BINDING OF COMPONENTS OF THE PROPERDIN SYSTEM TO CULTURED HUMAN LYMPHOBLASTOID CELLS AND B LYMPHOCYTES*

By ARGYRIOS N. THEOFILOPOULOS and LUC H. PERRIN‡

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

A receptor for fluid phase human complement (C) components C3 and C3b (1, 2) was found on the surface membranes of human lymphoblastoid cells. No other C proteins or serum factors were needed to mediate binding of C3 or C3b to this receptor. In studying its biological importance, we demonstrated that Raji cells cultured in medium containing fresh normal human serum (NHS)[1] and cobra venom factor (CoF) were lysed (1). Results were similar when C3b-bearing Raji cells were cultured in medium with fresh NHS. In other experiments, lysis was eliminated by inactivating serum factor B.[2] Therefore, we concluded that C3b bound to the Raji cells activated the C system through C3b-dependent C3 convertase (3, 4) which resulted in fixation of the later C components directly on the cell membranes, thereby leading to their damage and lysis.

Joseph et al. (5) observed that immune human serum, containing antimeasles virus antibody and C, lysed HeLa cells infected with measles virus via activation of the alternative C pathway (6). By using immunofluorescence, properdin (P) was detected on the surfaces of the measles virus-infected HeLa cells previously incubated with the immune serum (Perrin, L., and M. B. A. Oldstone, unpublished observations). Therefore we questioned, do components of the P system bind to cells, and if so, what events lead to their fixation on the cell surface? We now show that P, as well as factor B, can bind to surfaces of certain lymphoblastoid cells and of human B type peripheral lymphocytes. The fixation

* This is Publication No. 1020 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, Calif. This work was supported by grant number DADA 17-73-C-3137 from the U. S. Department of the Army and grant numbers AI-07007 and NS-12428 from the U. S. Public Health Service.

‡ Supported by a fellowship from the Swiss National Research Foundation.

[1] *Abbreviations used in this paper:* AHG, aggregated human IgG; BSA, bovine serum albumin; CoF, cobra venom factor; EAC, complement-coated sensitized sheep erythrocytes; EAC3d^{mo}, immune adherence negative EA coated with C1-C3 from mouse serum deficient in C5; EAC3b^{rab}, immune adherence positive EA coated with C1-C5 from rabbit serum deficient in C6; EGTA, ethyleneglycolbis (B-amino-ethylether) N,N'-tetraacetic acid; FITC, fluorescein isothiocyanate; HPL, human peripheral lymphocytes; MEM, minimum essential medium; NHS, normal human serum; P, properdin; PBS, phosphate-buffered saline; VBS, veronal-buffered saline; ZC, zymosan particles interacted with NHS.

[2] The symbols for the properdin system conform to those from the 2nd International Congress of Immunology, Brighton, England, July 1974.

of P and factor B on cells is mediated by binding of C3 or C3b to C3-C3b receptors. Furthermore, binding of P precedes that of factor B or stabilizes the binding of factor B to C3b.

Materials and Methods

Lymphoblastoid Cell Lines. The origins of the Raji, Daudi, Wil2WT, 8866, Sommer 8402 (2), P3HRl (7), and MOLT-4 (8) cell lines have been described. These cell lines were cultured in Eagle's minimum essential medium (MEM; Autopow; Flow Laboratories, Inc., Rockville, Md.) supplemented with glutamine, nonessential amino acids, pyruvate, 10% fetal bovine serum, penicillin, streptomycin, and fungizone (1).

Purified Proteins and C Reagents. Human C3 and C3b [1 mg/ml Veronal-buffered saline containing 1.5×10^{-4} M $CaCl_2$ and 5×10^{-4} M $MgCl_2$ (VBS)] were prepared as published (9, 10) and were a gift from Dr. V. A. Bokisch (Scripps Clinic and Research Foundation, La Jolla, Calif.). Of two preparations of P, the first was purified as described (11), and the second was purified by affinity chromatography with rabbit antiserum to human P coupled to Sepharose 4B (12, 13) (Pharmacia Fine Chemicals, Inc., Uppsala Sweden). A euglobulin pool made 0.01 M with EDTA (ethylenediaminetetraacetate) was used as the source of P. After prolonged washing with NaCl 0.5 M, EDTA 0.01 M, pH 7.2, P was eluted with potassium thiocyanate 2 M, pH 6.5; the eluted fraction was immediately dialyzed against VBS. Both preparations were adjusted to 200 μg P/ml VBS. P prepared by the first method was free of contaminating proteins when tested by Ouchterlony analysis and immunoelectrophoresis with antiwhole human serum, antihuman C3, C4, C5, and factor B, and migrated cathodally when reacted with rabbit antiserum to human P in immunoelectrophoresis. The second preparation of P presented the same characteristics, but a trace amount of IgG was detected by Ouchterlony analysis. Results obtained with both preparations were invariably the same. Factor B was purified either as published (6) or by affinity chromatography with rabbit antiserum to human factor B coupled to Sepharose 4B (14) as above. The isolated factor B preparations were concentrated to 500 μg/ml VBS. Both preparations were free of contaminating proteins as determined by Ouchterlony analysis and immunoelectrophoresis and behaved similarly in the experiments reported below. Factor D was partially purified by concentrating to one-fifth of their original volume the fractions containing factor D activity which were recovered after the exclusion peak of NHS filtered through a Sephadex G75 column. Factor D activity in the fractions was checked by a hemolytic radial immunodiffusion method.[3] Briefly, we used 0.6% agarose plates containing a final concentration of 5 mM Mg^{++}, 10 mM EGTA, the exclusion peak of a NHS pool filtered through a Sephadex G75 column (15) diluted 1/20, and 0.75% guinea pig erythrocytes. The fractions were applied in the wells, and the plates were incubated first at 4°C for 24 h and then at 37°C for 3 h before reading the hemolytic rings. Human sera, our source of C, were obtained from the blood of healthy laboratory personnel, clotted for 60 min at room temperature and then 30 min at 4°C, and subsequently centrifuged. Sera were used immediately or frozen at −70°C. Human serum genetically deficient in C2 was provided by Dr. C. Arroyave (Scripps Clinic). Aged human serum was obtained by incubating NHS for 10 days at 4°C. NHS made 0.01 M with respect to EDTA and adjusted to pH 7.5 by NaOH 0.1 N was selectively depleted of P by passage through a column of Sepharose 4B coupled to rabbit antiserum to human P. The serum was then concentrated by negative pressure to the original volume and dialyzed against VBS. Similarly, rabbit antiserum to human factor B coupled to Sepharose 4B was used to obtain a serum selectively depleted of factor B. The exclusion peak of NHS made 0.01 M with respect to EDTA, after filtration through a Sephadex G75, was concentrated by negative pressure to the original volume and dialyzed against VBS; this preparation was used as factor D-depleted serum (15, 16). Human serum was depleted of C3 as described by Lachmann by incubating two parts of purified $F(ab')_2$ goat antihuman C3 with one part of NHS in the presence of 0.01 M EDTA (17). The C3-depleted serum was dialyzed against VBS before use. The P, factor B, and factor D depleted sera each had a CH_{50} greater than 60 U (normal values ± 2 SD:65–100) and did not convert C3 or factor

[3] Martin, A., P. J. Lachman, L. Halbwachs, and M. J. Hobart. 1976. Haemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. Submitted for publication.

B after incubation with inulin (20 mg/ml final concentration). Each activity was restored by the respective addition of each purified, depleted component. Human IgA was a gift from Dr. H. Spiegelberg (Scripps Clinic). Human IgG was prepared as described (2). Human IgA and IgG were aggregated in samples heated at 63°C for 10 min and 30 min, respectively. EDTA and EGTA [ethyleneglycolbis (B-amino-ethylether) N, N^1-tetraacetic acid] were purchased from Sigma Chemical Co., St. Louis, Mo.

Activators of the Alternative C Pathway. Inulin was purchased from Pfanstiehl Labs., Inc., Waukegan, Ill., zymosan from Nutritional Biochemicals Corporation, Cleveland, Ohio, endotoxin from *S. marcescens* from Difco Laboratories, Detroit, Mich., and CoF containing 100 U/ml VBS from Cordis Laboratories Miami, Fla.

Activation of C in NHS. An appropriate amount of zymosan or inulin was washed twice with VBS. After centrifugation at 2,000 *g* for 10 min at 22°C, the supernate was removed. Prewarmed NHS (37°C) was added to 2 mg of the zymosan pellet/ml or 20 mg of the inulin pellet/ml serum, and the mixture was agitated. After incubation for 30 min at 37°C with periodic shaking (every 5 min), the serum was separated from the zymosan or inulin particles by centrifugation (2,000 *g*, 4°C, 15 min) and used immediately to appraise the effect of these activators on the binding of P and factor B to cells. NHS was also incubated for 30 min at 37°C with CoF (100 U/ml), endotoxin (1 mg/ml), isolated human C3b (1 mg/ml), aggregated human IgA (3 mg/ml), or aggregated human IgG (AHG; 5 mg/ml), and after centrifugation at 4°C for 15 min was used immediately for binding studies.

Antisera. Monospecific antisera (1) to human C3 were raised in rabbits and goats (10). Fab'_2 goat anti-C3 was prepared by pepsin digestion of human C3-goat antihuman C3 complexes (17). Antisera to human P and to human factor B were raised in rabbits by injecting 50 and 200 μg, respectively, of the isolated proteins with incomplete Freund's adjuvant three times at 2-wk intervals. These antisera had one precipitation line when reacted with the isolated respective proteins and whole NHS. Antisera to Bb and Ba fragments of human factor B were raised in rabbits (16). Rabbit antiserum to human immunoglobulins (IgG + IgA + IgM) was purchased from Cappel Laboratories, Downington, Pa. Antiserum to rabbit IgG was raised in sheep. The IgG fraction of these antisera was obtained by fractionation on a DEAE-52 cellulose column after elution with 0.015 M phosphate buffer, pH 7.2, and subsequently was conjugated with fluorescein isothiocyanate (FITC) by dialysis (18). The FITC-conjugated antisera were dialyzed versus phosphate-buffered saline (PBS) and used at a concentration of 10 mg/ml PBS.

Immunofluorescence. For the detection of cell bound C3 or C3b and P, direct immunofluorescence was used. 5×10^6 cells (washed three times with MEM or physiologic saline) in 25 μl of MEM, saline, or VBS and with various amounts of isolated human C components, NHS, human serum with activated C, or serum depleted of certain C proteins were incubated with gentle agitation at 37°C for 30 min. Subsequently, cells were washed three times with MEM and agitated during incubation for 30 min at 4°C with 25 μl of the FITC antiserum to C3 or to P.

Cell bound factor B was detected with indirect immunofluorescence. Cells were first incubated (30 min, 37°C) with various amounts of isolated factor B, NHS, human serum depleted of certain C proteins, or human serum which had first reacted with different activators of the C system. Subsequently, cells were washed as above and reacted (30 min, 4°C) with 25 μl of FITC antifactor B. The cells were then washed three times with MEM and incubated (30 min, 4°C) with 25 μl of a nonfluorescein-conjugated sheep antirabbit IgG (5 mg/ml) in order to cross-link molecules of antifactor B bound to cells. Finally in both direct and indirect techniques, cells were washed three times with MEM and resuspended in 10 μl of a 10% bovine serum albumin (BSA) in saline. Cell smears were prepared and observed as described (1). Since all cells in a given preparation stained to the same degree, we could semiquantitatively classify the intensity of stain from negative to 4+ for both P and factor B binding.

Erythrocyte-Antibody-Complement Complexes (EAC). EAC3b rabbit cells ($EAC3b^{rab}$) and EAC3d mouse cells ($EAC3d^{mo}$) were prepared from sheep erythrocytes (E) sensitized with rabbit antierythrocyte antibody (A) and incubated with rabbit serum genetically-deficient in C6 (19) or mouse serum genetically-deficient in C5 (20), respectively (2). $EAC3b^{rab}$ cells (immune adherence positive) and $EAC3d^{mo}$ cells (immune adherence negative) (2) were used in a rosette assay (1, 2) to demonstrate C3b and C3d receptors on cells. Receptors for C3b on human lymphoblastoid cell lines were also sought by using soluble human C3 or C3b in the direct immunofluorescence assay described above. It has been demonstrated that receptors for C3 and C3b on Raji cells are the same

or closely associated (1). The presence of receptors for C3-C3b and C3d on human lymphoblastoid cell lines studied here, except the MOLT-4 and P3HRl lines, was established previously (2) and is compared with the ability of these cells to fix P and factor B.

Rosette Formation of Lymphoblastoid Cells with Zymosan-Complement Complexes. Preparation of zymosan-C complexes (ZC) and detection of ZC rosette-forming lymphoblastoid cells were performed exactly as described by Mendes et al. (21).

Human Peripheral Lymphocytes (HPL). HPL from healthy persons were isolated by defibrination, dextran sedimentation, and Ficoll-Isopaque centrifugation (1). Monocytes were removed with a lymphocyte-separating reagent (Technicon Instrument Corp., Tarrytown, N. Y.) and a magnet (22) and then were washed three times and suspended in MEM. 97–99% of the cells obtained were lymphocytes. We removed C receptor-bearing cells by incubating samples of 2×10^6 HPL in 200 μl MEM for 30 min at 37°C with 25 μl of $EAC3b^{rab}$. Subsequently, 20 such samples were mixed carefully, layered over 2 ml of Ficoll-Isopaque, and centrifuged for 15 min at 1,000 *g*. C receptor-bearing HPL (rosetting) were found at the bottom and non-C receptor-bearing cells (nonrosetting) at the interface.

Immunoelectrophoresis. 50–200 μl of NHS were incubated alone or with 5×10^6 cells of different cell lines with gentle shaking for 30 min at 37°C. After centrifugation (1,000 *g*) for 10 min, the supernates were made 0.01 M with respect to EDTA before being immunoelectrophoresed. C3 and factor B were immunoelectrophoresed in 1.5% agar in diethylbarbiturate buffer 0.1 M, 0.005 M EDTA, pH 8.2, as was P in 1% agarose and the same buffer as above.

Trypsin Treatment. 5×10^6 cells were incubated with 0.01% trypsin in MEM alone for 15 min at 37°C and then washed twice with 0.1% soy bean trypsin inhibitor in MEM containing 10% fetal bovine serum.

Results

Binding of P to Raji Cells. When Raji cells were incubated in 25 μl MEM, saline, or VBS containing 100 μl of fresh NHS, nearly 100% of the cells were positive when subsequently stained with FITC anti-P (Fig. 1). Control Raji cells incubated with MEM only and stained were negative. The antiserum was specific since no staining occurred with cells incubated with NHS and then reacted with FITC anti-P previously absorbed with isolated P. In addition, cells incubated with NHS and then reacted with FITC anti-P previously absorbed with washed ZC particles did not stain. Furthermore, cells incubated with 200 μg of other proteins, such as C3, C3b, and 7S IgG, known to bind to Raji cells (1, 2), and reacted with FITC anti-P remained negative. Raji cells incubated with C2-deficient human serum and stained with FITC anti-P were immunofluorescence positive. In contrast, cells were negative when reacted with FITC anti-P after incubation with either (*a*) 50 μg of isolated P, (*b*) 100 μl aged human serum which contained cathodically migrating P (23), C3d, and C3c, but not C3 and C3b (24), or (*c*) heated (56°C, 30 min) serum. Fixation of P to cells was somewhat temperature dependent. Incubation with NHS at 4°C for 30 min gave weakly positive reactions; whereas incubation at 37°C for 30 min yielded maximum intensities of immunofluorescence.

The binding of P to cells, as graded by intensity of immunofluorescence, was enhanced after incubation of cells with human serum first reacted with inulin, endotoxin, or aggregated IgA (Table I). AHG did not appreciably increase the intensity of staining over that of NHS alone. Cells incubated with serum first reacted with zymosan (2 mg/ml) and subsequently stained with FITC anti-P showed very weak or no staining. EDTA (10 mM) added to serum before incubation with the cells or trypsin pretreatment of cells precluded binding of P. Yet EGTA (10 mM) added to serum only slightly decreased the binding of P to

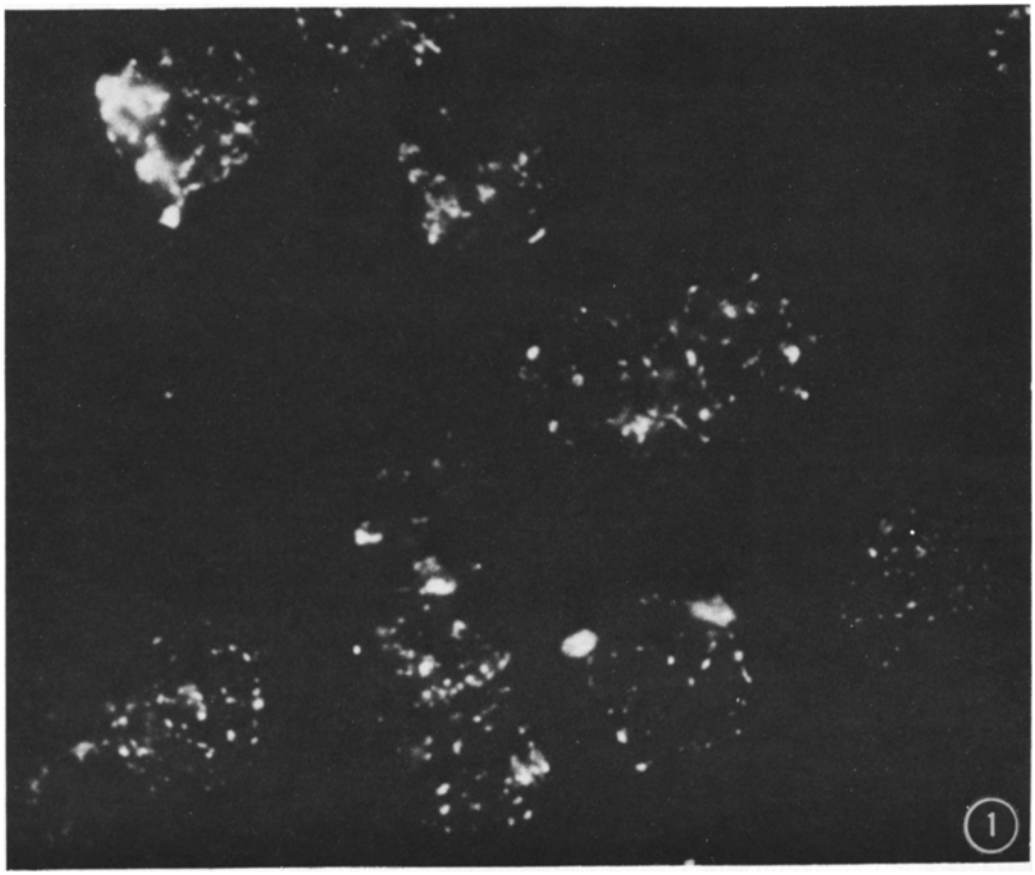

FIG. 1. P bound to Raji cells seen by immunofluorescence. Cells were incubated with NHS at 37°C for 30 min and then stained with FITC anti-P (4°C, 30 min). Magnification × 630.

Raji cells as judged by immunofluorescence. Finally, EDTA added to a serum already activated by inulin did not inhibit fixation of P to cells.

Immunoelectrophoretic analysis of NHS incubated at 37°C for 30 min without cells showed slow cathodally migrating P. In contrast, fast cathodally migrating P was detected in the supernate of 100 μl of the same serum after incubation with 5×10^6 Raji cells at 37°C for 30 min (Fig. 2). When 5×10^6 Raji cells were incubated (37°C, 30 min) with 50 μl of NHS, and the supernate was analyzed by immunoelectrophoresis, no P was found, indicating that all of the P in this amount of serum had been adsorbed to the cells.

Binding of Factor B to Raji Cells. When Raji cells were incubated in MEM, saline, or VBS containing 100 μl of fresh NHS or up to 250 μg of isolated factor B, then stained with FITC antifactor B, no cells immunofluorescence positive for factor B were observed. In contrast, incubation of cells with either 100 μl NHS or C2-deficient serum first treated with inulin resulted in nearly 100% immunofluorescence positive cells (Fig. 3). Neither incubation of cells with 100 μl of heated (50°C, 30 min) human serum nor with serum specifically depleted of C3, P, factor B, and factor D, but treated with inulin, resulted in factor B fixation to cells.

TABLE I

*Effect of Activators of the P System on the Binding of P to Raji Cells**

Incubation	Intensity‡ of immunofluorescence
NHS	1+
NHS + inulin	4+
NHS + endotoxin	2+
NHS + zymosan	± to negative
NHS + aggregated IgA	2+

* Raji cells were incubated with NHS or NHS first treated with the activators. Subsequently, the washed cells were stained with FITC anti-P.

‡ Graded from negative to 4+.

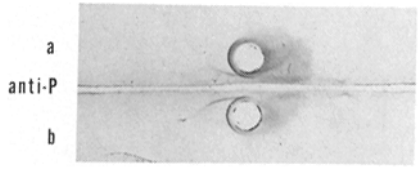

FIG. 2. Conversion of P after incubation of NHS with Raji cells. (a) The upper well contains NHS incubated at 37°C for 30 min without cells. (b) The lower well contains the supernate of the same NHS after incubation (37°C, 30 min) with cells. Trough was filled with rabbit antihuman P. The slide was stained with amido black. Cathode was on the left. Note the cathodal shift of the P arc in (b).

However, when physiologic amounts of the respective proteins were added to the depleted sera and incubated with inulin, factor B bound to cells. Factor B fixed to Raji cells with inulin-treated NHS and also with serum pretreated with zymosan, endotoxin, isolated human C3b, CoF, aggregated IgA, and AHG (Table II). The binding of factor B to cells was temperature dependent resulting in weak staining after incubation at 4°C with inulin-activated human serum and strong staining after incubation at 37°C. When Raji cells carrying factor B (after interaction with inulin-treated NHS) were stained with FITC antisera reacting preferentially with the Bb or the Ba fragments of factor B, immunofluorescent staining was intense with the former but very weak with the latter. The addition of 10 mM EDTA, but not EGTA, to NHS before incubation with inulin prevented binding of factor B to cells. However, when EDTA was added to serum already activated by inulin, fixation of factor B proceeded normally. Raji cells pretreated with trypsin could not fix factor B from inulin-treated human serum.

Immunoelectrophoretic analysis of the supernate obtained after incubating (30 min, 37°C) NHS (not treated with inulin) with Raji cells revealed conversion of factor B (Fig. 4). Control NHS incubated as above, but without cells, did not convert factor B.

Sequence of Events Leading to the Binding of P and Factor B to Raji Cells. Since P and factor B bound to Raji cells only in the presence of other

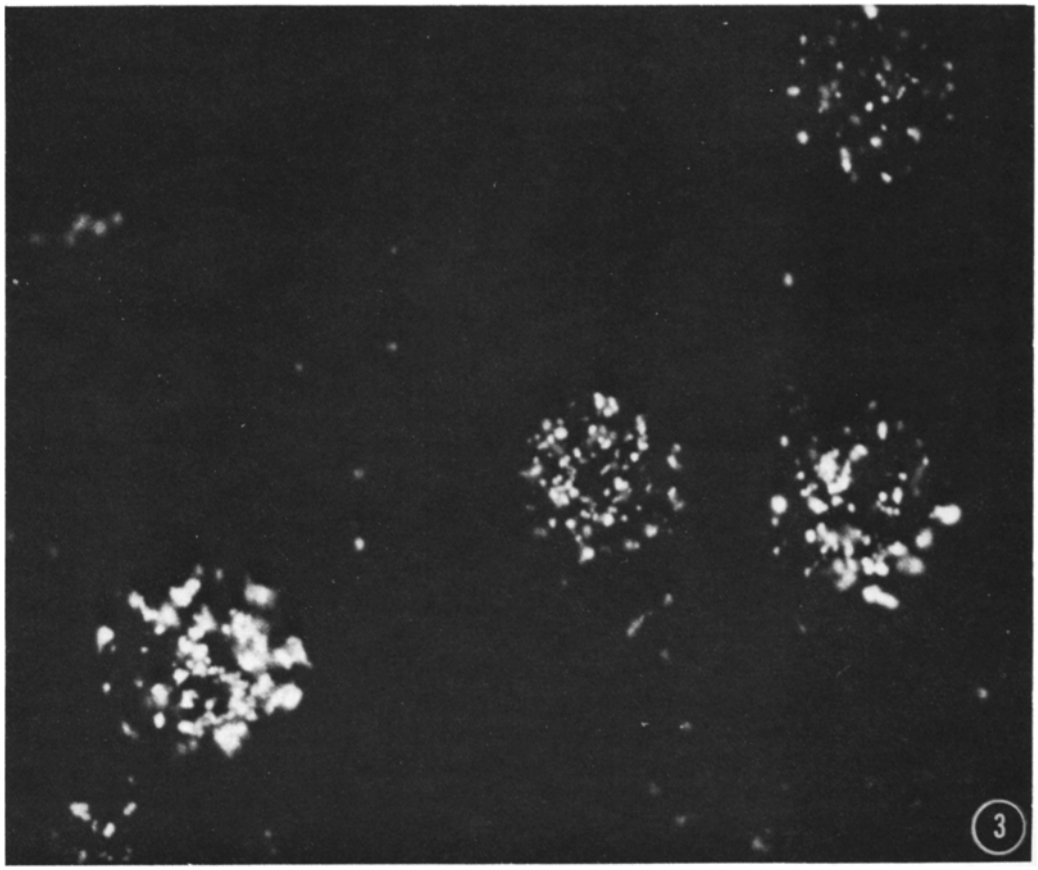

FIG. 3. Factor B bound to Raji cells shown by immunofluorescence. Cells were incubated with NHS which first had reacted with inulin. Subsequently, the washed cells were stained with FITC antifactor B and then reacted with sheep antirabbit IgG without FITC. Magnification × 630.

serum factors, it was important to identify these factors, their sequence of binding, and their structural element(s) which mediates this binding. As shown in Tables III and IV: (*a*) Raji cells incubated with C3-depleted human serum then reacted with FITC anti-P showed no immunofluorescence positivity. Other Raji cells incubated with C3-depleted serum first treated with CoF, then stained with FITC antifactor B, also were negative. Activation of factor B by CoF is known to be independent of the presence of C3 (4, 25). (*b*) One sample of Raji cells was incubated with a mixture of C3 or C3b and P and another was first reacted with C3 or C3b, washed three times with MEM, and then incubated with P. When both samples were stained with FITC anti-P, immunofluorescence appeared. Control cells similarly incubated with P alone and stained were negative. (*c*) C3b-bearing Raji cells were reacted with anti-C3 and then with P; no immunofluorescence-positive cells were observed after staining with FITC anti-P. (*d*) Raji cells were incubated with factor B or factor D-depleted serum, washed three times with MEM, and then stained with FITC anti-P. Almost

TABLE II
*Effect of Activators of the P System on the Binding of Factor B to Raji Cells**

Incubation	Intensity of immunofluorescense‡
NHS	Negative
NHS + inulin	4+
NHS + zymosan	3+
NHS + endotoxin	1+
NHS + C3b	1+
NHS + CoF	1+
NHS + aggregated IgA	1+
NHS + AHG	1+

* Raji cells were incubated with NHS or NHS first treated with the activators. Subsequently, the washed cells were incubated with FITC rabbit antifactor B and nonfluorescein-conjugated sheep antirabbit IgG.

‡ Graded from negative to 4+.

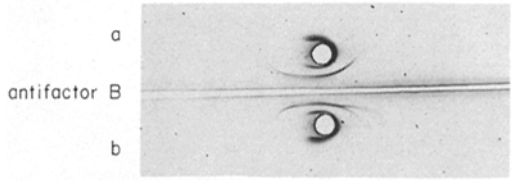

FIG. 4. Immunoelectrophoretic demonstration of Raji cell-induced conversion of factor B in NHS: (a) The upper well contains NHS incubated at 37°C for 30 min without cells. (b) The lower well contains the supernate of the same NHS after incubation (37°C, 30 min) with cells. Trough was filled with rabbit antihuman factor B. The slide was stained with amido black. The cathode was on the right. Note the appearance of a second arc in (b) representing the Bb fragment of factor B.

100% of cells were immunofluorescence positive. (*e*) Raji cells were first incubated with C3b, washed, and then reacted with NHS. After the washed cells were stained with FITC antifactor B, nearly all were weakly immunofluorescence positive. (*f*) Raji cells were incubated with a mixture of C3b, factor D, and factor B or they were first reacted with C3b, washed, and then incubated with a mixture of factor D and factor B. When the washed cells were subsequently stained with FITC antifactor B, no cells were immunofluorescence positive. (*g*) samples of cells were incubated with mixtures of C3b and P-depleted serum with or without isolated P. Activation of factor B by C3b is independent of P (15). When these cells were stained with FITC antifactor B, the former cells, but not the latter, were immunofluorescence positive. In summary, binding of P and factor B to cells evidently requires the presence of C3 or C3b, and binding of P seems to be necessary for the fixation or stabilization of cell-bound factor B.

Additional experiments were performed to demonstrate that binding of P and factor B to cells follows fixation of C3 or C3b to C3-C3b receptors. Several human lymphoblastoid cells lines were checked for C3-C3b receptors and binding of P

TABLE III
*Fixation of P to Raji Cells via C3-C3b Receptors and Independence of P Binding from Factor D and Factor B**

Cell type	First incubation	Second incubation	Third incubation	Immunofluorescence positive cells
				%
Raji	C3	FITC anti-C3	—	100
"	C3b	"	—	98
"	NHS	"	—	100
"	NHS	FITC anti-P	—	100
"	HS‡-depleted of C3	"	—	0
"	P	"	—	0
"	C3 + P	"	—	90
"	C3b + P	"	—	92
Raji + C3	P	"	—	100
Raji + C3b	P	"	—	95
Raji + C3b	Rab-anti-C3	P	FITC anti-P	0
Raji	HS-depleted of P	FITC anti-P	—	0
"	HS-depleted of factor D	"	—	92
"	HS-depleted of factor B	"	—	97

* 50 μg of C3 or C3b, 10μg of P, and 100 μl of NHS or human serum depleted of certain proteins were employed.
‡ HS, human serum.

TABLE IV
*Fixation of Factor B to Raji Cells via C3-C3b Receptors and Dependence or Stabilization of Its Fixation by P**

Cell type	Incubation	Immunofluorescence positive cells‡
		%
Raji	NHS	0
"	NHS + CoF	95
"	HS-depleted of C3 + CoF	0
"	C3b + NHS	89
Raji + C3b	NHS	93
Raji	C3b + factor D + factor B	5
Raji + C3b	factor D + factor B	2
Raji	C3b + HS-depleted of P	5
Raji	C3b + HS-depleted of P + P	95

* 50 μg of C3b, 50 μl of factor D, 50 μg of factor B, 10 μg of P, and 100 μl of NHS or human serum (HS) depleted of certain proteins were employed.
‡ Cells were stained with FITC rabbit antifactor B and then incubated with nonfluorescein-conjugated sheep antirabbit IgG.

TABLE V
Relationship of Receptors for C3-C3b and C3d to Fixation of P and Factor B on Human Lymphoblastoid Cell Lines

Cell type	Percent cells binding* C3 or C3b	Percent cells forming rosettes with:		Percent cells binding‡:	
		$EAC3b^{rab}$	$EAC3d^{mo}$	P	Factor B
Raji	100	96	98	100	100
Daudi	90	2	35	92	90
Wil2WT	2–3	1	0	0	0
8866	5	3	0	0	0
P3HR-1	0	0	0	0	0
MOLT-4	0	0	31	0	0
Sommer 8402	0	0	53	0	0

* The binding of soluble C3 or C3b was assessed by staining with FITC anti-C3.
‡ The binding of P and factor B was assessed by incubating the cells with NHS which had first reacted with inulin and by subsequent staining with FITC anti-P in the former and FITC rab-antifactor B and sheep antirabbit IgG in the latter.

and factor B. The results in Table V indicate an absolute correlation between the presence of receptors for C3-C3b and the ability of cells to fix P and factor B. MOLT-4 and Sommer 8402 cells which have C3d, but not C3-C3b receptors, were unable to bind P and factor B. Immunoelectrophoretic studies of the supernate obtained from incubating NHS with all cell lines studied, as done with Raji cells, revealed activation of P and factor B.

We (unpublished observations) and others (21) have observed that ZC particles incubated with lymphocytes bearing C receptors adhere to cells and form rosettes (Fig. 5). Surfaces of ZC particles carry fragments of C3, P, and factor B (26–29). However, preincubation of washed ZC with an antihuman C3 serum completely inhibited their adherence to Raji cells (Table VI). In contrast, treatment of ZC with anti-P or antifactor B had no effect on the adherence of ZC and rosette formation with Raji cells. Furthermore, addition of 200 μg of C3b to Raji-ZC rosettes dissociated the rosettes. Finally, preincubation of Raji cells with 100 μg C3b inhibited the adherence of ZC to cells (Table VI). These experiments showed conclusively that binding of P and factor B to cells was not mediated by specific cellular receptors for these proteins.

Binding of P and factor B to HPL. Inasmuch as some human lymphoblastoid cell lines bound P and factor B, we investigated whether HPL also bound these proteins (Table VII). HPL stained directly after the purification steps did not carry P or factor B. Furthermore, in contrast to lymphoblasts, HPL incubated with NHS did not bind P. However, incubating HPL with NHS pretreated with inulin and staining with FITC anti-P or FITC antifactor B resulted in approximately 20–25% immunofluorescence-positive cells. Next HPL were incubated with $EAC3b^{rab}$, then nonrosetting cells obtained at the interface after Ficoll-Isopaque gradient centrifugation were reacted with human serum pretreated with inulin and stained with FITC anti-P or FITC factor B, after which no immunofluorescence-positive cells were observed. In contrast, the majority of rosetted cells bearing C receptors (after lysis of the attached erythrocytes by

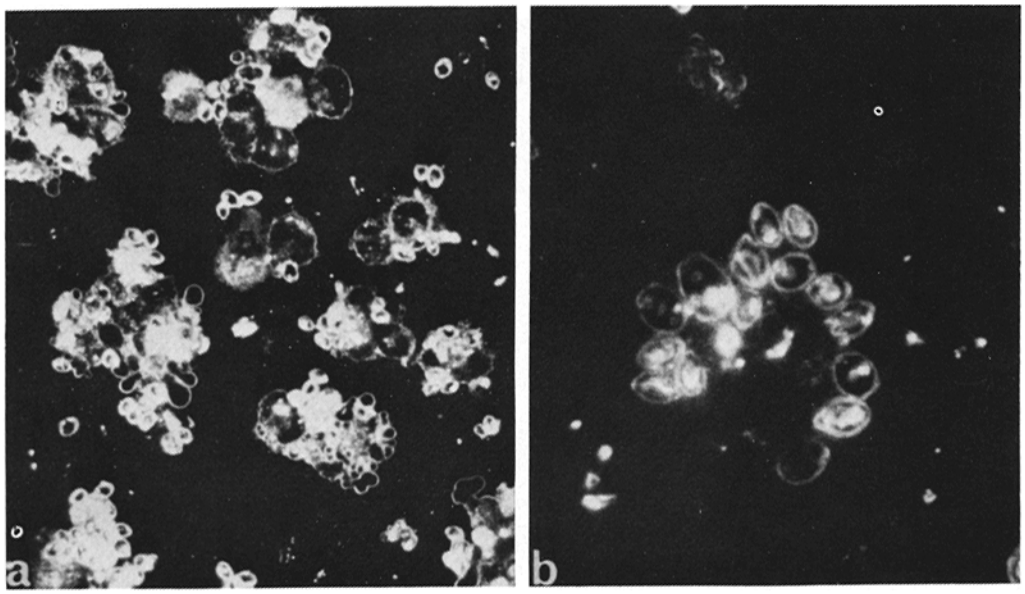

FIG. 5. Rosette formation between Raji cells and ZC. (a) Magnification × 250. (b) Magnification × 630.

TABLE VI
Adherence of ZC Particles Carrying C3b, P, and Factor B to Raji Cells by C3-C3b Receptors

Cell type	First incubation	Second incubation	Percent rosette-forming cells
Raji	ZC	MEM	100
"	ZC-anti-C3	"	0
"	ZC-anti-P	"	98
"	ZC-anti-factor B	"	100
"	ZC	C3b	5
Raji + C3b	ZC	MEM	3

brief exposure to 0.17 M NH_4Cl) incubated with NHS activated by inulin and subsequently stained possessed both P and factor B (Table VIII). These results show that HPL carrying C3b receptors, and which are considered of B type (30), are the cells that bind P and factor B.

Discussion

The present studies demonstrate that under appropriate conditions certain human lymphoblastoid cells as well as HPL of the B type bind P and factor B. Furthermore, fixation of these proteins to cells follows binding of C3 or C3b to C3-C3b receptors.

Immunoelectrophoretic studies of the supernate from incubating NHS with lymphoblastoid cells revealed activation of P and factor B. Furthermore, re-

TABLE VII
Binding of P and Factor B to HPL

Incubation	Staining procedure	Immunofluorescence positive cells
		%
MEM	FITC anti-P	0
NHS	"	0
NHS + inulin	"	19
MEM	FITC rab anti-factor B-sheep anti-rab IgG	0
NHS	"	0
NHS + inulin	"	25

TABLE VIII
Percentage of Unseparated HPL and Ficoll-Isopaque Separated EAC3b^{rab} Rosetted and Nonrosetted Cells which Bind P and Factor B

	Percent EAC3b^{rab} rosetting cells	Percent cells binding*	
		P	Factor B
Unseparated HPL	19	20	24
Interface	0	1	3
Pellet	58	52	61

* The binding of P and factor B was assessed by incubating the cells with NHS that had first reacted with inulin and by subsequent staining with FITC anti-P in the former and FITC rab-antifactor B and sheep antirabbit IgG in the latter.

moval of Ca^{++} and Mg^{++} by EDTA, but not removal of Ca^{++} alone with EGTA, from NHS inhibited fixation of P to Raji cells. Thus, human lymphoblastoid cells, like other particulate materials such as zymosan and inulin (26–29, 31, 32), can activate the P system. Since no P was detected by immunoelectrophoresis in the supernate obtained after incubation of 5×10^6 cells with 50 μl NHS, apparently about 2×10^5 molecules of P bound per Raji cell. The reason why lymphoblastoid cells in continuous culture activate the P system is not apparent. Possibly these cells are endowed with P system-activating enzymes or C-activating factors. Such factors have been found in supernates of HPL stimulated with mitogens (33). The possibility that endotoxin, a known activator of the P system (34), was present in the milieu cannot be excluded. Alternatively, the surfaces of these cells may carry virus or viral proteins capable of activating the C system. Others have found that virus-transformed murine lymphocytes fix C1 from serum (35), and that polyoma virus, Moloney virus, and vesicular stomatitis virus activate C in the absence of antibody (36, 37).

The enhanced binding of P to cells, as judged by the intensity of immunofluorescence after using inulin, endotoxin, or aggregated IgA-treated human serum, agrees with the previously shown activation of the P system by these substances

(31, 32, 34, 38, and footnote 4). Furthermore, this finding may indicate that the cells by themselves are not as potent activators of P or other serum factors, such as C3, potentiating the fixation of P to cells as the above substances. AHG could not enhance P binding just as AHGs were previously shown to be ineffective in triggering the P system[4]. Diminution of P fixation after incubation of cells with human serum pretreated with zymosan, under conditions which did not result in substantial removal of C3, indicated that most of the P adhered to these particles. Concentrations of zymosan similar to or smaller than those used in this study are known to remove the P from serum (32).

The presence of P on cells incubated with NHS or human serum with activated C and the absence of P on cells incubated with isolated P, aged human serum, and C3-depleted serum indicated that C3 or C3b were necessary for the binding of P to cells. This conclusion was reinforced by the fact that isolated P added with isolated C3 or C3b or added alone to Raji cells with bound C3 or C3b could fix to the Raji cells. Furthermore, cell lines devoid of C3-C3b receptors did not bind P. Recent studies have suggested that P in serum is a 6S molecule that forms weak complexes with native or altered C3 and possibly other serum factors, such as factor D and factor B (39). Fearon and Austen (40, 41), as well as Schreiber et al. (42), demonstrated that P binds to EAC43b, but not to EAC4, and that P bound to EAC43b does not decay significantly from the intermediate during 30-min incubation at 37°C. Fixation of P to Raji cells, as shown in the present study, was independent of factor B and factor D.

In contrast to the binding of P, factor B could not be demonstrated on Raji cells interacted with NHS or C2-deficient serum. However, factor B did bind after incubation of cells with serum first reacted with inulin or other activators of the P system. The reason why factor B activated by Raji cells, in constrast to factor B activated by inulin or the other activators, did not fix to the cells is not clear. Activators other than cells may generate more activated factor B and other serum factors needed for fixation or stabilization, such as C3b and activated P, than cells produce. Fearon et al. demonstrated that factor B decayed rapidly and was released into the milieu after EAC43b carrying factor D and factor B were incubated at 37°C. The half-life of the EAC43 bound convertase was only 4–6 min at 30°C (40, 41, 43). Brade et al. (26–28) found similar decay of factor B on zymosan particles and failure of factor B released from the particles to rebind. Conceivably then, the inability to detect factor B on cells incubated with NHS may be due to the decay and release of the bound factor B. Alternatively, as stated above, it may be that cells, in contrast to other activators, do not generate C3b and, therefore, factor B cannot bind to cells. In the case of P such generation of C3b, although potentiating its binding to cells, is not a prerequisite since activated P can combine with native C3 (15, 39, 42).

Since incubation of cells with CoF-treated C3-depleted human serum, in contrast to the CoF-treated NHS, did not result in factor B fixation, and since isolated factor B did not bind to cells, we concluded that C3b was necessary for the fixation of factor B to cells. Nicholson et al. (44) have already shown that binding of factor B to zymosan particles is mediated via C3b. However, incuba-

[4] Müller-Eberhard, H. J., O. Götze, and H. L. Spiegelberg. 1975. Activation of the alternative pathway by immunoglobulin aggregates and bacterial substances. Manuscript in preparation.

tion of soluble or cell bound C3b with factor D and factor B did not result in binding of factor B to Raji cells indicating that, apart from C3b, another serum factor(s) was needed for this binding. This other factor was identified as P, since incubation of C3b-bearing Raji cells with human serum depleted of P resulted in factor B fixation to cells only after reconstitution with P. Fearon and Austen have found that activated P greatly enhanced the binding of factor B to EAC43b, and after the addition of P with B and D to EAC43b, the hemolytic titer of either B or D increased 10–20-fold, and the half-life of the hemolytically active site rose from 4 up to 50 min (40, 41). Thus, C3b-dependent binding of P could either lead to more efficient interaction of factor D, factor B, and C3b or serve to stabilize the convertase once formed (40). After we incubated cells with zymosan-treated serum, from which most if not all of the P had been removed by the particles, factor B fixed to the cells. This fact indicates that factor B combined with large amounts of C3b can fix to cells without P. Binding of factor B onto EAC43b without P has been shown (43), but as stated, factor B decays rapidly in this situation. Therefore, one can envision three possible mechanisms for the binding of C3 or C3b, P, and factor B to cells or possibly other particles: (*a*) activated P in fluid phase combines with C3, factor D, and factor B, and the whole complex fixes to cells' C3-C3b receptors via its C3 moiety; (*b*) C3b in fluid phase generated by either the classical or alternative pathways combines with P, C3, factor D, and factor B and binds to C3-C3b receptors; or (*c*) C3 or C3b first fixes onto the C3-C3b receptor and thereafter interacts with P, factor D, and factor B. Since we showed that treatment of C3b-bearing Raji cells with anti-C3 abolished the binding of P, and others (44) showed that treatment of C3b-bearing zymosan particles with anti-C3 inhibited the binding of factor B to the particles, we must assume that both P and factor B bind directly to C3b. Thereafter the cell bound P + C3 + factor D + factor B or C3b + P + C3 + factor D + factor B complexes may act upon C3, releasing C3b which may enter the amplification loop or positive feedback mechanism of C activation (3, 4).

The interdependence of P and factor B binding with the presence of C3-C3b receptors was obvious in several cell lines. In addition, binding of P and factor B complexed with C3b clearly was not mediated via the P or factor B moiety of the complex but through C3b, since treatment of ZC particles with anti-C3, but not with anti-P or antifactor B, inhibited adherence of the ZC particles to Raji cells. Furthermore, addition of C3b to the Raji-ZC rosettes completely dissociated the rosettes and preincubation of cells with C3b halted Raji-ZC rosette formation. C3d receptors do not appear to participate in the binding of P and factor B to cells since the MOLT-4 and Sommer 8402 cells, which are devoid of C3-C3b receptors but have receptors for C3d, were unable to bind these proteins. Others (44) have used C3b inactivator to treat zymosan particles carrying C3b, thus releasing C3c, but leaving behind C3d (45) and have inhibited the binding of factor D and factor B to the particles.

Unlike human lymphoblastoid cells, HPL incubated with NHS did not fix P. This result suggests that transformed cells, in contrast to HPL, acquire factors that can induce activation and subsequent fixation of P. However, P, as well as factor B, were present in approximately 25% of HPL first incubated with inulin-treated human serum. Depletion of C3b receptor-bearing cells from HPL re-

moved the P- and factor B-fixing cells. Therefore, we concluded that the P- and factor B-fixing HPL were the same as the C3b receptor bearing cells which are considered as B type (30). This conclusion was verified when isolated C receptor bearing HPL were indeed found to have the capacity to bind P and factor B from inulin-activated human serum.

Binding of components of the P system to cells or other particles may be important in vivo. It is conceivable that the C system is activated by transformed cells or by other factors following which C3b binds to C3b receptor-bearing cells. Cell-bound C3b may then initiate an intense utilization of the terminal components of C by formation of the factor B-dependent convertase. Assembly of the terminal attack sequence of C directly on the cell membrane will then lead to cell lysis. This view is consistent with the following observations: (*a*) factor B-dependent C3 convertase bound to erythrocytes carrying C3b brought the hemolytic reaction to completion with an efficiency comparable to that of classical C3 convertase (43), (*b*) Raji cells that had absorbed C3b were susceptible to factor B dependent lysis (1), and a substantial percentage of ^{51}Cr-labeled Raji cells incubated with NHS were lysed,[5] (*c*) depletion of C receptor-bearing lymphocytes from a mixed lymphoid cell population occurred after treatment with CoF-activated serum (46), (*d*) a parallelism existed between antitumor effects of certain polysaccharides and their ability to activate the alternative C pathway (47), and (*e*) leukemia cells in mice were lysed after infusion of NHS, but not of heated or C5-deficient serum (48).

The binding of components of the P system to cells might not only be associated with lysis but might also influence other functions such as phagocytosis (49), lymphocyte proliferation and/or stimulation (50, 51), directional migration of cells and attraction of other cell types (52, 53), and responsiveness to external stimuli (52). Studies are under ways to investigate the influence on various cell activities of C3b, P, and factor B binding to C3-C3b receptor-bearing cells.

Summary

Immunofluorescence studies showed that properdin (P) and factor B bind to C3-C3b receptor bearing human lymphoblastoid cells (Raji, Daudi) and B type human peripheral lymphocytes (HPL). P bound to Raji cells first incubated with normal human serum (NHS). EDTA, but not EGTA, halted the binding of P to cells incubated with NHS. However, fixation of P to Raji cells, after incubation with NHS first reacted with inulin, was independent of Ca^{++} and Mg^{++} ions. Fixation of P to Raji cells depended on the presence of C3 or C3b and occurred in the absence of factor D and factor B. Binding of P to B type HPL was detectable only after incubation of these cells with NHS first reacted with inulin; under these conditions binding of P to Raji cells was also greatly enhanced. With both Raji cells and HPL, factor B was detectable on cell surfaces only after incubation of these cells with NHS first reacted with activators of the P system. Binding of factor B to cells required the presence of C3b and binding or stabilization of cell bound factor B necessitated the presence of activated P. P and factor B were

[5] Theofilopoulos, A. N., and L. H. Perrin. Lysis of C3b receptor bearing human cultured lymphoblastoid cells by NHS. Manuscript in preparation.

detectable only on cultured cells having C3-C3b receptors. However, incubation of NHS with all lymphoblastoid cell lines studied resulted in activation of P and cleavage of factor B. Binding of P and factor B to cells may follow one of three sequences: (*a*) activated P in fluid phase combines with C3, factor D, and factor B, and the whole complex fixes to cellular C3-C3b receptors via its C3 moiety; (*b*) C3b generated in fluid phase combines with P, C3, factor D, and factor B and binds to C3-C3b receptors; or (*c*) C3 or C3b first binds onto the C3-C3b receptors and thereafter interacts with P, factor D, and factor B.

Binding of components of the P system to cells or other particles may relate to such biological phenomena as lysis, phagocytosis, proliferation, attraction of other cell types, and alteration of responsiveness to external stimuli.

The authors wish to thank Dr. F. J. Dixon and Dr. H. J. Müller-Eberhard for their advice and guidance throughout this study and for reviewing the manuscript. The excellent technical assistance of Ms. Maryellen Masciangelo and the secretarial and editorial assistance of Mrs. Sally Hendrix and Mrs. Phyllis Minick is acknowledged.

Received for publication 8 September 1975.

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