# RELEASE OF ENDOGENOUS C3b INACTIVATOR FROM LYMPHOCYTES IN RESPONSE TO TRIGGERING MEMBRANE RECEPTORS FOR β1H GLOBULIN*

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In the past the complement (C)[1] system has been primarily characterized as the plasma cytotoxic effector mechanism of the immune response that complements the recognition function provided by specific antibody. Although this description is certainly applicable to the classical pathway of C activation, it is now recognized that the alternative pathway usually is not activated directly by immune complexes and has its own antibody-independent mechanism for recognition of non-self structures (1). With the finding of lymphocyte and macrophage synthesis of most of the components, inhibitors, and regulatory proteins of both the alternative and classical pathways of C activation (2–5), the question arises as to whether these components constitute a cell-bound system that is functionally analogous to the plasma C system, particularly with regard to the recognition of alternative pathway-activating surfaces.

Initiation of the alternative pathway involves the spontaneous activation of C3 (6) that binds randomly as C3b to nearby surface carbohydrate structures (7). Unbound C3b or C3b bound to normal tissue is rapidly cleaved into C3bi by C3b inactivator (C3bINA), whereas C3b bound to an activating surface resists C3bINA cleavage and

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[1] *Abbreviations used in this paper:* β1H or β1H globulin, essential cofactor for cleavage of fluid-phase C3b by C3b inactivator (11), and a potentiator of C3b inactivator cleavage of bound C3b (12); Bb, activated factor B; C, complement; BSA, bovine serum albumin; BDV, BSA dextrose veronal buffer; BDVA, BDV with 0.2% sodium azide; BDVE, BDV with 20 mM EDTA; C3b, 180,000-dalton fragment of C3; C3bi, C3b inactivator cleaved C3b; C3c, 140,000-dalton fragment of C3bi; C3d, 30,000-dalton fragment of C3bi that remains bound to surfaces after proteolysis of C3bi; C3bINA, C3b inactivator; $CR_1$, C receptor type one, the C4b-C3b receptor; $CR_2$, C receptor type two, the C3d-C3bi receptor; $CR_3$, C receptor type three, the C3bi receptor; E, erythrocyte; EA, IgM-antibody-sensitized E; EC3, sheep E coated with C3; EAC, EA coated with C; EACA, epsilon amino caproic acid; EDTA-DGVB, 20 mM EDTA in dextrose gelatin veronal buffer; FITC, fluorescein isothiocyanate; GVB, gelatin veronal buffer; Mg-BDV, BDV containing 10 mM magnesium chloride and 10 mM EGTA; NF, nephritic factor, human IgG antibody specific for the C3b,Bb complex; NP-40, Nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PBS, phosphate-buffered saline; PBS-BSA, PBS containing 1% BSA and 0.2% sodium azide; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TRITC, tetramethyl rhodamine isothiocyanate.

instead combines with activated factor B (Bb) and properdin (P) to form the amplification convertase (C3b,Bb,P). This surface-bound C3b,Bb,P rapidly cleaves more C3, generating a bound cluster of C3b that binds C5 and makes it susceptible to cleavage-activation by the C3b,Bb,P. Activated C5 (C5b) then combines with C6, C7, C8, and C9 to form the cytotoxic membrane attack complex. β1H globulin (β1H) has the recognition function that directs C3bINA to either unbound C3b or to C3b bound to normal tissue, thus preventing total consumption of plasma C and protecting normal tissue from cytolysis (8–11).

In our study, B lymphocytes were found to have membrane receptors for β1H. The binding of β1H to these receptors was shown to induce the release of endogenous C3bINA from lymphocytes, resulting in cleavage of EC3b into EC3bi. These resultant EC3bi then bound to B cell C receptor type two ($CR_2$, the C3d receptor) and did not bind to C receptor type one ($CR_1$, the C3b-C4b receptor). B cell-derived C3bINA thus produced $CR_2$-dependent rosette formation with EC3b.

## Materials and Methods

*Cell Isolation and Culture.* Normal blood and tonsil lymphocytes were isolated on Ficoll-Hypaque as previously described (12) and then monocytes were removed by absorption onto Sephadex G-10 (Pharmacia Fine Chemicals) (13). B lymphocyte-enriched fractions were prepared by depletion of T lymphocytes forming rosettes with sheep E (14). More than 75% of the cells in the tonsil B cell fraction were stained with $F(ab')_2$ anti-immunoglobulin (Ig)-tetramethyl-rhodamine isothiocyanate (TRITC). The Burkitt's lymphoma-derived lymphoblastoid cell lines known as Raji and Daudi, and the B. F. line derived from transformed normal lymphocytes (kindly provided by Dr. James Simmons, University of North Carolina at Chapel Hill, N. C.), were maintained in RPMI-1640 media supplemented with 10% fetal calf serum, penicillin, and streptomycin.

*Purified C Components and Enzymes.* Plasminogen, C4, C2, C3, C5, factor B, β1H, and C3bINA were isolated from a single 3-liter pool of fresh citrated plasma. Plasminogen was isolated from protease inhibitor-treated plasma by affinity chromatography on lysine-agarose (15). After precipitation of fibrinogen by addition of 5 g solid polyethylene glycol 4000 (PEG) (Sigma Chemical Co., St. Louis, Mo.) per 100 ml plasma, the plasma was diluted to 2 mS conductivity (4°C) with 5 mM Tris/HCl, pH 7.8, that contained 50 mM epsilon amino caproic acid (EACA) (Sigma Chemical Co.), 5 mM EDTA, 10 mM benzamidine (Sigma Chemical Co.), and 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.). After concentration to 3 liters with a Millipore Pellicon concentrator with 5 square feet of PSED membrane (Millipore Corp., Bedford, Mass.), the sample was loaded onto a 10- × 120-cm column of DEAE-Sephacel (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) equilibrated with 10 mM Tris/HCl pH 7.8, conductivity 2 mS (4°C), that contained 50 mM EACA, 5 mM EDTA, and 10 mM benzamidine. After a 12-liter wash with starting buffer, the column was eluted with a 30-liter linear NaCl gradient progressing to 0.3 M NaCl. C3bINA eluted at 2.7 mS conductivity (4°C), C2 at 3.4 mS, factor B at 4.0 mS, β1H at 5.2 mS, C5 at 6.2 mS, C3 at 8.0 mS, and C4 at 10.6 mS.

One pool that contained C3bINA and one-half the C2, and a second pool that contained factor B and the remaining C2, were each adjusted to pH 7.0 with 1 N HCl, diluted to 1.3 mS (4°C) with veronal buffer, pH 7.0, and loaded onto two 4- × 60-cm columns of Bio-Rex 70 (Bio-Rad Laboratories, Inc., Richmond, Calif.) equilibrated with veronal buffer, pH 7.0, conductivity 1.3 mS (4°C), containing 50 mM EACA and 5 mM EDTA. After a 1.5-liter wash with this buffer, each column was eluted with a 3 liter linear NaCl gradient progressing to 0.3 M NaCl. The three separate activity peaks of C3bINA, C2, and factor B that eluted at 1.6 mS, 9.2 mS, and 14.6 mS respectively, were each concentrated to 15 ml with a PM-10 membrane (Amicon Corp., Scientific Systems Div., Lexington, Mass.) and chromatographed on a 5- × 90-cm column of Sephadex G-150 equilibrated with 100 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 50 mM EACA, and 5 mM EDTA. Both the C3bINA and the factor B were

then chromatographed on a 2- × 30-cm column of C3b-agarose containing 3 mg of cyanogen bromide-linked C3b (16, 17)/ml of gel. C3bINA bound to C3b-agarose in 30 mM NaCl, 5 mM phosphate pH 7.0, and was eluted with PBS. Factor B bound to C3b-agarose in 10 mM $MgCl_2$ in veronal buffer, pH 7.2, and was eluted with 10 mM EDTA in veronal buffered saline. Common contaminants of the C3bINA, factor B, and C2 were then removed by immunoabsorption with agarose linked antibodies specific for $\beta$1H, C3, albumin, transferrin, hemopexin, IgG, and IgA.

The $\beta$1H, C5, C3, and C4 each eluted in distinct activity peaks from DEAE-Sephacel, making subsequent hydroxylapatite chromatography unnecessary (15). Each was precipitated from column fractions by addition of solid PEG to 16% (wt:vol), redissolved in 40–60 ml of 100 mM sodium phosphate buffer pH 7.4 containing 150 mM NaCl, 50 mM EACA, 5 mM EDTA, and 0.2% sodium azide, and chromatographed on a 10- × 120-cm column of Sepharose CL-6B equilibrated in the same buffer. The $\beta$1H was then immunoabsorbed with agarose conjugated to IgG antibodies specific for IgA, IgG, C3, C5, $\alpha_2$-macroglobulin, and C3bINA. The $\beta$1H was homogenous by both sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) and no contaminants were detected by immunodiffusion analysis over a dilution range of 20 mg/ml to 50 $\mu$g/ml with a variety of different antisera. The purified $\beta$1H did not cleave fluid-phase C3b at a 1:1 (wt:wt) ratio after 20 h at 37°C. By comparison, C3b was completely cleaved to C3bi after 20 h at 37°C by a mixture of 0.5% $\beta$1H and 0.2% C3bINA (wt:wt). The C4 was further purified by chromatography on QAE-Sephadex (Pharmacia Fine Chemicals) and DEAE Bio-Gel A (Bio-Rad Laboratories, Inc.) as described by Bolotin et al (19). Both the C3 and C5 were immunoabsorbed with agarose conjugated to antibodies specific for IgG, IgA, and $\beta$1H, after which the C3 was absorbed with agarose-anti-C5, and the C5 was absorbed with agarose-anti-C3. The C4, C3, and C5 were each judged to be >95% pure by SDS-PAGE and immunodiffusion analysis with a variety of antisera over a dilution range of 20 mg/ml to 50 $\mu$g/ml.

C3 nephritic factor (NF) was partially purified (20) from the plasma of a patient with partial lipodistrophy (kindly provided by Dr. W. Ray Gammon, University of North Carolina at Chapel Hill). Functionally pure factor D was isolated from 20 ml of twofold concentrated (PM-10 membrane) whole serum by chromatography on a 5- × 90-cm column of Sephadex G-75 in veronal-buffered saline.

*Antibodies to C Components and C Receptors.* Rabbits were immunized with C3c and C3d fragments prepared by trypsin cleavage of purified C3 (16). In immunodiffusion tests, anti-C3c formed a precipitin line of identity with C3, C3b, and C3c, but was unreactive with C3a, C3d, or $\beta$1H. Anti-C3c had an agglutinating titer of 2,400 with either EC3b or EC3bi ($1 \times 10^8$/ml), but did not agglutinate EC3d. Anti-C3d formed a precipitin line of identity with C3, C3b, and C3d, but formed no line with C3c, $\beta$1H, or C3a. Anti-C3d had an agglutinating titer of 400 with either EC3b or EC3bi, and a titer of 800 with EC3d. Fab fragments of these two antibodies were isolated by papain cleavage and chromatography on CM-52 cellulose (Whatman Chemicals, Division of W and R Balston, Maidstone, Kent, England) (21). Two different goat anti-C3bINA sera were used. One was a gift from Dr. Robert D. Schreiber, Scripps Clinic and Research Foundation, La Jolla, Calif., and the other was a gift from Dr. Brian F. Tack, Harvard Medical School, Boston, Mass., that was produced by immunization with the purified C3bINA described in the preceding section. When tested by immunodiffusion, both anti-C3bINA sera formed a single precipitin line of identity between purified C3bINA and whole human serum, and were unreactive with various dilutions of purified C3, IgG, $\beta$1H, factor B, and P. The IgG fraction of one anti-C3bINA serum was isolated by chromatography on DEAE-Sephacel in 0.02 M Tris/HCl pH 8.0, and after concentration to 34 mg/ml, was shown to be free of goat C3bINA activity. Goat anti-$\beta$1H was a gift from Dr. Brian F. Tack produced by immunization with the purified $\beta$1H described above. $F(ab')_2$ fragments of anti-$\beta$1H were produced by trypsin cleavage of DEAE-isolated IgG (22), followed by column chromatography on Sephadex G-150.

$CR_1$ was isolated from $1 \times 10^{14}$ erythrocyte membranes by modification of a technique, originally described by Fearon (23), that will be described in more detail elsewhere (N. J. Dobson, J. D. Lambris, and G. D. Ross. Manuscript in preparation.). Anti-$CR_1$ was produced by weekly immunization of rabbits with 100 $\mu$g of pure $CR_1$ emulsified in complete Freund's

adjuvant and had the same specificity as that described by Fearon (24). $CR_2$ was isolated from 15 liters of Raji cell culture supernate by ammonium sulfate precipitation, followed by sequential steps of column chromatography on DEAE-Sephacel, Sephadex G-150, and C3d-agarose.[2] Anti-$CR_2$ was produced by weekly intramuscular immunization of rabbits with 100 μg of pure $CR_2$ emulsified in complete Freund's adjuvant. Anti-$CR_2$ immunoprecipitated a single membrane glycoprotein of 72,000 mol wt from Nonidet P-40 (Particle Data Laboratories, Inc., Elmhurst, Ill.) solubilized B lymphoblastoid lines grown in a combination of $^{14}C$- and $^{35}S$-labeled amino acids. $F(ab')_2$ fragments of anti-$CR_1$ and anti-$CR_2$ were prepared by pepsin digestion and chromatography on Sephadex G-150 (25). Monovalent Fab' fragments were produced from $F(ab')_2$ fragments by mild reduction with 0.1 M cysteine (26).

*Labeling of Proteins with $^3H$, $^{125}I$, Fluorescein, or TRITC.* Purified C3 and β1H were labeled with $^3H$ by reductive methylation (27). For C3 this resulted in a $0.5 \times 10^6$–$2.5 \times 10^6$ dpm per μg sp act, and no detectable change in either hemolytic activity or binding site affinities for $CR_1$, $CR_2$, $CR_3$, or β1H. $^3H$-labeled β1H, $3.6 \times 10^6$ dpm per μg, had normal cofactor activity with C3bINA, and also normal activity in decay-dissociation of the amplification convertase (EC3b,Bb,P). Fab' fragments of anti-$CR_1$ were labeled with $^{125}I$ (28) and had a $6 \times 10^7$ cpm per μg sp act. $F(ab')_2$ fragments of anti-$CR_1$, anti-$CR_2$, and anti-β1H were each coupled to either fluorescein isothiocyanate (FITC) or TRITC as previously described (25). Alternatively, native IgG antibodies were coupled to either FITC-protein A (Pharmacia Fine Chemicals) or TRITC-Protein A (Zymed Laboratories; Burlingame, Calif.), before use for immunofluorescence assay to block any possible Fc receptor interaction with the antibody. Rabbit $F(ab')_2$ anti-Ig-TRITC (specific for μ, δ, α, and γ Ig heavy chains) was prepared as previously described (29).

*C-coated Erythrocytes (EAC and EC).* Sheep erythrocytes (E) were coated with IgM antibody (Cordis Laboratories, Miami, Fla.) and purified human C components to form EAC (30). $CR_1$-reactive EAC14b were prepared with 100 μg of C4 per $1 \times 10^9$ EACl. EC3b were prepared with $^3H$-labeled C3 as described by Pangburn and Müller-Eberhard (9), then a portion of the EC3b was subsequently converted to EC3bi by treatment with β1H and C3bINA, and finally some of the EC3bi was degraded to EC3d with plasmin. For $CR_1$ assay, 40 μg of $^3H$-labeled C3 were added per $1 \times 10^9$ EC3b,Bb,NF, resulting in a total of $1.5 \times 10^4$–$1.8 \times 10^4$ molecules of bound C3b per cell (determined by scintillation counting of solubilized cells). Such EC3b contained three to four times more bound C3b than was required to obtain plateau values for human E $CR_1$-dependent rosette formation, and conversion to EC3bi or EC3d also resulted in plateau values for Daudi cell $CR_2$-dependent rosette formation. For generation of EC3bi, EC3b at $5 \times 10^8$/ml in 20 mM EDTA-DGVB (3.8 mS at 22°C) was mixed with an equal volume KSCN-treated human serum (previously dialyzed against 20 mM EDTA-DGVB) (30), and incubated at 37°C for 1 h. The presence of any residual uncleaved C3b on EC3bi was detected by hemolytic assay for C3b sites capable of forming C3 convertase with purified factors B and D. For conversion to EC3d, 5 ml of EC3bi at $5 \times 10^8$/ml in GVB were mixed with 5 ml of freshly-activated plasmin (176 μg/ml) and incubated at 37°C for 30 min. Plasminogen was activated by mixing 2.5 ml of plasminogen at 352 μg/ml in 40 mM Tris/lysine-buffered saline, pH 9.0, with 20 μl of urokinase at 100,000 U/ml (kindly provided by Dr. Peter Harpel, Cornell University Medical College, New York) and incubating at 37°C for 30 min. Before addition to EC3bi, the pH of the plasmin was lowered to 8.0 by addition of 2.5 ml of 100 mM phosphate buffer, pH 7.0, in 50 mM NaCl. Complete cleavage of all C3bi to C3d was confirmed by absence of agglutination by either anti-C3c or purified bovine conglutinin (kindly provided by Dr. Robert Eisenberg, University of North Carolina at Chapel Hill).

Bovine E were also coupled to purified β1H with tannic acid (31) for assay of β1H receptors by rosette formation. Control bovine E coupled to bovine serum albumin (BSA) were prepared in parallel.

*C-Receptor Assay.* Rosette assays were performed in 10- × 75-mm plastic tubes as previously described by mixing 100 μl of EC or EAC at $2 \times 10^8$/ml with 100 μl of C-receptor cells at $4 \times 10^6$/ml and then incubating on a tube rotator for 15 min at 37°C (30). The assay media used

[2] Lambris, J. D., N. J. Dobson, and G. D. Ross. Isolation of lymphocyte membrane complement receptor type two (the C3d receptor) and preparation of receptor-specific antibody. Manuscript submitted for publication.

in all cases had a low ionic strength (6 mS conductivity at 22°C), as this was found to potentiate both $CR_1$ and $CR_2$ activity by ~50%. This media (BDV) consisted of 1% BSA and 3.2% dextrose in veronal buffer pH 7.2 with enough added NaCl to raise the conductivity to 6 mS. In some cases as noted, BDV was supplemented with either 20 mM EDTA (BDVE), 10 mM $MgCl_2$ in 10 mM EGTA (Mg-BDV), or 0.2% $NaN_2$ (BDVA), and then with these supplements, less NaCl was added so that the final conductivity was always 6 mS. In experiments that examined the kinetics of rosette formation, samples or rosette-assay mixture were examined at timed intervals.

Direct immunofluorescence of $CR_1$ and $CR_2$ was performed in the same way as for other surface markers such as Ig (25). $\beta$1H receptors were detected by immunofluorescence after incubation of cells with unlabeled $\beta$1H. A pellet of $5 \times 10^5$ cells was resuspended in 25 $\mu$l of $\beta$1H at 100 $\mu$g/ml in 1% BSA/PBS/0.2% $NaN_3$ (PBS-BSA) and incubated at 37°C for 10 minutes. Next, after only one washing step, 25 $\mu$l of fluorochrome-anti-$\beta$1H [$F(ab')_2$ or IgG-protein A] at 1 mg/ml was added to the cell pellet and incubation was continued for 20 min at 37°C. Cells were examined for surface fluorescence after three washes in PBS-BSA.

$CR_1$ and $\beta$1H receptors were also detected by radioimmune assay with $^{125}$I-Fab'-anti-$CR_1$ or [$^3$H]$\beta$1H, respectively. Nonspecific uptake was assessed by measuring the uptake of radiolabeled ligand in the presence of a 1,000-fold molar excess of the homologous unlabeled ligand. Graded amounts of [$^3$H]$\beta$1H or $^{125}$I-Fab'-anti-$CR_1$ in BDVA were added to 400 $\mu$l conical plastic centrifuge tubes that contained $1 \times 10^6$ cells and the total volume was adjusted to 100 $\mu$l with BDVA. After 30 min at 37°C, the cells were pelleted by centrifugation through oil at 8,000 *g* for 2 min (32). The floating aqueous phase was aspirated for determination of unbound ligand, and the cell pellet was either counted directly for $^{125}$I, or for $^3$H, solubilized with NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, Ill.) and counted in OCS Scintillation Fluid (Amersham Corp.).

*Rosette Inhibition Assays.* Cell pellets of either $2 \times 10^7$ EC3 or $4 \times 10^5$ C-receptor cells were resuspended in 100 $\mu$l of either BDV or various IgG antibodies or their $F(ab')_2$ (or Fab') fragments diluted in BDV. After 30 min at room temperature, treated cells were either tested without washing, or where indicated, washed three times in BDV, and then assayed for rosette formation. In assays for inhibition by EDTA, Mg-EGTA, or azide, both C-receptor cells and EC3 were suspended in BDVE, Mg-BDV, or BDVA, respectively.

*Assay for C3bINA Synthesis.* 100 ml of Raji cells at $1 \times 10^6$/ml were cultured for 16 h in leucine-free RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2% fetal calf serum, penicillin-streptomycin, and 5 mCi of [$^3$H]leucine (Amersham Corp.). Alternatively, $2.2 \times 10^8$ Raji cells were cultured in 110 ml of media supplemented with 50 $\mu$Ci each of $^{14}$C-labeled leucine, phenylalanine, and lysine, and 250 $\mu$Ci of [$^{35}$S]methionine. The harvested cells were washed three times in PBS and solubilized with 1% Nonidet P-40 (NP-40) in 50 mM Tris/HCl pH 7.5 that contained 100 mM KCl. The culture supernate was concentrated 50-fold (PM-10 membrane), dialyzed against phosphate-buffered saline (PBS), and then centrifuged at 40,000 *g* for 16 h. Both solubilized cells and culture supernate were then mixed with an equal volume of human serum and analyzed by immunodiffusion for C3bINA in barbital-buffered 1% agarose, pH 8.6. After 2 d at 4°C, soluble proteins were allowed to diffuse out of the agarose by immersion in 1% Triton X-100 (Sigma Chemical Co.) in PBS for 7 d. Detergent and then salts were eluted by 1-d immersions in PBS followed by deionized water. The agarose was dried, stained with amido black, impregnated with Enhance scintillant (New England Nuclear, Boston, Mass.), and then applied to X-Omat RP film (Eastman Kodak Co., Rochester, N. Y.) for 6 d at −85°C.

The presence of C3bINA antigen was also assessed by the capacity of Raji cells to absorb anti-C3bINA antibodies. A 1.0-ml pellet of washed Raji cells was lysed by freezing and thawing to expose cytoplasmic components, and then fixed in 1% glutaraldehyde-PBS for 15 min at 37°C. After two washes with PBS-BSA and two washes with PBS, 100 $\mu$l of packed cells was dispensed into four 1-ml plastic conical tubes and all supernatant fluid was aspirated. Next, 300 $\mu$l of goat IgG anti-C3bINA at 10 mg/ml ($OD_{280}$ = 14) was absorbed in succession with each of the four cell pellets for 30 min at 37°C. The absorbed anti-C3bINA had an $OD_{280}$ of 10.2, indicating a dilution of ≤25%. The concentration of anti-C3bINA antibody was determined by reverse Mancini radial immunodiffusion assay in which 10% human serum, as source

of C3bINA antigen, was incorporated into 1% agarose. The diameter of precipitin rings in dried and amido black-stained agarose were measured and the amount of anti-C3bINA was computed by comparison to a standard curve established with known amounts of anti-C3bINA. An allowance for 25% dilution was included in calculations of the amount of antibody absorbed.

*Assays for C3bINA Activity.* C3bINA activity released from β1H-treated lymphoid cells was measured by two different methods: (*a*) cleavage of $^{3}$H-labeled C3b on EC3b to EC3bi as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and fluorography; (*b*) generation of EC3b-Raji cell rosette formation inhibitable by anti-C3bINA.

For demonstration of EC3b conversion to EC3bi by lymphoid cell C3bINA, EC3b that contained $2.5 \times 10^{4}$ molecules of $^{3}$H-labeled C3b per cell were prepared. These EC3b[$^{3}$H] at $2 \times 10^{8}$/ml in BDVA were mixed with an equal volume of β1H-induced cell supernate and incubated overnight at 37°C. Controls included untreated EC3b[$^{3}$H], and EC3b[$^{3}$H] treated with either unstimulated-cell supernate or anti-C3bINA-treated supernate from β1H-triggered cells. After four washes with 0.15 M phosphate-buffered 0.3 M NaCl pH 7.5, the EC3b[$^{3}$H] cell pellet was lysed with 2% SDS, 0.1 M dithiothreitol in 62 mM Tris/HCl, pH 6.8, heated at 100°C for 5 min, and analyzed by SDS-PAGE using 7% polyacrylamide. After staining with Coomassie brilliant blue and photography to record the positions of known molecular weight markers, the gel slab was impregnated with Enhance scintillant and applied to X-Omat RP film for fluorography. The film was developed after 6 d exposure at −85°C and the molecular weight of each radioactive band was determined from its relative mobility by reference to a standard curve established with the known molecular weight markers.

C3bINA conversion of EC3b to EC3bi was also assayed by Raji cell rosette formation in the presence of EDTA. EDTA inhibited Raji cell C3bINA release (Results), so that Raji cell rosette formation in the presence of EDTA only occurred with EC3bi or EC3d. The specificity of this assay for C3bINA was confirmed with each different lymphoid cell type by inhibition studies with anti-C3bINA. Cell-free supernates derived from various β1H-triggered cell types were incubated with EC3b for 30 min at 37°C. Next, all β1H that was bound to the EC3b was eluted by six washing steps at 37°C with 1% BSA in 50 mM PBS (0.25 M NaCl), pH 7.5 (30 mS conductivity at 22°C). Finally, the supernate-treated and washed EC3b were examined for Raji rosettes in BDVE. Complete removal of β1H bound to EC3b by this washing procedure was confirmed in pilot studies with $^{3}$H-labeled β1H. As an additional control for possible β1H carryover on EC3b that might have occurred when testing supernates from β1H-treated cells, an equivalent amount of β1H was added to cell-free supernates derived from cells incubated without β1H.

## Results

*Specificity and Sensitivity of Assays for C Receptors; Absence of $CR_1$ on Raji and Daudi Cells.* Assay of lymphoid cells and erythrocytes for C receptors by immunofluorescence with $CR_1$- and $CR_2$-specific antibodies gave results similar to those obtained by conventional rosette assay procedures with C-coated sheep E (Table I). The EC3b rosette assay was the most sensitive for detection of $CR_1$, as EC3b formed rosettes with 75% of human E that are known to express only 900 $CR_1$ per cell (24), whereas EAC14b formed rosettes with only 60% of human E, and anti-$CR_1$ fluorescence staining was undetectable on human E. The cell lines Raji and Daudi expressed $CR_2$ but lacked detectable $CR_1$ by both fluorescence and rosette assays. In addition, a radioimmune assay with $^{125}$I-labeled-Fab′-anti-$CR_1$ was negative with these two cell lines. After subtraction of nonspecific uptake, human E bound twentyfold more $^{125}$I-Fab′-anti-$CR_1$ than did either Raji cells or sheep E (data not shown).

The specificity of $CR_1$ and $CR_2$ for the C3c and C3d portions of C3, respectively, was examined by assays for inhibition of EC3 rosette formation by Fab antibody fragments specific for C3c, C3d, $CR_1$, and $CR_2$ (Table II). Complete inhibition of EC3b rosette formation by either anti-C3c or anti-$CR_1$, indicated that EC3b were bound exclusively to $CR_1$ at a binding site within the C3c region of C3b. Anti-C3d

TABLE I

*Assay of $CR_1$ and $CR_2$ by C-dependent Rosette Formation Versus Direct Immunofluorescence with C-Receptor-specific Antibodies*

| Cell type | $CR_1$ markers | | | $CR_2$ markers | | |
|---|---|---|---|---|---|---|
| | EAC14b rosettes | EC3b rosettes | anti-$CR_1$ FITC | EC3bi rosettes | EC3d rosettes | anti-$CR_2$ FITC |
| | % | | | | | |
| Erythrocytes | 60 | 75 | 0 | 10 | 0 | 0 |
| Normal lymphocytes | | | | | | |
| Blood | 14 | 17 | 15 | 9 | 8 | 9 |
| Tonsils | | | | | | |
| Unseparated | 55 | 69 | 65 | 74 | 70 | 65 |
| E-rosette negative | 70 | 90 | 85 | 92 | 84 | 81 |
| Lymphoblastoid cell lines | | | | | | |
| Raji | 0 | 0 | 0 | 98 | 98 | 90 |
| Daudi | 0 | 0 | 0 | 85 | 80 | 75 |
| B.F. | 49 | 65 | 57 | 95 | 93 | 85 |

TABLE II

*Inhibition of EC3 Rosette Formation by Antibodies Specific for C3 Fragments and C Receptors*

| EC3 type | C receptor cell | C receptor type | Inhibition by antibody specific for | | | |
|---|---|---|---|---|---|---|
| | | | C3c | C3d | $CR_1$ | $CR_2$ |
| | | | | % | | |
| EC3b | Erythrocyte | $CR_1$ | 100 | 0 | 100 | 0 |
| | Tonsil B cell | $CR_1$, $CR_2$ | 100 | 0 | 100 | 0 |
| EC3bi | Raji or Daudi | $CR_2$ | 100 | 100 | 0 | 60 |
| | Tonsil B cell | $CR_1$, $CR_2$ | 100 | 100 | 0 | 50 |
| EC3d | Raji or Daudi | $CR_2$ | 0 | 100 | 0 | 100 |
| | Tonsil B cell | $CR_1$, $CR_2$ | 0 | 100 | 0 | 100 |

did not inhibit EC3b rosette formation with cells bearing both $CR_1$ and $CR_2$. EC3bi rosette formation was not inhibited by anti-$CR_1$, indicating that EC3bi did not bind to $CR_1$, despite the observation of occasional human E rosettes with EC3bi (Table I). Complete inhibition of EC3bi rosettes by anti-C3d and partial inhibition by anti-$CR_2$ indicated that at least some EC3bi were bound to $CR_2$. However, complete inhibition of EC3bi rosette formation by anti-C3c and only partial inhibition by anti-$CR_2$ suggested that EC3bi might also be bound to a third type of C receptor (Table II). EC3d were not inhibited from forming rosettes by either anti-C3c or anti-$CR_1$, whereas anti-C3d and anti-$CR_2$ completely inhibited EC3d rosette formation with all cell types.

*$\beta$1H-induced EC3b Rosette Formation with $CR_1$-Negative Lymphoid Cells.* Even though EC3b did not bind to Raji cells, small amounts of purified $\beta$1H induced dose-dependent EC3b rosette formation with Raji cells (Figure 1). Kinetic studies of Raji cell-EC3b rosette formation induced by 30 $\mu$g/ml $\beta$1H, indicated that maximum

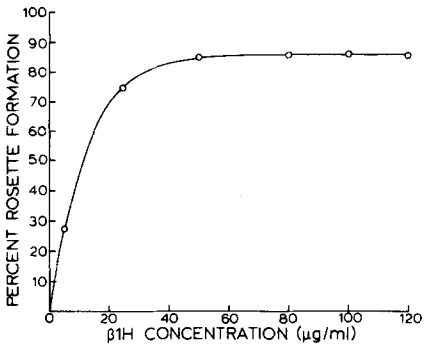


FIG. 1. Dose-dependence of β1H-induced EC3b rosette formation with Raji cells. EC3b was resuspended ir increasing concentrations of purified β1H in BDV, and then assayed for rosette formation with Raji cells for 30 min at 37°C.

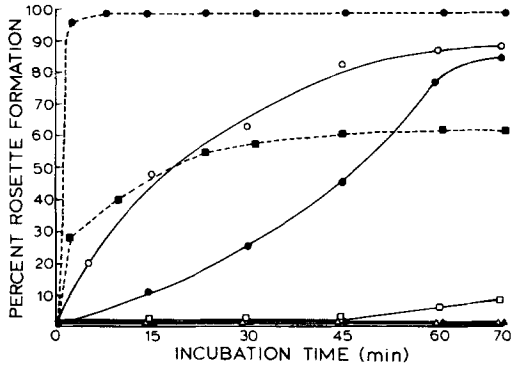


FIG. 2. Kinetics of EC3 rosette formation with Raji and Daudi lymphoblastoid cells. EC3bi rosette formation with Raji cells (●) and Daudi cells (■) occurred more rapidly than did β1H-induced (30 μg/ml) EC3b rosette formation with Raji cells (○) and Daudi Cells (□). No EC3b rosette formation was observed in the absence of β1H with either Raji cells (▲) or Daudi cells (△). Pretreatment of Raji cells with IgG anti-C3bINA followed by two washing steps and resuspension with BDV, reduced the rate of β1H-induced EC3b rosette formation (●).

rosette formation required 45–60 min, as compared to only 2 min for maximum rosette formation with EC3bi (Fig. 2). A few Daudi cell-EC3b rosettes were also observed after 60 min with 30 μg/ml β1H; however, neither Daudi nor Raji formed rosettes with EC3b after 60 min in the absence of β1H. Raji and Daudi cells also formed rosettes with preformed EC3b-β1H complexes prepared with $^3$H-labeled β1H. Maximum Raji cell rosette formation was achieved in 10 min with $8.8 \times 10^3$ β1H molecules bound per EC3b-β1H. Removal of all bound [$^3$H]β1H from EC3b-β1H by six washes with high ionic strength (30 mS conductivity at 22°C) buffer, resulted in complete loss of the ability of the EC3b to form rosettes with Raji cells.

*Specificity of β1H-induced Rosette Formation.* The binding of EC3b-β1H to Raji cells was not inhibited by the incubation of Raji cells in 5.5 mg/ml of β1H, both before and during the rosette assay with EC3b-β1H. β1H-induced EC3b rosette formation was completely inhibited by the presence of anti-C3c or anti-C3d in the assay mixture, but little or no inhibition was observed if either Raji or EC3b were first treated with these same antibodies and washed before rosette assay (Table III). Studies with $^3$H-labeled β1H indicated that the amounts of anti-C3c and anti-C3d used for rosette

TABLE III

*Inhibition of β1H-induced Raji Cell-EC3b Rosette Formation by Antibodies Specific for C3 Fragments, C Receptors, and C3bINA*

| Antibody | Inhibition of rosette formation |
|---|---|
| | % |
| Anti-C3c | 100 |
| Anti-C3d | 100 |
| Anti-$CR_1$ | 0 |
| Anti-$CR_2$ | 100 |
| Anti-C3bINA | 100 |

inhibition did not inhibit the uptake of small amounts of β1H onto EC3b. β1H-induced EC3b rosette formation was also completely inhibited with anti-$CR_2$-treated and washed Raji cells, whereas there was no inhibition when Raji were treated with anti-$CR_1$ both before and during EC3b-β1H rosette assay.

The finding of complete inhibition by anti-C3c as well as by anti-C3d, indicated that β1H-induced EC3b rosettes resembled EC3bi rosettes (Table II). Thus, the process of rosette formation might involve C3bINA. This hypothesis was confirmed by demonstration that anti-C3bINA completely inhibited Raji cell EC3b-β1H rosette formation, whereas the same antibody had no effect on either EC3bi or EC3d rosette formation with Raji cells and did not inhibit EC3b rosettes with $CR_1$-bearing cells. Complete inhibition occurred only when anti-C3bINA was present in the rosette assay mixture. Pretreatment of Raji cells with anti-C3bINA followed by washing steps to remove unbound antibody, produced a lag period in subsequent β1H-induced EC3b rosette formation, but did not diminish the maximum level of rosette formation observed after 60 minutes (Fig. 2). These data indicated that Raji cells released C3bINA that converted EC3b-β1H to EC3bi, and that these EC3bi and not the EC3b were bound subsequently to $CR_2$.

*Synthesis of C3bINA by Lymphocytes.* Raji cells cultured in media supplemented with radiolabeled amino acids, were examined by immunoprecipitation for incorporation of radiolabel into C3bINA antigen. By immunodiffusion analysis, radiolabeled C3bINA was detected in both culture supernates and detergent-solubilized cell pellets (Fig. 3). By a reverse Mancini radial immunodiffusion assay, it was also shown that absorption of anti-C3bINA with Raji cells removed >50% of the antibody activity directed to serum C3bINA.

*β1H-induced Release of Lymphocyte C3bINA.* Raji cells were incubated in BDV buffer either with or without 30 μg/ml of β1H for 60 min at 37°C, and then the cell-free supernates were harvested after centrifugation and tested for their ability to cleave cell bound C3b into C3bi. When the supernate derived from β1H-treated Raji cells was incubated overnight at 37°C with EC3b prepared with $^3$H-labeled C3, a portion of the bound C3b was converted to C3bi as shown by SDS-PAGE and fluorography of the solubilized EC3b cells (Fig. 4). Appearance of the 43,000-dalton α′-chain fragment after reduction of disulfide bonds is characteristic of bound C3bi (33). C3bi formation by supernates of β1H-treated cells was completely prevented by the presence of anti-C3bINA (Fig. 4).

The most sensitive assay for C3bINA was the induction of EC3b rosette formation with Raji cells. Studies of EC3bi prepared with increasing amounts of $^3$H-labeled C3

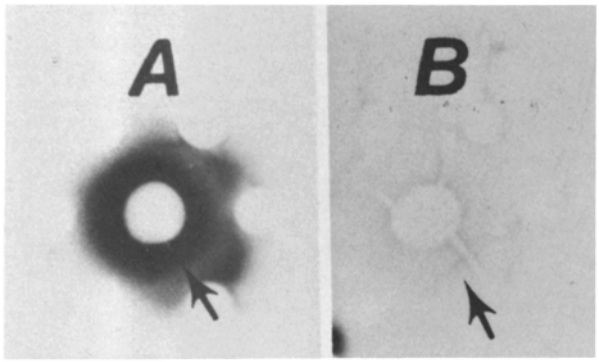


FIG. 3. Demonstration of Raji cell $^3$H-leucine endogenously-labeled C3bINA by immunodiffusion and fluorography. Goat anti-C3bINA was placed in the outer well and solubilized Raji cells were placed in the center well of panel A and culture supernate in the center well of panel B. The positions of the radioactive precipitin lines (indicated with arrows) exactly corresponded to the position of the amido black-stained serum C3bINA precipitin line. Other outer wells contained antibodies specific for human serum proteins other than C3bINA, and formed only unlabeled precipitin lines (anti-IgG and anti-ceruloplasmin).

demonstrated that plateau values for Raji rosette formation could be achieved with as few as $1.5 \times 10^3$ molecules of bound C3bi per EC3bi. Thus, with the usual EC3b that contained $1.5 \times 10^4$ molecules of C3b per cell, maximum Raji rosette formation required cleavage of only 10% of bound C3b to C3bi. Using this Raji cell assay for C3bINA, normal blood and tonsil lymphocytes, and various B lymphoblastoid cell lines were analyzed for C3bINA release after treatment with buffer either that contained or lacked 30 μg/ml of β1H (Table IV). β1H-induced C3bINA release was detected with all lymphoid cell types examined. In addition to fluid-phase β1H, bovine E coated with β1H (but not with BSA) induced C3bINA release. Treatment of all β1H-induced cell supernates with anti-C3bINA completely blocked their ability to generate EC3b rosette formation with Raji cells, confirming the C3bINA specificity of this assay with each cell type. Where noted in Table IV, β1H was added to control cell-free supernates to assure that low amounts of C3bINA were not overlooked because of missing β1H potentiation of C3bINA activity.

Sucrose density-gradient analysis of various purified β1H preparations demonstrated that 5%–50% of the β1H was aggregated into hexomers of $\sim 9 \times 10^5$ mol wt, whereas the remaining β1H was monomeric and $1.5 \times 10^5$ mol wt. Aggregation apparently occurred during concentration of purified β1H by high pressure ultrafiltration, as β1H aggregation was undetectable by gel filtration chromatography of solubilized β1H following polyethylene glycol precipitation. When Raji cells were treated with 30 μg/ml of a β1H preparation that was estimated to be 90–95% monomeric β1H and 5–10% hexomeric β1H by sucrose gradient analysis, C3bINA release was detectable in the cell-free supernate by induction of Raji cell-EC3b rosette formation. Monomeric β1H taken directly from sucrose density fractions could not be used in rosette assays without prior dialysis, as high sucrose concentrations lysed Raji cells. Dialysis against BDV resulted in trace amounts of β1H precipitation, and presumably, also some soluble β1H aggregates, as 30 μg/ml of the dialyzed and centrifuged β1H induced C3bINA release.

*Demonstration of β1H Receptors.* Raji cells exhibited a dose-dependent and saturable

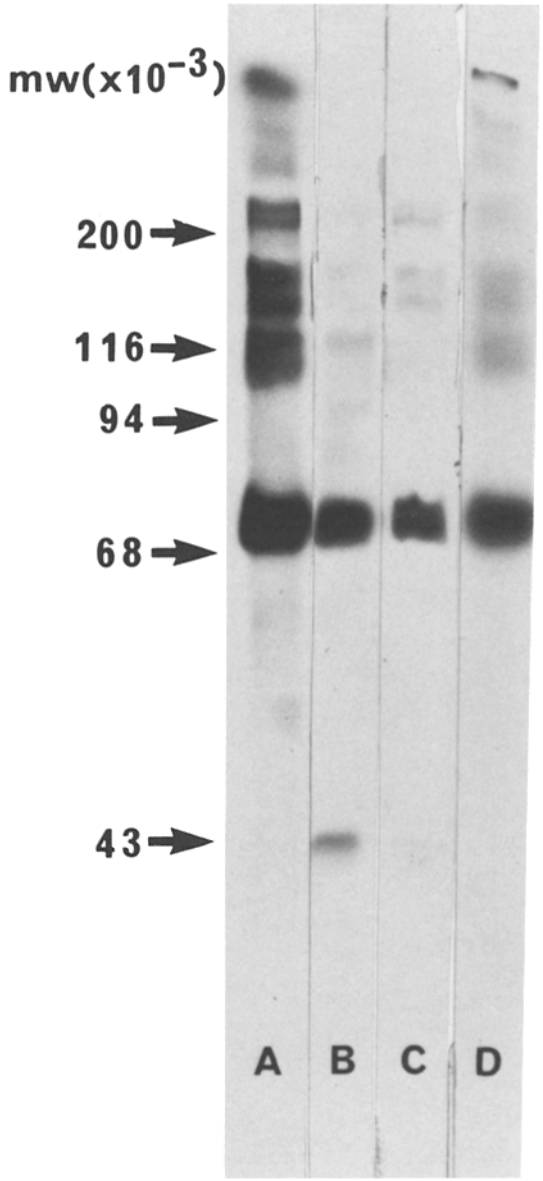


FIG. 4. Demonstration of EC3b conversion into EC3bi by cell-free and serum-free supernates obtained from β1H-triggered Raji cells. Track A was overloaded with untreated EC3b[$^3$H] and shows no detectable contaminating C3bi on the EC3b before treatment with Raji supernatant. In track A the β-chain of C3b (75,000 daltons) is just above the position of the BSA marker (68,000 daltons). The higher molecular weight bands in track A represent covalent complexes of the α′ chain of C3b and various sheep E membrane proteins (33). Track B contained EC3b treated with the supernate of Raji cells that had been incubated with 30 μg/ml of β1H for 60 min at 37°C. The 43,000-dalton fragment of the α′-chain of C3bi appears at the same position as the parallel chicken ovalbumin marker and below the intact β-chain (33). The larger 68,000-dalton α′-chain fragment of C3bi remains covalently-complexed to several different molecular weight sheep E membrane proteins and is represented by the faint higher molecular weight bands positioned above the β-chain. Track C contained EC3b[$^3$H] treated with the supernate of Raji cells incubated in β1H-free BDV buffer for 60 min at 37°C. Only trace amounts of the C3bi-specific 43,000 dalton α′-chain fragment were observed, indicating only low levels of spontaneous C3bINA release in the absence of β1H-triggering. Track D contained EC3b[$^3$H] treated with the cell-free supernate of Raji cells incubated with 30 μg/ml of β1H and goat IgG anti-C3bINA.

TABLE IV

*β1H-induced Release of C3bINA from Normal Lymphocytes and Various Lymphoblastoid Cell Lines as Detected by Conversion of Raji Rosette-Negative EC3b to Raji Rosette-Positive EC3bi*

| Cell | Cell treatment | Addition to harvested supernate | Raji rosettes with EC3b that were treated with harvested supernate |
|---|---|---|---|
| | | | % |
| Blood lymphocytes | Buffer | β1H | 15 |
| | β1H | Buffer | 85 |
| | β1H and anti-C3bINA | Buffer | 0 |
| Tonsil lymphocytes | Buffer | β1H | 10 |
| | β1H | Buffer | 90 |
| | β1H and anti-C3bINA | Buffer | 0 |
| Lymphoblastoid cell lines | | | |
| Raji | Buffer | β1H | 0 |
| | β1H | Buffer | 85 |
| | β1H and anti-C3bINA | Buffer | 0 |
| | β1H-coated E | Buffer | 28 |
| | BSA-coated E | Buffer | 0 |
| Daudi | Buffer | β1H | 0 |
| | β1H | Buffer | 62 |
| | β1H and anti-C3bINA | Buffer | 0 |
| B.F. | Buffer | β1H | 5 |
| | β1H | Buffer | 60 |
| | β1H and anti-C3bINA | Buffer | 0 |

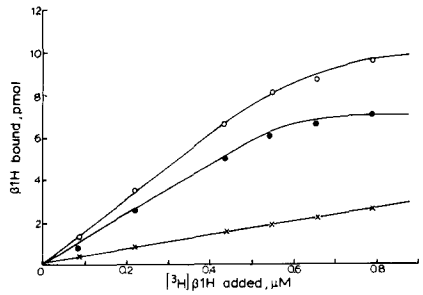


FIG. 5. Demonstration of β1H-specific membrane receptors by assay for binding of $^3$H-labeled β1H to Raji cells. The total uptake of $^3$H-labeled β1H is shown by the top curve (○). Nonspecific uptake of $^3$H-labeled β1H was determined with a 1,000-fold molar excess of unlabeled β1H (×). Specific uptake of β1H (●) was calculated by subtraction of nonspecific uptake from total uptake.

uptake of $^3$H-labeled β1H (50% hexomeric) that was inhibitable by excess unlabeled β1H (95% monomeric) (Figure 5). Individual β1H-binding cells were identified by immunofluorescence after incubation of cells in unlabeled β1H. Approximately 10–15% of Raji cells were stained brightly and an additional 30–40% of Raji cells were stained weakly by this technique. Double-label immunofluorescence assay of normal peripheral blood lymphocytes for β1H receptors with fluorescein and for surface Ig

with rhodamine, demonstrated that nearly all cells that bore $\beta$1H receptors also expressed surface Ig. Assay of $\beta$1H receptors by rosette assay with $\beta$1H-coated erythrocytes (34) detected <10% as many $\beta$1H-receptor bearing cells as did the immunofluorescence assay.

*Energy and Metal Ion Requirements.* Raji cell $\beta$1H-induced EC3b rosettes were completely inhibited by the presence of either EDTA or Mg-EGTA. Resuspension of EDTA-treated Raji cells in buffer lacking EDTA restored $\beta$1H-induced EC3b rosetting. By comparison, EDTA and Mg-EGTA produced very little inhibition of either $CR_1$-or $CR_2$-dependent rosettes with EC3b and EC3d respectively. EDTA neither inhibited the uptake of $\beta$1H onto $\beta$1H receptors nor inhibited the cleavage of C3b by C3bINA. Sodium azide was also observed to completely inhibit $\beta$1H-induced EC3b rosette formation, whereas removal of azide restored $\beta$1H-induced rosetting capacity.

## Discussion

The major conclusion derived from these studies is that B lymphocytes and B-type lymphoblastoid cell lines express membrane receptors for $\beta$1H that trigger the release of endogenous C3bINA. B cells, exposed to highly purified $\beta$1H, converted nearby EC3b to EC3bi, and then these EC3bi were subsequently bound to $CR_2$ receptors. Synthesis of C3bINA was confirmed by lymphocyte incorporation of radiolabeled amino acids into C3bINA antigen, and by inhibition of lymphocyte cleavage of C3b into C3bi by anti-C3bINA. Little, if any, spontaneous release of C3bINA was detected when lymphocytes were incubated in buffer lacking $\beta$1H. Lymphocyte receptors for $\beta$1H were detected by the uptake of $^3$H-labeled $\beta$1H, and individual B cells with $\beta$1H receptors were identified by immunofluorescence following treatment of lymphocytes with unlabeled $\beta$1H.

In previous studies, complex bound C3 has been shown to bind to B lymphocytes by way of two distinct types of membrane C receptors that are designated $CR_1$ and $CR_2$ (35). Each of these two types of C receptors has been isolated and specific antibodies have been prepared by immunization with the purified receptors. $CR_1$ was isolated from erythrocyte membranes and shown to be a 205,000 mol wt single-chain glycoprotein (23, 24) that bound to both C3b and C4b, but not to C3bi or C3d.[3] Fab-anti-$CR_1$ inhibited EC3b (24) and EAC14b rosettes with all leukocyte types, but had no effect on EC3bi or EC3d rosettes (Table II). The finding of inhibition of EC3b-$CR_1$ rosettes by Fab-anti-C3c, but not by Fab-anti-C3d (Table II), indicated that the binding site for $CR_1$ was in the c region of C3b and not close enough to the d region for steric hindrance by Fab-anti-C3d. $CR_2$ was isolated from spent media used to culture Raji cells and was shown to be a 72,000-mol wt single-chain glycoprotein that bound to C3bi and C3d, but not to C3b or C4b.[2] Anti-$CR_2$ was shown to be specific for a single endogenously $^{14}$C-labeled 72,000-dalton protein obtained from an NP-40 lysate of Raji cells. Fab-anti-$CR_2$ inhibited EC3d rosette formation with all B-type lymphoid cells examined, but had no effect on EC3b or EAC14b rosette formation (Table II).

Tests of C receptor reagents for specificity and sensitivity suggested that lymphocytes might also express a third type of C3 receptor that was distinct from $CR_1$ and $CR_2$, and possibly the same as myeloid cell $CR_3$ (35). EC3bi were apparently bound

[3] Dobson, N. J., J. D. Lambris, and G. D. Ross. Characteristics of isolated erythrocyte complement receptor type one ($CR_1$, C4b-C3b receptor) and $CR_1$-specific antibodies. *J. Immunol.* In press.

to B cells by two different types of receptors. Excess and saturating amounts of $F(ab')_2$-anti-$CR_2$ that totally inhibited all EC3d-$CR_2$ rosettes produced only 50–60% inhibition of EC3bi rosettes. Further studies will attempt to characterize the specificity of lymphocyte $CR_3$ more precisely and determine the relationship of lymphocyte $CR_3$ to myeloid cell $CR_3$.

$CR_1$ was undetectable on the surface of Raji and Daudi cells when analyzed by three different procedures, including a radioimmune assay with $^{125}I$-labeled Fab-anti-$CR_1$. EC3b that contained three times more bound C3b than was required to obtain plateau values for $CR_1$-dependent human E rosette formation did not form rosettes with Raji cells (Table I). Raji cell-EC3b rosettes were induced, however, by the addition of as little as 5 μg/ml of purified β1H (representing 1% of plasma β1H concentration). Rosette formation also occurred with preformed EC3b-β1H complexes, whereas removal of all bound β1H from EC3b completely inhibited subsequent rosette formation.

β1H-induced EC3b rosettes with Raji cells were bound to $CR_2$ and not to $CR_1$ or to β1H receptors. Pretreatment of Raji cells with $F(ab')_2$-anti-$CR_2$ totally inhibited β1H-induced EC3b rosettes, whereas the presence of Fab-anti-$CR_1$ or concentrations of fluid-phase β1H ≤5.5 mg/ml had no effect on subsequent β1H-induced rosettes. Rosette formation was also inhibited completely by the presence of either Fab-anti-C3c or Fab-anti-C3d. However, pretreatment of EC3b with either of these two antibodies had no effect on β1H-induced rosettes. By comparison, pretreatment of EC3b with Fab-anti-C3c completely inhibited $CR_1$-dependent rosettes, and pretreatment of EC3bi or EC3d with Fab-anti-C3d completely inhibited $CR_2$-dependent rosettes (Table II). These observations indicated that the C3 determinants that bound to Raji cells were not exposed on EC3b but were uncovered by Raji cells in the presence of β1H. Furthermore, the inhibition by anti-C3c as well as by anti-C3d suggested that the specificity of β1H-induced EC3b rosette formation resembled that observed with EC3bi rosette formation. Control studies with $^3H$-labeled β1H demonstrated that the anti-C3c and anti-C3d used for rosette inhibition did not block the uptake of β1H onto EC3b. These results indicated that Raji cells might release a C3bINA-like enzyme that converted EC3b into EC3bi in the presence of β1H, and that these EC3bi were bound subsequently to Raji cell-$CR_2$ forming rosettes. This hypothesis was confirmed by the finding that β1H-induced EC3b rosettes were completely inhibited by the presence of anti-C3bINA. Thus, Raji cells did not bind EC3b-β1H to either $CR_2$ (36) or to β1H receptors (34), but instead converted EC3b-β1H into EC3bi that bound to $CR_2$. The finding that β1H-induced EC3b rosettes were completely inhibited by anti-$CR_2$, whereas EC3bi rosettes were inhibited only 50–60% by anti-$CR_2$ suggests that only small amounts of C3bi were formed on the EC3b and that this C3bi was bound preferentially to $CR_2$ rather than to $CR_3$. The finding of complete inhibition by anti-C3c suggests that little, if any, of the bound C3bi was fully degraded to C3d, as anti-C3c had no effect on EC3d rosettes (Table II).

Lymphocyte synthesis of C3bINA was confirmed by immunodiffusion and fluorography analysis of spent media and solubilized Raji cells obtained from overnight culture in radiolabeled amino acids. Anti-C3bINA formed a single precipitin line of identity with serum C3bINA and a radiolabeled C3bINA protein synthesized by Raji cells. Identity of lymphocyte C3bINA with serum C3bINA was also indicated by

antibody absorption experiments. Absorption of anti-C3bINA with Raji cells removed >50% of the antibody directed to human serum C3bINA. In addition to detection of the incorporation of labeled amino acids into C3bINA antigen, C3bINA activity was demonstrated in serum-free lymphocyte supernates by conversion of EC3b to EC3bi detected by SDS-PAGE and by conversion of Raji-rosette-negative EC3b into Raji-rosette-positive EC3bi. Each assay was shown to be specific for C3bINA by parallel controls that demonstrated that anti-C3bINA completely inhibited the activity measured.

C3bINA was released from lymphocytes in response to $\beta$1H, and very little spontaneous release of C3bINA occurred in the absence of $\beta$1H. Cell-free supernates from Raji cells treated with $\beta$1H converted EC3b to EC3bi as determined by SDS-PAGE analysis. Very little C3bi was generated on EC3b treated with cell-free supernates obtained from Raji cells incubated in $\beta$1H-free buffer.

Normal lymphocytes from blood and tonsils also released C3bINA after exposure to $\beta$1H. However, it was only possible to measure $CR_2$-specific $\beta$1H-induced EC3b rosettes with normal lymphocytes that had been pretreated with Fab'-anti-$CR_1$, because untreated normal lymphocytes bound EC3b to $CR_1$ in the absence of $\beta$1H. After Fab'-anti-$CR_1$ pretreatment, normal lymphocyte $\beta$1H-induced $CR_2$-dependent rosettes with EC3b were nearly equivalent to EC3bi rosettes (data not shown). $\beta$1H-induced normal lymphocyte release of C3bINA was more easily measured by testing cell-free supernates of $\beta$1H-treated cells by a Raji cell assay for conversion of rosette-negative EC3b into rosette-positive EC3bi (Table IV). With both normal lymphocytes and cells from two other B lymphoblastoid lines, C3bINA release was stimulated by the presence of $\beta$1H, and little C3bINA release occurred spontaneously in the absence of $\beta$1H.

B lymphocytes expressed $\beta$1H-specific receptors of relatively low affinity. $\beta$1H receptors appeared to be saturable and reversible, however, this was difficult to confirm with the [$^3$H]$\beta$1H used for this study as it contained ~50% hexomeric $\beta$1H. Scatchard plot analysis of Raji cell $\beta$1H uptake demonstrated the presence of at least two different binding species of this [$^3$H]$\beta$1H preparation, and from this it is assumed that the hexomeric [$^3$H]$\beta$1H had a higher affinity for $\beta$1H receptors than did the monomeric [$^3$H]$\beta$1H in the labeled $\beta$1H preparation (Scatchard plot not shown). Thus far it has been difficult to isolate monomeric $\beta$1H that is totally free of hexomeric $\beta$1H. By criteria other than Scatchard analysis, [$^3$H]$\beta$1H did appear to have a much lower affinity for Raji cell $\beta$1H receptors that it had for EC3b. Elution of [$^3$H]$\beta$1H from EC3b-$\beta$1H required six washes with warm high-ionic strength buffer, whereas [$^3$H]$\beta$1H was easily eluted from Raji cells by two washes with ice-cold isotonic buffer. $\beta$1H receptors were also difficult to detect by rosette assay with $\beta$1H-coated erythrocytes (34). Individual cells with $\beta$1H receptors were more easily identified with an immunofluorescence assay for the uptake of unlabeled $\beta$1H. Double-label studies of normal lymphocytes indicated that $\beta$1H receptors were expressed only on B cells.

$\beta$1H receptors presumably have to be crosslinked on the cell surface to trigger C3bINA release. However, at this time it is unclear whether this requires either aggregated or complex-bound $\beta$1H. Treatment of lymphocytes with 30 $\mu$g/ml of $\beta$1H that was ~90% monomeric and 10% hexomeric induced C3bINA release. In the future, attempts will be made to isolate 100% monomeric $\beta$1H, because it is presumed that plasma $\beta$1H is monomeric and does not trigger $\beta$1H receptors. Alternatively,

β1H isolation procedures may generate an activated form of β1H similar to that which has been described for P (37).

β1H receptor-mediated release of C3bINA may involve a calcium- and energy-dependent transport of C3bINA through the lymphocyte membrane. EDTA did not inhibit the uptake of β1H onto lymphocyte receptors, but β1H-induced C3bINA release was inhibited completely in the presence of either EDTA, magnesium-EGTA, or sodium azide. Because the C3bINA releasing activity of EDTA-treated cells was restored by washing with EDTA-free buffer, it is possible that intracellular stores of calcium restored membrane calcium following removal of EDTA. Alternatively, EDTA and EGTA may inhibit directly by binding to some membrane constituent required for release of C3bINA.

The immunologic significance of β1H-induced release of C3bINA from lymphocytes remains to be established by future investigation. Because β1H and C3bINA are both synthesized and released by lymphocytes (38), it is possible that these two components may play some role in antigen recognition and the immune response. In this regard it should be noted that C3bINA does not cleave all bound C3b molecules with equal efficiency and that C3b bound to alternative pathway-activating surfaces is particularly resistent to C3bINA. This increased resistence of C3b to C3bINA is determined by the relative binding affinity of β1H for C3b, which is regulated by the physicochemical properties of the surface to which C3b is bound (8, 9). Many different strains of bacteria and yeast, as well as certain human tumor cells, have outer wall or membrane structures that function as activating surfaces for the C alternative pathway. When C3b is bound to an alternative pathway-activating surface, β1H has a reduced affinity for C3b that results in diminished C3b cleavage by C3bINA. This allows the uncleaved C3b to become a site for assembly of the amplification convertase, C3b,Bb,P. By contrast, when C3b is bound to normal tissue surfaces, it has a high affinity for β1H and is rapidly degraded by C3bINA to C3bi. Thus, the plasma C system has this mechanism of distinguishing self from nonself, whereby C3b deposited onto self surfaces is degraded into C3bi, whereas C3b deposited onto non-self surfaces is allowed to form the amplification convertase that activates the cytolytic terminal C components. Cells or other C-fixing complexes that are not lysed by this mechanism would then presumably circulate with either C3bi (self) or C3b,Bb,P (non-self) bound to exposed surfaces. Recent studies have suggested that cells of the immune system may have membrane recognition systems capable of responding to C3b,Bb,P-coated non-self substances, and likewise also possess a control mechanism to prevent a response to C3bi-coated self substances. Macrophages have been shown to be activated by factor Bb (39) through cleavage of their surface-bound C5 (40). In addition, macrophages synthesize and release β1H and C3bINA (4) that might potentially degrade the C3b,Bb,P enzyme on normal tissue, thus limiting cell activation to non-self surfaces. Preliminary evidence has now been obtained that B lymphocytes may possess a similar membrane recognition system to distinguish C-activating surfaces (38). Not only do lymphocytes synthesize C3bINA, but they also synthesize β1H (38), factor B, and P (5), and express C5 as an exposed membrane-bound component (3, 38). Cleavage of this surface-bound C5 by exposure to EAC14$^{oxy}$23b or EC3b,Bb,P leads to release of both β1H and C3bINA that convert EC3b to EC3bi (38). Present evidence indicates that this surface C5-mediated mechanism of C3bINA release is probably independent of the β1H receptor-mediated mechanism of C3bINA release.

The same magnesium EGTA buffer that totally inhibited $\beta$1H receptor-mediated C3bINA release had no effect on C5-mediated C3bINA release. Sundsmo (3), who first described lymphocyte surface C5, has recently reported that $F(ab')_2$ anti-C5 induced B cell blastogenesis, whereas Fab-anti-C5 inhibited mixed lymphocyte reactions (41). The relationship of this surface C5-dependent blastogenic response to C5-mediated release of $\beta$1H and C3bINA is presently being investigated.

The possible involvement of the C system in the various stages of antigen trapping, recognition, and lymphocyte activation has been suggested by several previous investigators (42–44). In particular, a role for endogenous factor B and C3bINA was hypothesized by Hartman and Bokisch (45, 46) before the synthesis of these components by lymphocytes had been demonstrated. Future studies will be aimed at further characterizing the components and reactions of the lymphocyte and macrophage C system and how this system relates to the overall functions of these cells in the host defense mechanism.

## Summary

Human bone marrow-derived lymphocytes and cells from B lymphoblastoid lines were shown to have specific membrane receptors for $\beta$1H globulin. Lymphocytes responded to the presence of $\beta$1H by releasing endogenously-synthesized C3b-inactivator. Very little spontaneous release of C3b-inactivator occurred in the absence of $\beta$1H. $\beta$1H-treated lymphocytes that either lacked complement receptor type one ($CR_1$, the C4b-C3b receptor) or had their $CR_1$ blocked with Fab′-anti-$CR_1$ formed rosettes with C3b-coated sheep erythrocytes (EC3b) by adherence to complement receptor type two ($CR_2$, the C3d-C3bi receptor). The mechanism of this $\beta$1H-induced EC3b rosette formation was shown to involve the release of lymphocyte C3b-inactivator that cleaved bound C3b into C3bi forming EC3bi. This lymphocyte-generated EC3bi then bound to $CR_2$, forming rosettes. $\beta$1H-induced EC3b rosettes were completely inhibited by the presence of either anti-C3b-inactivator, $F(ab')_2$-anti-$CR_2$, Fab-anti-C3c, or Fab-anti-C3d, but were unaffected by the presence of fluid-phase concentrations of $\beta$1H up to 5.5 mg/ml or Fab′-anti-$CR_1$. Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography demonstrated that cell-free supernates of $\beta$1H-treated lymphocytes cleaved $^3$H-labeled C3b on EC3b into C3bi. Inhibition studies with chelating agents and sodium azide suggested that the release of C3b inactivator might involve a calcium and energy-dependent transport of this enzyme across the membranes of $\beta$1H-triggered cells. Because plasma $\beta$1H and C3b-inactivator are known to have important functions in the distinction of alternative pathway-activating substances from normal tissue, it is possible that this $\beta$1H receptor-C3b-inactivator releasing system in lymphocytes may have an analogous function.

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