IDENTIFICATION OF A C3bi-SPECIFIC MEMBRANE COMPLEMENT RECEPTOR THAT IS EXPRESSED ON LYMPHOCYTES, MONOCYTES, NEUTROPHILS, AND ERYTHROCYTES*

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Membrane complement (C)[1] receptors specific for different parts of the C3 molecule and for β1H, C5a, and Clq have been described on a variety of different cell types (1). CR_1, specific for C3b and C4b, and CR_2 for C3d have been isolated and shown to be glycoproteins of 205,000 M_r and 72,000 M_r, respectively (2–4). Recently, the β1H receptor also was isolated successfully using an anti-β1H idiotypic antibody (5).[2] Specific antibodies to isolated C receptors indicated that a common structure for CR_1 is shared with erythrocytes, lymphocytes, monocytes, and neutrophils (2, 3) and that CR_2 is restricted to B lymphocytes (4). Because earlier studies had shown that monocytes (6, 7) and neutrophils (8) bound EAC1-3d, it was thought that these phagocytic cell types expressed a C3d-specific receptor that was similar to lymphocyte CR_2. However, it now appears likely that the EAC1-3d reagents used in these previous studies contained bound C3bi and little or no bound C3d. Previously, EAC1-3d were prepared by treatment of EAC1-3b with purified C3b inactivator (C3bINA) because it was believed that C3bINA removed C3c from the complexes, leaving only bound C3d. Subsequently, it was demonstrated that cleavage of fluid-phase C3b with purified C3bINA (9) resulted in formation of the C3bi fragment, and that EACl-3b or EC3b treated with purified C3bINA contained only C3bi (EAC1-3bi or EC3bi) and no C3d (10). Further cleavage of C3bi into C3c and C3d required trypsin (9) or

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[1] *Abbreviations used in this paper:* β1H, essential cofactor for cleavage of fluid-phase C3b by C3b-inactivator (C3bINA) and a potentiator of C3bINA cleavage of bound C3b; BDVEA, 1% bovine serum albumin (BSA), 3.2% dextrose, 35 mM veronal buffer, pH 7.2, with 20 mM EDTA, and 0.2% sodium azide; C; complement; C3b, 181,000 M_r fragment of C3; C3bi, C3bINA-cleaved C3b; C3c, 140,000 M_r fragment resulting from proteolysis of C3bi; C3d, 30,000 M_r fragment of C3bi that remains bound to complexes following proteolysis of bound C3bi; C3e, 10,000 M_r acidic fragment derived from extensively trypsinized C3; C3-ms, C3-coated microspheres; CR_1, C-receptor type one, the C3b-C4b receptor; CR_2, C-receptor type two, the C3d-C3bi receptor; CR_3, C-receptor type three, the C3bi receptor; EAC1-3, antibody-coated sheep erythrocytes containing C3 fixed by way of the classical pathway of C activation; EC3, sheep erythrocytes containing C3 fixed by way of the alternative pathway of C activation; FITC, fluorescein isothiocyanate; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; VBS, veronal-buffered saline.

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plasmin (11), suggesting that if these enzymes were present in serum, EAC prepared with serum might contain bound C3d as well as C3bi or only bound C3d. (Terms are defined in abbreviations list.)

In the present study, various cell types were examined for binding of complexes that contained only C3bi or only C3d. Neutrophils, monocytes, and erythrocytes were found to bind C3bi but not C3d and to express a receptor for C3bi (CR_3) that was distinct from CR_2 and specific for a site contained in the C3bi molecule that was outside of the d region.

Materials and Methods

Leukocytes and Erythrocytes. Heparinized blood was obtained from normal volunteers or patients with leukemia. Tonsils were obtained from patients undergoing routine tonsillectomy. Normal blood mononuclear cells and neutrophils were isolated on a two-step Ficoll-Hypaque density gradient (1.08 g/ml and 1.105 g/ml) (3, 8), and monocytes were either depleted from mononuclear cell fractions with Sephadex G-10 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) (12) or purified on Percoll gradients (13). Immature myeloid cells were isolated from leukemic blood on a six-step Ficoll-Hypaque gradient (8). After two washes with phosphate-buffered saline (PBS), erythrocytes and each leukocyte type were resuspended at 4×10^6 cells/ml in 35 mM veronal buffer, pH 7.2, containing 1% bovine serum albumin (BSA), 20 mM EDTA, 3.2% dextrose, and 0.2% sodium azide (BDVEA; 6 mS at 22°C). Raji and Daudi leukemic lymphoblastoid cells and the BF lymphoblastoid line derived from normal lymphocytes were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics.

Purification of C Components and Preparation of C3 Fragments. C3, factor B, β1H, nephritic factor, and C3bINA were purified as previously described (14, 15). Factor D was purified as described by LaSavre et al. (16). C3b-Sepharose was generated by mixing together 600 mg of C3 in 10 mM EDTA-PBS with a 0.24% weight ratio of trypsin in the presence of 75 ml of activated-thiol Sepharose (Pharmacia Fine Chemicals) in a total volume of 150 ml (17). After 15 min at 37°C, trypsin was inhibited by addition of a threefold molar excess of soybean trypsin inhibitor (STI) and then the disulfide-linked C3b-sepharose was washed three times by centrifugation with ice-cold 10 mM EDTA-PBS. Elution of the C3b-Sepharose with *l*-cysteine demonstrated 6 mg of C3b per ml of gel. C3bi-Sepharose was formed by treatment of the C3b-Sepharose in 20 mM EDTA-DGVB (3.7 mS at 22°C), with a weight ratio of 50% βIH and 4% C3bINA for 6 h at 37°C, followed by four washes with 1.0 M NaCl in PBS. C3d-Sepharose was formed by treatment of C3b-Sepharose with trypsin (17) or elastase. With elastase, 8.5 ml of C3b-Sepharose was treated with an 8% weight ratio of purified porcine elastase (18) in 20 mM Tris/HCl, pH 8.7, for 3 h at 37°C, followed by a second addition of 8% elastase and another 3 h at 37°C. The C3d-Sepharoses formed with each enzyme were then washed three times with ice-cold veronal-buffered saline (VBS). The wash supernatants were then concentrated to 10 ml with a UM-2 membrane (Amicon Corp., Scientific Sys. Div. Lexington, Mass.) and chromatographed on a 5×90-cm column of Sephadex G-75 (Pharmacia Fine Chemicals) in VBS. Four protein (A_{280}) peaks were detected, separately pooled, and concentrated with a UM-2 membrane. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (19) demonstrated that the first peak from the G-75 column contained C3c (20) and the second peak contained trypsin or elastase, whereas no Coomasie Blue-stained protein bands were detectable in the third and fourth peaks with 15% polyacrylamide. These last two small C3 fragment pools contained no detectable intact C3c or C3d by SDS-PAGE. A small C3 fragments preparation was also generated by treatment of water-lysed EC3bi stroma with plasmin-Sepharose. Purified plasminogen (21) was coupled to 5 ml of Sepharose CL-4B (22) at a ratio of 10 mg enzyme per ml of gel and then after activation of a 50% gel suspension with urokinase (14), was mixed on a tube rotator for 60 min at 37°C with 4×10^{10} EC3bi stroma that contained 1.2×10^5 C3bi molecules per cell. The supernatant was then collected by centrifugation, concentrated with a UM-2 membrane, and chromatographed on Sephadex G-75 in a similar manner as were the trypsin and elastase C3 fragments. The C3b-, C3bi-, and C3d-Sepharoses were each eluted with

20 mM cysteine and the liberated C3 fragments dialyzed against BDVEA (prepared without BSA). Analysis of each C3 fragment by SDS-PAGE under reducing conditions with 12% polyacrylamide demonstrated the known M_r chain structures (23):[3] C3b, 115,000 α' and 75,000 β; C3bi, 80% iC3b$_3$ (41,000 α'_4) and 20% iC3b$_2$ (75,000 β, 68,000 α'_1, and 43,000 α'_2); C3c (from elastase digest only), 75,000 β, 43,000 α'_2, and 29,000 α'_3; and C3d, 30,000 α'_5. Furthermore, no C3b was detected in the C3bi nor in the C3c, and no C3d was detected in the C3bi.

Preparation of C3-coated Sheep Erythrocytes (EC3) and Fluorescent Microspheres (C3-ms). EC3b, EC3bi, and EC3d were prepared as previously described (14, 24) and contained 1.5 to 2.5 × 10^4 bound C3 molecules per cell, as determined by the uptake of ^{125}I-monoclonal anti-C3 (Bethesda Research Laoraories, Rockville, Md.). Coumarin (green) and rhodamine (red) stained fluorscent 0.9-μm Diam microspheres (Covalent Technology Corp., Redwood City, Calif.) were coated with isolated C3b, C3bi, and C3d fragments forming C3b-ms, C3bi-ms, and C3d-ms (25). 300 μl of a 1.4% suspension of microspheres in PBS were mixed with 100 μl of C3b, C3bi, or C3d (400 μg/ml) and incubated at 25°C for 1 h on a tube rotator. The C3-ms were then washed three times with 1% BSA/PBS by centrifugation for 10 min in a Beckman Microfuge (Beckman Instruments, Inc., Palo Alto, Calif.), resuspended in 3.0 ml of BDVEA containing 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated briefly until a single particle suspension was obtained.

Preparation of Antibodies Specific for CR$_1$, CR$_2$, C3c, and C3d. Rabbits were immunized with purified CR$_1$ (26), CR$_2$ (4), trypsin-generated C3c and C3d (14), and the F(ab')$_2$, Fab', or Fab fragments of the isolated IgG antibodies were prepared by pepsin or papain cleavage (3, 4, 14).

Assay of C Receptors. C receptors were assayed by rosette formation with EC3 (1) or C3-ms in BDVEA. For C3-ms rosette assay, 100 μl of cells at 4 × 10^6 cells/ml were mixed with 100 μl of C3-ms in a 10 × 75-mm plastic tube and placed on a tube rotator with horizontal axis for 15 min at 37°C. Alternatively, the 200-μl mixture of cells and C3-ms were pelleted together at 1,000 *g* for 5 min and incubated as a pellet for 5 min at 37°C. Next, the unbound C3-ms were removed from the cell suspension (or resuspended cell pellet) by layering the 200 μl of cells onto 4 ml of 6% BSA in PBS in another 10 × 75-mm plastic tube and centrifuging at 200 *g* for 10 min at room temperature. After aspiration of the supernatant, the cell pellet was resuspended in residual wash fluid by shaking the tube gently, and the cells were examined for bound fluorescent beads by standard fluorescence microscopy techniques. With leukocytes, cells binding five or more C3-ms were considered positive, whereas with erythrocytes, a positive cutoff of three or more bound C3-ms was used.

For assay of the morphology of immature myeloid cell EC3 rosettes, Wright-Giemsa stained smears of rosette suspensions were prepared and analyzed as previously described (8).

Assay for C Receptor Specificity. A pellet of 4 × 10^5 C-receptor cells in a 10 × 75-mm plastic tube was resuspended in 100 μl of either BDVEA or F(ab')$_2$ anti-CR$_1$ (1 mg/ml), F(ab')$_2$-anti-CR$_2$ (3 mg/ml), C3b (1.0 mg/ml), C3bi (0.7 mg/ml), elastase-generated C3c (2.5 mg/ml), or trypsin-generated C3d (0.5 mg/ml) diluted in BDVEA, incubated at 37°C for 10 min, and assayed for C-receptors by addition of 100 μl of EC3 or C3-ms in BDVEA. Alternatively, pellets of 2 × 10^7 EC3 were treated with 100 μl of BDVEA or Fab anti-C3c (100 μg/ml) or Fab anti-C3d (3 mg/ml) in BDVEA, incubated for 20 min at 37°C, and then tested for rosette formation with 100 μl of C-receptor cells.

Assay for Enhancement of EC3bi *Rosette Formation by Protease Inhibitors and Anti-Elastase.* Cell pellets of 4 × 10^5 leukocytes in 10 × 75-mm plastic tubes were resuspended in 100 μl of various concentrations of either protease inhibitors or rabbit IgG anti-human neutrophil elastase (kindly provided by Dr. John Spitznagel, Emory University, Atlanta, Ga.), previously absorbed six times with a 10% packed volume of sheep erythrocytes. Next, each inhibitor or anti-elastase treated cell suspension was assayed for EC3bi rosette formation in the usual manner.

Double-Label Assay of Lymphocytes for CR$_3$ and Surface Immunoglobulin (Ig), or Leu-1 and 3A1 T Cell Antigens, or OKM-1 Monocyte-Null Cell Determinant. A pellet of 1 × 10^6 lymphocytes was treated simultaneously for 20 min at room temperature with 25 μl of F(ab')$_2$-anti-CR$_2$ (3 mg/ml) and

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[3] Ross, G. D., and J. D. Lambris. Identification of three forms of iC3b that have distinct structures and binding site properties. Proceedings of IX International Complement Workshop. *J. Immunol.* In press.

a fluorescein isothiocyanate (FITC)-linked stain specific for either surface Ig, Leu-1 or 3A1 T cell antigens, or OKM-1 monocyte-null cell determinant, and then examined for rosette formation with rhodamine-stained C3bi-ms. For Ig staining, cells were treated with 25 μl of F(ab')$_2$-anti-IgM, IgD, IgA, IgG-fluorescein (N. L. Cappel Laboratories, Cochranville, Pa.). For T cell staining, cells were treated with a 45-μl mixture containing 2.0 μg of protein-A-FITC (Pharmacia Fine Chemicals) and 1.0 μg of either mouse IgG-anti-Leu-1 (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, Calif.), mouse IgG-anti-3A1 (27) (kindly donated by Dr. George Eisenbarth, Duke University, Durham, N. C.), or mouse IgG-anti-OKM-1 (Ortho Pharmaceutical, Raritan, N. J.). Next, to enhance the monoclonal antibody FITC staining, the washed cells were stained in addition with FITC-F(ab')$_2$-anti-mouse IgG (N. L. Cappel Laboratories). Finally, the fluorescein-stained cells were resuspended in 100 μl of BDVEA and assayed for CR$_3$ by addition of 100 μl of rhodamine-C3bi-ms. Cells were examined sequentially for fluorescein and/or rhodamine staining. In each case the anti-CR$_2$-treated cells were also tested for complete absence of the ability to rosette with C3d-ms.

Results

Binding of C3bi Complexes to Various Leukocyte Types and Erythrocytes. Both EC3bi and C3bi-ms bound to a proportion of lymphocytes, erythrocytes, neutrophils, and monocytes (Table I). In all cases, C3bi-ms bound to a greater percentage of cells than did EC3bi. The increased binding of C3bi-ms was particularly apparent with erythrocytes, 92% of which bound C3bi-ms, and 10% or less bound EC3bi. Only lymphoid cells bound C3d complexes. Immature monocytes and myeloid cells isolated from patients with either acute monocytic leukemia or chronic myelogenous leukemia were also negative for binding of C3d complexes.

Specificity of C3bi-dependent Rosette Formation. The specificity of C3bi complex binding was examined by assays for inhibition of rosette formation, either by treatment of the C receptor cells with Fab' anti-C-receptor antibodies or fluid-phase C3 fragments (Table II) or by treatment of the C3 complexes with Fab anti-C3c or Fab anti-C3d antibodies (Table III). With all cell types, C3b-ms rosettes were inhibited by anti-CR$_1$, fluid-phase C3b and fluid-phase C3c but not by anti-CR$_2$, fluid-phase C3bi, or fluid-phase C3d. By contrast, C3bi-ms rosettes were not inhibited by anti-CR$_1$, fluid-phase C3b, or fluid-phase C3c. Thus, neither C3bi-ms nor fluid-phase C3bi bound to CR$_1$ on any cell type. With erythrocytes, neutrophils, and monocytes, C3bi-ms rosettes were inhibited by fluid-phase C3bi but not by anti-CR$_1$, anti-CR$_2$, fluid-phase C3b, C3c, or C3d. Therefore, with these nonlymphoid cell types, C3bi-ms were bound to

TABLE I

EC3 and C3-ms Rosette Formation with Lymphocytes, Erythrocytes, Neutrophils, and Monocytes

	EC3b	C3b-ms	EC3bi	C3bi-ms	EC3d	C3d-ms
	%	%	%	%	%	%
Blood lymphocytes	16	17	10	12	7	9
Lymphoblastoid lines						
Raji	0	0	99	100	98	100
Daudi	0	0	84	96	86	95
BF	98	100	98	99	97	98
Erythrocytes	75	95	10	92	0	0
Neutrophils	95	100	75*	89*	0	0
Monocytes	85	95	84	91	0	0

* Assayed in the presence of 1.0 mg/ml of STI.

TABLE II

Inhibition of C3-ms Rosettes by Anti-C3 Receptor Antibodies and Fluid-Phase C3 Fragments

C receptor cell type	C3 complex	Inhibition of rosette formation by					
		Anti-CR_1	Anti-CR_2	Fluid-phase*			
				C3b	C3bi	C3c	C3d
		%	%	%	%	%	%
Erythrocytes	C3b-ms	100	0	100	0	48	0
	C3bi-ms	0	0	0	85	0	0
Neutrophils	C3b-ms	100	0	62	0	32	0
	C3bi-ms	0	0	0	80	0	0
Monocytes	C3b-ms	100	0	60	0	27	0
	C3bi-ms	0	0	0	50	0	0
Raji lymphoblasts	C3bi-ms	0	78	0	100	0	71
	C3d-ms	0	100	0	100	0	100

* C3b, 500 μg/ml; C3bi, 350 μg/ml; C3c (elastase), 1.25 mg/ml; C3d (trypsin), 250 μg/ml.

TABLE III

Inhibition of EC3 Rosettes with Fab-Anti-C3c and Fab-Anti-C3d

Cell type	EC3 type	Inhibition of rosettes by	
		Anti-C3c	Anti-C3d
		%	%
Erythrocyte	EC3b	100	0
	EC3bi	100	100
Neutrophils and	EC3b	100	0
Monocytes	EC3bi	100	100
Raji or Daudi	EC3bi	100	100
Lymphoblasts	EC3d	0	100

a receptor that was distinct from CR_1 and CR_2, herein designated CR_3. Lymphocytes differed from other cell types in that C3bi-ms rosettes were partially inhibited by anti-CR_2 and fluid-phase C3d. Also, lymphocyte-C3d-ms rosettes were completely inhibited by fluid-phase C3bi as well as by anti-CR_2 or fluid-phase C3d. Thus, with lymphocytes that expressed CR_2, C3bi complexes were bound to CR_2 by way of the d region of the intact C3bi molecule. However, with concentrations of up to 5 mg/ml of Fab′ anti-CR_2 or 1.0 mg/ml of fluid-phase C3d, lymphocyte C3bi complex rosettes were never inhibited completely. This indicated that lymphocytes expressed CR_3 in addition to CR_2 and that both C receptor types were responsible for binding C3bi complexes to lymphocytes.

Fab anti-C3c and Fab anti-C3d both inhibited EC3bi rosette formation with all cell types (Table III). Fab anti-C3d did not inhibit EC3b rosette formation, despite the finding that Fab anti-C3d inhibited the agglutination of EC3b by IgG anti-C3d and thus bound to the d region of intact C3b.

Because CR_3-dependent rosettes were not inhibited by fluid-phase C3c or C3d, other smaller C3 fragments generated by proteolysis of C3b or C3bi were examined for inhibition of C3bi-ms rosette formation. Inhibition of neutrophil-EC3bi rosettes and human E-C3bi-ms rosettes was observed with the fluid-phase small C3 fragments pool generated with plasmin or trypsin but not with elastase.

Inhibition of C3bi-dependent Neutrophil Rosette Formation by Secreted Neutrophil Elastase. Because of the known proteolytic sensitivity of C3bi (9) and because the bound product of C3bi digestion, C3d, was unreactive with neutrophils and monocytes, protease inhibitors were added to rosette assays to protect the C3bi-complexes from proteolysis into CR_3-unreactive C3d complexes (Table IV). Both STI and PMSF enhanced neutrophil-EC3bi rosette formation from 5% up to 76–89%, whereas benzamidine and epsilon amino caproic acid caused no rosette enhancement. These same protease inhibitors had no effect on EC3bi rosette formation with blood monocytes or lymphocytes. Because neutrophils were known to secrete elastase in response to opsonized bacteria (28) and because human neutrophil elastase was known to cleave C3bi into C3c and C3d (20), an antibody to human neutrophil elastase was examined for its ability to enhance neutrophil-EC3bi rosette formation (Table IV). Anti-elastase produced the same rosette enhancement as did STI and PMSF. In the absence of protease inhibitors or anti-elastase, EC3bi that had been incubated with neutrophils for 60 min at 37°C were no longer agglutinated by anti-C3c, whereas agglutination by anti-C3d and Raji rosette formation were undiminished.

Acquisition of Elastase-secreting Ability with Neutrophil Maturation. Neutrophils from the blood of a patient with chronic myelogenous leukemia and blood count of 2×10^5 neutrophils per μl were fractionated into immature (band-form nucleus) and mature polymorphonuclear cells and examined for EC3bi rosette formation with increasing concentrations of STI (Table V). In the absence of STI, 49% of band-form neutrophils formed rosettes with EC3bi, whereas high-density polymorphs did not form EC3bi

TABLE IV

Enhancement of Neutrophil-EC3bi Rosette Formation with Protease Inhibitors and Anti-Elastase

Protease inhibitor	Neutrophil-EC3bi rosette formation
	%
Buffer control	5
STI	
1.0 mg/ml	88
0.5 mg/ml	89
0.25 mg/ml	70
0.10 mg/ml	35
0.05 mg/ml	25
PMSF	
2.0 mM	36
1.0 mM	76
0.5 mM	40
Anti-elastase	
1/5	87
1/10	87
1/20	79
1/40	38

TABLE V

Neutrophil Cell Density and Maturation-linked Requirement for Increased Amounts of STI to Allow EC3bi Rosette Formation

	Neutrophil density in g/ml (predominant morphology)			
	1.07 (bands)	1.09 (PMN)	1.105 (PMN)	1.12 (PMN)
	*%R**	*%R*	*%R*	*%R*
Buffer control	49	40	10	0
STI				
25 μg/ml	51	54	20	10
50 μg/ml	50	70	25	19
200 μg/ml	50	69	49	39
400 μg/ml	51	68	92	68
800 μg/ml	49	71	95	97
1 mg/ml	51	69	94	98

* Percent EC3bi rosette formation.

TABLE VI

Expression of CR_3 on Lymphocytes Detected by C3bi-ms Rosette Formation with Anti-CR_2-treated Cells

Cell type	Rosette formation with	
	C3d-ms	C3bi-ms
	%	%
Blood lymphocytes (8)	9	12
+ anti-CR_2	0	3.5
Tonsil lymphocytes (3)	54	59
+ anti-CR_2	0	27
Raji lymphoblasts	99	99
+ anti-CR_2	0	40
Daudi lymphoblasts	95	96
+ anti-CR_2	0	32
BF lymphoblasts	59	56
+ anti-CR_2	0	9

rosettes. With the band-form neutrophils, STI did not increase the proportion of EC3bi rosettes. However, with neutrophils isolated at a 1.09 g/ml density, 50 μg/ml STI was required for maximum enhancement of EC3bi rosette formation, whereas with 1.105 g/ml neutrophils and 1.12 g/ml neutrophils, 400 μg/ml and 800 μg/ml of STI were required, respectively (Table V).

Expression of CR_3 on Different Lymphocyte Subsets. Lymphocytes from blood and tonsils and various B type lymphoblastoid lines were treated with sufficient anti-CR_2 to inhibit C3d-ms binding completely and then assayed for binding of C3bi-ms (Table VI). The majority of C3bi-ms-binding cells expressed only CR_2 and did not express CR_3, as anti-CR_2 treatment of cells produced 54–84% inhibition of C3bi-ms rosette formation. Among normal blood lymphocytes, only 1.5–4.5% (average 3.5%) of cells expressed CR_3, and these were apparently distinct from the CR_2-bearing cells that represented 9.0% of peripheral lymphocytes. Double-label assays with blood lymphocytes from six normal individuals demonstrated that 86% of cells bearing CR_2 or CR_3 also expressed membrane Ig detectable with $F(ab')_2$-anti-Ig. No CR_2-positive cells were detected that expressed either Leu-1 or 3A1 T cell determinants. Among the CR_3-positive blood lymphocytes, 5% of cells stained with either anti-Leu-1 or anti-3A1, and 16% of cells stained with anti-OKM-1. Tonsils contained a considerably higher proportion of CR_3-bearing cells than did peripheral blood. However, unlike blood lymphocytes, the majority of tonsil CR_3-positive cells expressed CR_2 because the percentage of cells binding either C3bi-ms or C3d-ms was nearly equal. All three B type lymphoblastoid cell lines examined expressed CR_3 on a proportion of the cells.

Discussion

The major finding in the present study is that lymphocytes, monocytes, neutrophils, and erythrocytes express a C3bi-specific membrane C receptor (CR_3) that is distinct from CR_1 and CR_2. CR_3 is specific for C3bi and unreactive with C3b, C3c, and C3d. Neutrophils and monocytes lack detectable CR_2 at all stages of maturation and bind C3bi complexes exclusively to CR_3. Neutrophils begin to express CR_3 at the myelocyte stage and the receptor is fully expressed on polymorphs. Peripheral blood lymphocytes bind C3bi complexes primarily to CR_2, and the cells that express CR_3 are a separate B cell subset from the CR_2-bearing B cells.

C receptors were assayed with either sheep erythrocytes or fluorescent microspheres coated with specific C3 fragments (EC3 or C3-ms). C3-ms had distinct advantages over EC3. First, probably because of their smaller size, C3-ms were more sensitive to cells known to have a low number of C-receptors per cell. C3b-ms and C3d-ms bound to nearly all human erythrocytes and Daudi cells respectively, whereas EC3b and EC3d bound to fewer of these two cell types. Also, C3-ms could be prepared with very small amounts of pure C3 fragments that had been previously characterized fully by SDS-PAGE.

The receptor specificity of C3 complex binding to different cell types was investigated by rosette inhibition studies in which either the C receptor cells were treated with anti-CR_1, anti-CR_2, fluid-phase C3b, C3bi, C3c, or C3d, or alternatively the C3 complexes were treated with anti-C3c or anti-C3d. In experiments that examined the binding specificity of C3b complexes or fluid-phase C3b, it was essential to use inhibitors of proteolysis of C3b in the rosette assay buffer. Lymphocytes (14, 29), monocytes (30, 31), and neutrophils (32) secrete endogenous β1H and C3bINA that may convert bound or fluid-phase C3b into C3bi. Neutrophils also secrete elastase that may cleave C3b into C3d (20). EDTA and sodium azide were used in the rosetting buffer (BDVEA) because they had been shown previously to inhibit the release of B cell C3bINA and β1H (14) and also seemed to inhibit monocyte and neutrophil release of these components. In addition, STI was added to the BDVEA buffer to inhibit neutrophil elastase activity. C3bi complexes did not bind to CR_1 because C3bi-ms rosettes were not inhibited by anti-CR_1, fluid-phase C3b, or fluid-phase C3c, whereas these same materials did inhibit C3b-ms binding to CR_1. In addition, isolated fluid-phase ^{125}I-labeled CR_1 does not bind to EC3bi, whereas fluid-phase CR_1 does bind to EC3b (3). The C3bi complex binding activity of erythrocytes, monocytes, and neutrophils was also distinct from CR_2 activity because these cell types did not bind C3d complexes, nor were C3bi-ms rosettes inhibited by anti-CR_2 or fluid-phase C3d. Lymphocytes differed from these other cell types in that C3bi complexes were bound primarily to CR_2. Lymphocyte binding of C3bi-ms was inhibited by anti-CR_2 and fluid-phase C3d as well as by fluid C3bi. Furthermore, lymphocyte-C3d-ms rosette formation was inhibited completely by fluid-phase C3bi as well as by fluid C3d and anti-CR_2. A portion of lymphocytes did bind C3bi complexes independently of CR_2 because treatment of lymphocytes with amounts of $F(ab')_2$ anti-CR_2 or fluid-phase C3d that were twofold to fourfold greater than that required for complete inhibition of C3d-ms binding only produced 50–84% inhibition of C3bi-ms binding. Thus, lymphocytes bound C3bi complexes either to CR_2 by way of the d region of the intact C3bi molecule or by way of a distinct C3bi-specific receptor that was distinct from CR_2. The C3bi-specific binding activity that was distinct from CR_2 was designated CR_3 with all cell types.

Because C3b does not bind to CR_3, the CR_3 binding site in the C3bi molecule must be exposed by cleavage of C3b with the C3bINA. Thus, CR_3 has a similar binding specificity as bovine conglutinin (33). Unlike conglutinin (34) however, CR_3 activity was not inhibited by EDTA or *n*-acetyl-D-glucosamine (G. D. Ross, unpublished observation). Because neither fluid-phase C3b, C3c, nor C3d inhibited the CR_3-binding activity of C3bi-ms, the CR_3 binding site in the C3bi molecule must be either destroyed, liberated, or covered by proteolysis of C3bi. To determine whether a small CR_3-specific fragment could be generated that was distinct from intact C3c and C3d, bound C3b or C3bi was digested into C3c and C3d with elastase, trypsin, or plasmin, and then after removal of the intact C3c and C3d fragments, the remaining small fragment pools were examined for inhibition of C3bi complex binding to neutrophil or erythrocyte CR_3. Inhibition of CR_3 was demonstrated by the small C3 fragments pool generated with plasmin and trypsin but not with elastase. Elastase is known to have a more limited number of digestion sites in the C3 molecule than has either plasmin or trypsin (11, 20, 23). In particular, elastase digests only the α chain of C3 or C3b (20), whereas trypsin cleaves both the α and the β chains of C3 or C3b, and plasmin cleaves both the α and β chains of C3bi (11). Because none of the three proteases digest C3d, the data suggests the possibility that the CR_3 binding site may be folded within the intact C3c fragment and not exposed until cleaved from the molecule with trypsin or plasmin. Likewise, the CR_1 binding site is apparently folded within the C3bi molecule and then re-exposed in the C3c fragment that is excised by proteolysis of C3bi. This is because fluid C3c but not fluid C3bi inhibited CR_1-C3b-ms rosette formation. Available data (35, 36) suggest the possibility that the C3e fragment may contain the CR_3 binding site. C3e was shown to be removed from C3c by extensive trypsin digestion, and ^{125}I-C3e was shown to bind to neutrophils (36).

Previously, it had been shown (8) that neutrophils acquired the ability to bind EAC1-3bi at approximately the myelocyte stage of maturation, and then as the cells matured into polymorphonuclear cells this ability was lost. In these former studies, an EAC1-3d reagent was used that is now recognized to have actually been an EAC1-3bi reagent because it was prepared with purified C3bINA without the additional proteolysis required to cleave the bound C3bi fully to C3d. The present study demonstrated that the loss of ability of mature neutrophils to rosette with EC3bi (or EAC1-3bi) was not due to a loss of CR_3 but rather to the maturation-linked acquisition of the ability to secrete elastase that cleaved the reagent EC3bi into CR_3-unreactive EC3d. Several lines of evidence supported this conclusion. First, EC3bi rosette formation with mature neutrophils was generated with an antibody directed to human neutrophil elastase. STI and PMSF also allowed EC3bi rosette formation with mature neutrophils. Second, in the absence of protease inhibitors, EC3bi that had been incubated with neutrophils lost all detectable C3c antigens while retaining C3d antigens and the ability to bind to lymphocyte CR_2. This indicated that neutrophil enzymes cleaved EC3bi to EC3d. Finally, when neutrophils were fractionated into cells with band form nucleus and polymorphonuclear cells, it was found that STI did not enhance EC3bi rosette formation with band form cells, whereas with polymorphs, more STI was required to allow EC3bi rosettes with high density (1.12 g/ml) mature polymorphs than with low density (1.09 g/ml) less mature polymorphs. Elastase has been detected in azurophilic myeloid cell granules by immunofluorescence at the promyelocyte stage of maturation (37). However, promyelocyte elastase is probably not secreted, and polymorphs apparently release azurophilic granule enzymes only at the site of contact with serum-opsonized bacteria (28). Because binding of opsonized bacteria probably involves Fc receptors, CR_1, and CR_3, one of these three types of receptors on mature cells may have the ability to trigger elastase secretion. Because of this elastase-secreting activity, neutrophils in vivo are probably unable to bind particles that contain only C3bi, and therefore it is presumed that CR_3 is not important for neutrophil phagocytosis. Because CR_3 apparently binds a small trypsin- or plasmin-derived C3bi fragment, it is possible that such an active fragment may be generated by C3bi proteolysis in vivo, and that this fragment may be responsible for triggering some neutrophil function other than phagocytosis.

Monocytes resembled neutrophils in that they expressed CR_1 and CR_3 and lacked detectable CR_2. Unlike neutrophils, monocytes did not require protease inhibitors to allow EC3bi rosette formation. Furthermore, other studies have demonstrated that human macrophages (31) and rat mast cells (38) ingest C3bi complexes much more efficiently than C3b complexes. Thus, these other phagocyte types differ from neutrophils in that CR_3 appears to be more important than CR_1 for phagocytosis in vivo.

CR_3 were also detected on the majority of human erythrocytes. In the past, human erythrocytes were thought to express only CR_1 and not to bind C3bi or C3d complexes. Gaither et al. had noted reduced human erythrocytes immune adherence with EAC43bi as compared with EAC43b (39). However, it was not clear whether this C3bi-dependent immune adherence was due to a distinct C3bi-specific receptor or rather a low affinity binding of C3bi to CR_1. Indeed, human E-rosette formation with EC3bi or EAC1-3bi is difficult to demonstrate, as it is such a weak reaction. In the present study, C3bi-ms were prepared with purified C3bi fragments containing no detectable C3b by SDS-PAGE and were shown to bind to nearly all human erythrocytes in the presence of amounts of anti-CR_1 that were sufficient to inhibit C3b-ms binding completely.

Cells of the human renal glomerulus apparently also express both CR_1 and CR_3. Carlo et al. have demonstrated that kidney cells bind both EAC43b and EAC43bi but not EAC43d (40). Also, it has recently been demonstrated that renal epithelial cells are fluorescence stained with $F(ab')_2$ anti-CR_1 (M. Papamichail, J. D. Lambris, and G. D. Ross, unpublished observation).

Lymphocytes differed from all the other cell types examined in that they expressed CR_2 in addition to CR_3, and C3bi complexes were primarily bound to CR_2 rather than to CR_3. For this reason, specific assay of lymphocyte CR_3 required complete blockade of membrane CR_2 with anti-CR_2 before assay of CR_3 with EC3bi or C3bims. With peripheral blood lymphocytes, only 3.5% of anti-CR_2-treated cells bound C3bi-ms. This finding indicated that the majority of the 12.0% of C3bi-ms-binding cells did not express CR_3 and expressed only CR_2. Parallel assay of C3d-ms-binding cells confirmed that 9.0% of cells expressed CR_2, so that the 12.0% C3bi-ms-binding cells consisted of 8.5% CR_2^+ CR_3^- cells, 3.0% $CR_2^-CR_3^+$ cells, and only 0.5% CR_2^+ CR_3^+ cells. Thus, CR_2^+ and CR_3^+ cells represented nearly distinct subsets. Previous double-label studies with EAC1-3bi (41) and present studies with C3bi-ms indicated that the majority of CR_2^+ and/or CR_3^+ peripheral blood cells expressed membrane Ig detectable with $F(ab')_2$-anti-Ig. The same finding was made when CR_2^+ cells or CR_3^+ cells were examined individually for Ig with C3d-ms or anti-CR_2 and C3bi-ms, respectively. Among CR_3^+ blood nonadherent lymphoid-appearing cells, an average of 86% of cells expressed membrane Ig, whereas only 5% of these cells expressed either Leu-1 or 3A1 T cell-specific determinants, and 15% expressed OKM-1 determinants. The OKM-1 staining CR_3^+ cells probably represented either third population lymphocytes (null cells), myeloid precursors, or promonocytes (42). Thus, the majority of CR_3-bearing blood lymphocytes are B cells (3.0%), whereas only 0.2% express T cell determinants and 0.6% express a monocyte-null lymphocyte determinant. Tonsil lymphocytes differed from blood lymphocytes in that one-half of the CR_3^+ cells also expressed CR_2. This was because the proportions of C3bi-ms- and C3d-ms-binding cells were nearly equal, and anti-CR_2 produced only 50% inhibition of C3bi-ms rosette formation. As with blood lymphocytes, the majority of CR_3^+ tonsil cells expressed membrane Ig. Three different B type lymphoblastoid lines also expressed CR_3^+ cells, though with all three lines, C3bi complexes were primarily bound to CR_2. In previous studies of three different T cell lymphoblastoid lines and several different lines of normal activated T cells maintained in T cell growth factor, no cells binding C3b, C4b, C3bi, or C3d complexes were observed (G. D. Ross and G. D. Bonnard, unpublished observation). The only exception was the MOLT-4 T cell lymphoblastoid line (43). Taken together, the various data indicate that CR_3 is primarily a B cell marker. Recently, Perlmann et al. (44) have reported that the activity of lymphocytes functional in antibody-dependent cellular cytotoxicity (ADCC) was greatly enhanced by target cell-bound C3bi, whereas less enhancement was observed with bound C3b and C3d. Because it was also shown in other studies that ADCC lymphocytes lacked detectable membrane Ig determinants (45) and expressed either T cell (46, 47) or null cell markers (48), it appears possible that the small number of $Ig^-CR_3^+$ cells detected in the present study may represent the cells functional in ADCC.

The significance of a C3bi-specific receptor is not fully understood. Subsequent to C activation, both bound and fluid-phase C3b are rapidly converted into C3bi, and some of this C3bi apparently persists in serum for several hours (10) before being degraded into smaller C3 fragments. Macrophages (31) and mast cells (38) ingest C3bi complexes much more efficiently than C3b complexes. Also, bound C3bi enhances both neutrophil phagocytosis of IgG-coated particles and the neutrophil superoxide burst (32). However, this C3bi-dependent enhancement of neutrophil function requires addition of an inhibitor of elastase, suggesting that bound C3bi may not have these functions in vivo. With the exception of ADCC cells, no function of lymphocyte CR_3 has yet been demonstrated. Because C3bi can bind to either CR_2 or CR_3, it may be possible that C3bi complexes can simultaneously crosslink B cell surface CR_2 and CR_3 and thereby induce some particular cell function. Future studies in which lymphocytes are cultured with C3bi-ms with or without Fab′-anti-CR_2 might be able to answer this question.

Summary

Cells expressing a membrane C receptor (CR_3) specific for C3b-inactivator-cleaved C3b (C3bi) were identified by rosette assay with C3bi-coated sheep erythrocytes (EC3bi) or C3bi-coated fluorescent microspheres (C3bi-ms). C3bi-ms, probably because of their smaller size, bound to a higher proportion of cells than did EC3bi. C3bi-ms bound to >90% of mature neutrophils, 85% of monocytes, 92% of erythrocytes, and 12% of peripheral blood lymphocytes. Binding of C3bi-ms to neutrophils, monocytes, and erythrocytes was inhibited by fluid-phase C3bi, Fab anti-C3c, or Fab anti-C3d but was not inhibited by $F(ab')_2$ anti-CR_1 (C3b receptor) or $F(ab')_2$ anti-CR_2 (C3d receptor) nor by fluid-phase C3b, C3c, or C3d. This indicated that monocytes, neutrophils, and erythrocytes expressed C3bi receptors (CR_3) that were separate and distinct from CR_1 and CR_2 and specific for a site in the C3 molecule that was only exposed subsequently to cleavage of C3b by C3b inactivator and that was either destroyed, covered, or liberated by cleavage of C3bi into C3c and C3d fragments. Lymphocytes differed from these other cell types in that they expressed CR_2 in addition to CR_3. Lymphocyte C3bi-ms rosettes were inhibited from 50 to 84% by $F(ab')_2$-anti-CR_2 or fluid-phase C3d, whereas C3d-ms rosettes were inhibited completely by $F(ab')_2$ anti-CR_2, fluid-phase C3bi, or fluid-phase C3d. Thus, with lymphocytes, C3bi was bound to CR_3, and in addition was bound to CR_2 by way of the intact d region of the C3bi molecule. In studies of the acquisition of C receptors occurring during myeloid cell maturation, the ability to rosette with C3bi-coated particles was detected readily with immature low-density cells, whereas this ability was nearly undetectable with high density mature polymorphonuclear cells. This absence of C3bi binding to polymorphs was not due to a loss of the CR_3 but instead was due to the maturation-linked acquisition of the abiity to secrete elastase that cleaved reagent particle-bound C3bi into CR_3-unreactive C3d. Neither neutrophils nor monocytes bound C3d-coated particles at any stage of maturation. Assay of CR_3 with mature neutrophils required inhibition of neutrophil elastase with either soybean trypsin inhibitor or anti-elastase antibodies, and the amounts of these elastase inhibitors required to allow EC3bi rosette formation increased with neutrophil maturation. Because lymphocytes bound C3bi to CR_2 as well as to CR_3, specific assay of lymphocyte CR_3 required saturation of membrane CR_2 with Fab′ anti-CR_2 before assay for rosettes with C3bi-ms. Only 3.5% of anti-CR_2-treated peripheral blood lymphocytes bound C3bi-ms. Therefore, among normal blood lymphocytes the majority of the 12% C3bi-ms-binding cells expressed only CR_2 (8.5%), and the small proportion of C3bi-ms-binding cells that expressed CR_3 (3.5%) represented a distinct subset from the CR_2^+ cells. Double-label assay indicated that 3.0% out of 3.5% of these CR_3-bearing lymphocytes were B cells because they expressed membrane immunoglobulins. Of the remaining CR_3^+ cells, 0.2% expressed either Leu-1 or 3A1 T cell antigens, and 0.6% expressed the OKM-1 monocyte-null lymphocyte determinant.

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