activation. To check the susceptibility of Raji cells to lysis by the alternate complement pathway, complement was activated by CoF (26) in cultures. Raji cells were suspended at a concentration of 2×10^5 cells/ml in medium containing 10% fresh or heat-inactivated human serum (50°C , 20 min). Viable cells were counted at different time intervals and the number was expressed as the percentage of the number at 0 time. Addition of 200 μg CoF to 20 ml of cell culture containing fresh human serum resulted in loss of 75% of cells in 24 h and 100% in 48 h (Fig. 5 A). Cells removed from culture two hours after the addition of CoF

² C3b inactivator used in this study was a gift from Dr. Neil Cooper. The activity of C3bina was assessed by its ability to render EAC14oxy23 cells negative in immune adherence tests using O group human erythrocytes.

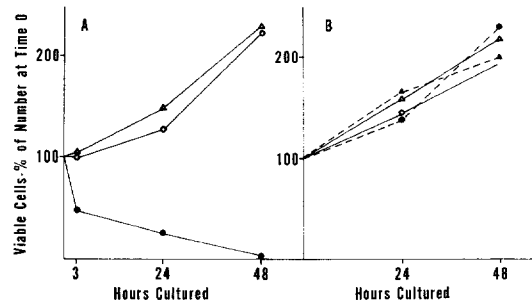


FIG. 5. (A) Effect of complement activation by CoF on Raji cells in culture. 2×10^5 cells/ml were cultured in medium containing fresh human serum (○—○) or in medium containing CoF-treated fresh (●—●) and heat-inactivated (50°C, 20 min) human serum (△—△). (B) Effect of complement activation by CoF on 8866 and Daudi cells in culture. 2×10^5 cells/ml of each cell line were cultured in medium containing fresh human serum (open signs) or medium containing CoF-treated fresh human serum (closed signs).

lacked the C3a determinant on their surface. CoF had no effect on Raji cells cultured in medium containing heat-inactivated human serum. In contrast to Raji cells, CoF had no effect on 8866 and Daudi cells cultured in medium containing fresh human serum (Fig. 5 B).

Effect of Receptor Bound C3b on Raji Cell Viability.—To test the effect of Raji cell bound C3b on cell viability, C3b-bearing Raji cells were cultured in medium containing fresh or heat-inactivated (50°C, 20 min) human serum. In these experiments batches of 5×10^6 Raji cells were incubated for 30 min at 37°C in 100 μ l MEM containing 100 μ g C3b or 100 μ g human serum albumin. Incubation in albumin served as control. Thereafter, the cells were washed three times and resuspended in 20 ml of the appropriate culture medium. Fig. 6 depicts the growth curve of C3b-bearing Raji cells cultured in medium containing fresh or heat-inactivated human serum and that of albumin-treated Raji cells in medium containing fresh human serum. A 70% loss of viable C3b-bearing Raji cells cultured in fresh human serum was observed after 24 h. In contrast, no effect on the growth of C3b-bearing cells cultured in heated human serum or albumin-treated Raji cells cultured in fresh human serum was noted. The rapid loss of viable C3b-bearing Raji cells in the presence of fresh serum but not in the presence of heated serum (50°C, 20 min) suggested the participation of C3PA in cell lysis.

Participation of C6 in Raji Cell Lysis.—Participation of C6 in the C3b initiated lysis of Raji cells was studied by culturing C3b-bearing Raji cells (2×10^5 /ml) in medium containing 10% of either C6 deficient or normal rabbit serum (NRS) (Fig. 7). The number of viable C3b-bearing Raji cells was drastically reduced in the presence of NRS, whereas no reduction of cell numbers was observed in the presence of C6 deficient serum. Raji cells without C3b on their surface grew normally in either serum. Hence destruction by C3b requires C6.

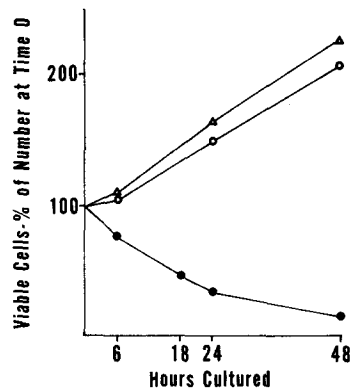


FIG. 6. Effect of bound C3b on Raji cells in culture. 5×10^6 C3b-bearing Raji cells were cultured in 20 ml medium containing either fresh (●—●) or heat-inactivated (50°C, 20 min) human serum (○—○). Control cells pretreated with human serum albumin were cultured in fresh human serum (△—△).

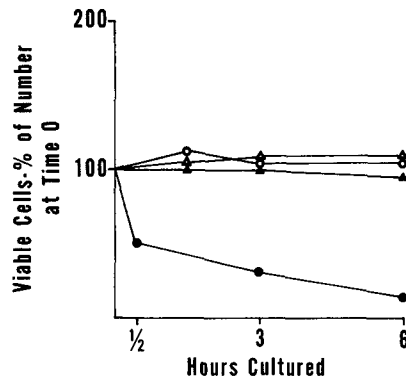


FIG. 7. Culture of C3b-bearing Raji cells (2×10^5 cells/ml) in medium containing normal (●—●) or C6-deficient (○—○) rabbit serum. Raji cells without surface-bound C3b were used as controls in normal (▲—▲) and C6-deficient (△—△) rabbit serum.

DISCUSSION

This study describes the binding properties and a biologic function of the C3 receptor on Raji cells. The binding of soluble C3 and C3b to Raji cells was demonstrated by the detection of these proteins on the cell surface with fluoresceinated anti-C3 serum and by the inhibition or dissociation (in the presence of C3 and C3b) of rosettes formed between Raji cells and EAC1423. The binding of soluble C3 and C3b was directly shown with radiolabeled proteins. Our experiments with human peripheral lymphocytes also showed that soluble C3b can bind to the C3b receptor present on a portion of those cells. Such binding of soluble proteins to receptors for C3 or C3b has hitherto not been

observed, although Eden and associates (4) postulated the binding of guinea pig C3b to the C3 receptor of mouse B lymphocytes. They showed that guinea pig serum treated with EAC142 inhibited rosette formation between mouse B lymphocytes and EAC1423. In the present study it was found that there was no requirement for other complement proteins or serum factors to mediate the binding of C3 or C3b to the receptor, since the isolated proteins bind to the receptor. However, it also had to be investigated whether a membrane-bound enzyme or cellular proteases released in the medium during the time of incubation could cleave C3 into the fragments C3b and C3a before binding to the receptor. If this was so, it was possible, as in the case of the human peripheral lymphocytes, that only C3b would bind to the Raji cell receptor. The possibility of enzymatic cleavage of C3 by released or cell bound enzyme was, however, ruled out by demonstrating the presence of antigenically intact C3 on Raji cells. The ability to bind C3 and C3b distinguishes the C3 receptor on Raji cells from the C3b receptor on human peripheral lymphocytes which only bind C3b.

Inhibition and dissociation of Raji cell-EAC14oxy23 rosettes by C3 and C3b indicated that both molecules were binding to the same receptor or to closely associated receptors on the cell membrane. The possibility that C3b generated from Raji cell-bound C3, by the C4oxy2 enzyme on red cells, was responsible for the inhibition of Raji-EAC14oxy23 rosettes was ruled out by the use of EAC1423 cells. Rosettes of Raji cells with EAC1423 and EAC14oxy23 were inhibited to the same degree by C3.

The fact that C3b-bearing Raji cells are immune adherence negative suggests that C3b binding to the C3 receptor is brought about through the immune adherence region of the molecule and not the C3d portion. It has been previously shown that C3b is bound to EAC142 cells through the C3d portion of the molecule. The C3c portion is exposed and mediates immune adherence (29).

The receptor for C3 and C3b on Raji cells is trypsin sensitive like the C3b receptor on peripheral lymphocytes (1), and binding of C3 and C3b to Raji cells is temperature-dependent. It has been previously shown that rosette formation between B cells and EAC' is greatly inhibited at 4°C (1). It was demonstrated by staining of C3- or C3b-bearing cells at 4°C that the C3 receptor on Raji cell membranes is uniformly distributed. Cap formation in some cells when staining was carried out at 37°C indicated that the C3 receptor can move in the membrane plane. Similar patterns of staining have been observed with B type lymphocytes carrying Ig on their membranes (30). Raji cells used in this study were lacking membrane-associated Ig (31) and therefore the possibility of the receptor being some class of Ig is excluded.

Uptake experiments showed that approximately 4×10^5 molecules of radio-labeled C3 or C3b bind per Raji cell. This number is 20 times larger than the number of membrane associated IgG molecules on other lymphoblastoid cells; for example, the number for Wil₂WT cells has been calculated to be 1.8×10^4

(31). The same experiments also indicated that the receptor has a higher affinity for C3 than C3b, since almost two times more C3b than C3 had to be offered to the cells in order to bind the same number of molecules. The higher affinity of C3 for the receptor was also shown by the fact that C3 was more potent than C3b in inhibiting Raji cell-EAC1423 rosette formation.

In addition to the described C3 receptor, another receptor specific for C3b inactivator-cleaved C3b (C3d) bound to red cells was shown to be present on Raji cells. The presence of a receptor for C3d was demonstrated by the formation of rosettes between Raji cells and EAC14oxy23 which had been previously treated with C3b inactivator and were immune adherence negative. In addition, C3b inactivator did not dissociate preformed rosettes and its presence did not have any effect on rosette formation between Raji cells and EAC-14oxy23. Finally, EAC' prepared with whole C5-deficient mouse serum in which C3b inactivator is present continued to form rosettes with Raji cells; preincubation of Raji cells with human or mouse serum did not inhibit rosette formation between Raji cells and EAC' made with C5-deficient mouse serum (unpublished observations). These findings are in accordance with Ross and associates, who have recently reported the presence of two different complement receptors on human lymphocytes, one specific for C3b and one specific for C3b inactivator-cleaved C3b (28). In contrast with the rosette formation between Raji cells and C3bina treated EAC14oxy23, binding of the soluble C3d and C3c, present in aged human serum, could not be detected by the immunofluorescence method.

C3b together with C3PA and C3Pase activates the alternate pathway of complement which in turn leads to cleavage of C3 into C3a and C3b (7). Since our experiments have shown that Raji cells bind a large number of C3b molecules, it is conceivable that in the presence of whole human serum cell-bound C3b could activate the alternate pathway of complement and thereby lead to membrane damage. The fact that Raji cells are susceptible to lysis by the alternate complement pathway was demonstrated in experiments in which CoF, which activates the alternate complement pathway (26), was added to Raji cells cultured in medium containing fresh human serum. It was found that after 24 h of incubation 75% of the cells were lysed. In contrast, 8866 and Daudi cells were not lysed under the same experimental conditions. Therefore, experiments were designed to assess a possible effect of Raji cell bound C3b on cell viability in the presence of fresh human serum. When C3b-bearing cells were cultured in medium containing fresh serum, lysis of these cells ensued. Lysis was abolished by heat inactivation of serum C3PA (50°C, 20 min), indicating that activation of the alternate complement pathway by cell-bound C3b was responsible for cell destruction. Furthermore, lysis of C3b-bearing Raji cells in culture medium containing normal but not C6-deficient rabbit serum indicated the participation of C5 and C6 in this reaction.

It is conceivable that the described mechanism of lysis of C3b-bearing cells

under culture conditions is also operative *in vivo*. C3 antigenic determinants have been found on Raji cells obtained directly from patients (32). Occupation of the C3 receptor by C3b could be achieved in two ways. Cell-bound C3 could either be converted to C3b on the cell surface or be replaced by C3b generated in plasma. In effect, our experiments with cultured Raji cells showed that C3-bearing cells in fresh human serum converted to C3b-bearing cells after addition of CoF. Cell-bound C3b could then lead to membrane damage through complement activation.

In this context it should also be mentioned that other cell lines exhibited properties similar to Raji cells as far as C3 binding is concerned. The cells from three lymphoblastoid lines, established by Dr. B. Croker from peripheral lymphocytes of two patients with disseminated lupus erythematosus and one patient with Sjögren's syndrome, were able to bind soluble C3 and C3b.³ It remains to be established if in these patients their peripheral lymphocytes themselves can bind C3.

Although cell destruction by this mechanism may play an important role in controlling cell growth, it probably constitutes only one consequence of the presence of the C3 receptor on cell membranes. It is likely that in other C3 receptor-bearing cells resistant to lysis by the alternate complement pathway, activation of complement on membrane surfaces may result in changes of cell activity instead of cell death. In this connection it has been postulated, for example, that binding of C3b to the C3b receptor of B lymphocytes has a controlling influence on antibody production and that complement receptor plays an important role in T- and B-cell interaction (33-35). Studies are under way to investigate the influence on various cell activities of C3b binding to C3 receptor-bearing cells.

SUMMARY

This study describes the presence of a receptor for fluid phase human C3 and C3b on Raji cell membranes. The binding of C3 and C3b was demonstrated indirectly by a fluoresceinated anti-C3 serum and directly by using radioiodinated proteins. No other complement proteins or serum factors were needed to mediate binding of C3 and C3b to the receptor. The possibility of enzymatic cleavage of C3 before or after its attachment on the cell membrane was ruled out by the demonstration of antigenically intact C3 on Raji cells. Inhibition and dissociation of Raji cell-EAC1423 rosettes by C3 and C3b indicated that both of these proteins bind to the same receptor site or closely associated receptor sites on Raji cells. C3b-bearing Raji cells were immune adherence negative, indicating that C3b binding to the receptor is brought about through the immune adherence region of the molecule and not the C3d portion. The C3 receptor on Raji cell membranes is uniformly distributed and can move on the

³ A. N. Theofilopoulos, V. A. Bokisch, and F. J. Dixon. Manuscript in preparation.

membrane plane. Approximately 4×10^5 molecules of C3 or C3b bind per Raji cell. The receptor had a higher affinity for C3 than C3b, as was shown by uptake experiments and inhibition of Raji cell-EAC1423 rosette formation. Apart from the described receptor for C3 and C3b another specific receptor for C3b inactivator-cleaved C3b (C3d) bound to red cells was shown to be present on Raji cells.

Raji cells cultured in medium containing fresh normal human serum and cobra venom factor were lysed. Similar results were obtained when C3b-bearing Raji cells were cultured in medium with fresh normal human serum. The lytic effect could be abolished by inactivating serum C3 proactivator (C3PA) and required C6. It was concluded that C3b bound to the Raji cell membrane activates the complement system through the alternate pathway and results in membrane damage and cytolysis. It is postulated that cell destruction by this mechanism may play an important role in vivo in controlling cell growth.

The authors wish to thank Dr. Müller-Eberhard for his advice and guidance throughout this study. The skillful technical assistance of Mrs. Mary Brothers is gratefully acknowledged.

REFERENCES

1. Lay, W. H., and V. Nussenzweig. 1968. Receptors for complement on leukocytes. *J. Exp. Med.* **128**:991.
2. Muller-Eberhard, H. J., A. P. Dalmaso, and M. A. Calcott. 1966. The reaction mechanism of β_{1c} -globulin (C'3) in immune hemolysis. *J. Exp. Med.* **123**:33.
3. Nelson, R. A., Jr. 1953. The immune adherence phenomenon. An immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis. *Science (Wash. D.C.)* **118**:733.
4. Eden, A., C. Bianco, V. Nussenzweig, and M. M. Mayer. 1973. C3 split products inhibit the binding of antigen-antibody-complement complexes to B lymphocytes. *J. Immunol.* **110**:1452.
5. Huber, H., M. J. Polley, W. D. Linscott, H. H. Fudenberg, and H. J. Muller-Eberhard. 1968. Human monocytes: Distinct receptor sites for the third component of complement and for immunoglobulin G. *Science (Wash. D.C.)* **162**:1281.
6. Perlmann, P., H. Perlmann, H. J. Muller-Eberhard, and T. A. Manni. 1969. Cytotoxic effects of leukocytes triggered by complement bound to target cells. *Science (Wash. D.C.)* **163**:937.
7. Muller-Eberhard, H. J., and O. Götze. 1972. C3 proactivator convertase and its mode of action. *J. Exp. Med.* **135**:1003.
8. Nicol, P. A. E., and P. J. Lachmann. 1973. The alternate pathway of complement activation. The role of C3 and its inactivator (KAF). *Immunology*. **24**:259.
9. Pulvertaft, R. J. V. 1965. A study of malignant tumours in Nigeria by short-term tissue culture. *J. Clin. Pathol.* **18**:261.
10. Klein, E., G. Klein, J. S. Nadkarni, J. J. Nadkarni, H. Wigzell, and P. Clifford. 1968. Surface IgM-kappa specificity on a Burkitt lymphoma cell in vivo and in derived cultured lines. *Cancer Res.* **28**:1300.

11. Finegold, I., J. L. Fahey, and H. Granger. 1967. Synthesis of immunoglobulins by human cell lines in tissue culture. *J. Immunol.* **99**:839.
12. Levy, J. A., M. Virolainen, and V. Defendi. 1968. Human lymphoblastoid lines from lymph node and spleen. *Cancer* **22**:517.
13. Lerner, R. A., and L. D. Hodge. 1971. Gene expression in synchronized lymphocytes: studies on the control of synthesis of immunoglobulin polypeptides. *J. Cell. Physiol.* **77**:265.
14. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* (Suppl.) **97**:77.
15. Greenwalt, T. J., M. Gajewski, and J. L. McKenna. 1962. A new method for preparing buffy coat-poor blood. *Transfusion* (Phila.) **2**:221.
16. Nilsson, V. R., and H. J. Müller-Eberhard. 1965. Isolation of β_{if} globulin from human serum and its characterization as the fifth component of complement. *J. Exp. Med.* **122**:277.
17. Bokisch, V. A., H. J. Müller-Eberhard, and C. G. Cochrane. 1969. Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med.* **129**:1109.
18. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**:185.
19. Müller-Eberhard, H. J., and K. E. Fjellström. 1971. Isolation of the anti-complementary protein from cobra venom and its mode of action of C3. *J. Immunol.* **107**:1666.
20. Clark, H. F., C. C. Shepard. 1963. A dialysis technique for preparing fluorescent antibody. *Virology.* **20**:642.
21. West, C. D., N. C. Davis, J. Forristal, J. Herbst, and R. Spitzer. 1966. Antigenic determinants of human β_{ic} -globulins. *J. Immunol.* **96**:650.
22. Nelson, R. A., Jr., J. Jensen, I. Gigli, and N. Tamura. 1966. Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry.* **3**:111.
23. Polley, M. J., and H. J. Müller-Eberhard. 1968. The second component of human complement: its isolation, fragmentation by C'1 esterase and incorporation into C'3 convertase. *J. Exp. Med.* **128**:533.
24. Polley, M. J., and H. J. Müller-Eberhard. 1967. Enhancement of the hemolytic activity of the second component of human complement by oxidation. *J. Exp. Med.* **126**:1013.
25. Müller-Eberhard, H. J., and C. E. Biro. 1963. Isolation and description of the fourth component of human complement. *J. Exp. Med.* **118**:447.
26. Götze, O., and H. J. Müller-Eberhard. 1971. The C3 activator system: an alternate pathway of complement activation. *J. Exp. Med.* **134**:90s.
27. Müller-Eberhard, H. J. 1969. Complement. *Ann. Rev. Biochem.* **38**:389.
28. Ross, G. D., M. J. Polley, E. M. Rabellino, and H. M. Grey. 1973. Two different receptors on human lymphocytes. One specific for C3b and one specific for C3b inactivator-cleaved C3b. *J. Exp. Med.* **138**:798.
29. Ruddy, S., and K. F. Austen. 1971. II. Fragments produced by C3b inactivator cleavage of cell-bound or fluid phase C3b. *J. Immunol.* **107**:742.
30. Taylor, R. B., W. P. H. Duffus, M. C. Raff, and S. de Petris. 1971. Redistribution

- and pinocytosis of lymphocytes surface immunoglobulin molecules induced by antiimmunoglobulin antibody. *Nat. New Biol.* **233**:225.
31. Lerner, R. A., P. J. McConahey, and F. J. Dixon. 1971. Quantitative aspects of plasma membrane-associated immunoglobulin in clones of diploid human lymphocytes. *Science (Wash. D.C.)* **173**:60.
 32. Povlsen, C. O., P. J. Fialkow, E. Klein, G. Klein, J. Rygaard, and F. Wiener. 1973. Growth and antigenic properties of a biopsy-derived Burkitt's lymphoma in thymus-less (nude) mice. *Int. J. Cancer* **11**:30.
 33. Bitter-Suermann, D., P. Dukor, R. H. Gisler, G. Schumann, M. Dierich, W. Konig, and V. Hadding. 1973. C3-dependence of B-cell activation? *J. Immunol.* **111**:301.
 34. Dukor, P., and K. V. Hartmann. 1973. Bound C3 as the second signal for B-cell activation. *Cell. Immunol.* **7**:349.
 35. Pepys, M. B. 1972. Role of Complement in Induction of the Allergic Response. *Nat. New Biol.* **237**:157.