MEMBRANE DISTRIBUTION AND ADSORPTIVE
ENDOCYTOSIS BY C3b RECEPTORS ON HUMAN
POLYMORPHONUCLEAR LEUKOCYTES*

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A most critical reaction in the complement system for the elaboration of its biologic effects is the cleavage of the third component, C3, whose major fragment, C3b, can covalently bind (1) to target particles and soluble complexes. The bound C3b, or its degradation products, C3bi and C3d, can then mediate attachment of the target material to erythrocytes (2) and immunologic effector cells, such as B lymphocytes (3, 4), macrophages (5, 6), and polymorphonuclear leukocytes (PMN),[1] which have specific membrane receptors for these fragments of C3. These adherence reactions have been shown to promote, but generally not to mediate, the phagocytosis of bound material by PMN and macrophages (7–10), to induce secretion of a chemotactic lymphokine by B lymphocytes (11), and to have roles in the development of B lymphocytes into memory cells (12) and antibody-secreting cells (13–15).

The receptor for C3b on human erythrocytes has been isolated and identified as a glycoprotein of ~205,000 mol wt (16, 17), a finding that has recently been confirmed (18). Its identity as the C3b receptor also of PMN, monocytes, and B lymphocytes was established when monospecific rabbit antibody to the purified erythrocyte glycoprotein inhibited the capacity of these cells to form rosettes with sheep erythrocytes bearing C3b by binding to an externally oriented membrane protein of 205,000 mol wt on each of these cell types (17, 18). The possibility that the C3b receptor was associated with specialized regions of the plasma membrane was suggested by the clustered distribution of fluorescein-conjugated Fab' anti-C3b receptor on PMN and monocytes, and by the apparent absence of lateral diffusive motion of the receptors as assessed by fluorescence photobleaching recovery experiments (19). Because other types of membrane receptors that exhibit clustered distributions, such as low density

[1] *Abbreviations used in this paper:* C3b-OR, the major cleavage fragment of C3 that is bound to constitutents of sheep erythrocyte membranes; DGVB⁺⁺, GVB containing 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and an equal volume of 5% dextrose; DMSO, dimethyl sulfoxide; E, erythrocyte; GVB, veronal-buffered saline containing 0.1% gelatin; HBSS-BSA, Hanks' balanced salt solution containing 2 mg/ml bovine serum albumin; P, properdin; PMN, polymorphonuclear leukocyte; TRITC, tetramethylrhodamine isothiocyanate.

lipoprotein receptors on fibroblasts (20), mediate adsorptive endocytosis of their ligands, a similar functional capacity for C3b receptors on PMN was sought.

Materials and Methods

Preparation of Antibodies.   Antibody to human C3b receptor was prepared by immunizing a rabbit with C3b receptor that had been purified to homogeneity from detergent-solubilized human erythrocyte membrane proteins (16, 17). The monospecificity of this rabbit antibody for the C3b receptor has been previously established and is based on the following observations. The antibody inhibits the capacities of human erythrocytes, PMN, monocytes, and B lympho-cytes to form rosettes with sheep erythrocytes (E) bearing C3b, and has no effect on C3bi and C3d receptor-dependent reactions; there is specific binding of the antibody by these four cell types expressing C3b receptors and not by peripheral blood T lymphocytes; and the antibody immunoprecipitates a single membrane protein of 205,000 mol wt from cells expressing C3b receptors (17). Antiserum to C3 was prepared by immunization of a rabbit with purified C3 (21). The IgG fractions of the rabbit antisera and of nonimmune rabbit serum were isolated by ammonium sulfate precipitation and anion-exchange chromatography. After digestion with pepsin (Worthington Biochemical Corp., Freehold, N. J.; 22), the $F(ab')_2$ fragments were purified by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) and adsorption with Sepharose-protein A (Sigma Chemical Co., St. Louis, Mo.). Fab' anti-C3b receptor and anti-C3 were prepared by mild reduction of the $F(ab')_2$ fragments with 2-mercaptoethanol and alkylation with iodoacetamide (22). Analysis of $F(ab')_2$ and Fab' anti-C3b receptor and anti-C3 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (23) showed them to be free of detectable intact IgG. These antibody fragments and goat IgG anti-rabbit $F(ab')_2$ (N. L. Cappel Laboratories, Inc., Cochranville, Pa.) were iodinated with ^{125}I and ^{131}I (New England Nuclear, Boston, Mass.) to specific activities ranging from 150,000 cpm/μg to 600,000 cpm/μg, using immobilized lactoperoxidase and glucose oxidase (Bio-Rad Laboratories, Richmond, Calif.). Goat IgG anti-rabbit $F(ab')_2$ was also conjugated with tetramethylrhodamine isothiocyanate (TRITC; Baltimore Biological Lab., Cockeysville, Md.; 24).

Preparation of ^{125}I-C3b Bound to Constituents of Sheep E Membranes (^{125}I-C3b-OR).   Human C3 (21), factor B (25), factor D (26), and properdin (P; 26) were purified to homogeneity, and C3 was trace-labeled with ^{125}I (New England Nuclear) using iodogen (Pierce Chemical Corp., Rockford, Ill.; 27) to specific activities ranging from 7.2×10^4 cpm to 3.8×10^5 cpm/μg without loss of functional activity. Veronal-buffered saline, pH 7.5, containing 0.1% gelatin (GVB), GVB mixed with an equal volume of 5% dextrose in water, and containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ ($DGVB^{++}$) were used as diluents (28).

The major cleavage fragment of C3, C3b-OR, that is bound to sheep E membrane constituents, was prepared by a two-step procedure (29). 2×10^{10} sheep E were incubated for 30 min at 30°C with 5,600 μg C3, 80 μg ^{125}I-C3, 1,200 μg factor B, 10 μg factor D, and 10 μg P in 2 ml $DGVB^{++}$ containing 5 mM $MgCl_2$. The cells bearing C3b were washed five times with $DGVB^{++}$, and then incubated with 200 μg B, 10 μg D, and 10 μg P in 1 ml $DGVB^{++}$ for 30 min at 30°C to form cellular intermediate-bearing P-stabilized amplification C3 convertase sites, C3b,Bb,P. These cells were centrifuged, resuspended in 1 ml cold $DGVB^{++}$ containing 600 μg ^{125}I-C3, incubated for 30 min at 30°C for the second stage of ^{125}I-C3b deposition, and were washed three times with GVB. Solubilized membrane-bound ^{125}I-C3b-OR was prepared by lysing 1×10^{10} sheep E ^{125}I-C3b in 5 ml of ice-cold 5 mM $NaPO_4$, pH 6.9, containing 1 mg/ml soybean trypsin inhibitor and 2 mg/ml lima bean trypsin inhibitor. The lysates were centrifuged at 149,000 g for 60 min at 4°C in a Beckman ultracentrifuge, model L2-65B (Beckman Instruments Inc., Fullerton, Calif.), the pelleted membranes were resuspended in 5 ml of the same buffer and centrifuged again. The washed membranes were incubated for 30 min at 30°C in 4 ml veronal-buffered saline containing 0.25% Nonidet P-40 (BDH Chemical Ltd., Poole, England) and were then subjected to centrifugation at 149,000 g for 60 min at 4°C. The supernate-containing solubilized ^{125}I-C3b-OR was concentrated fourfold by negative pressure filtration with a collodion bag (Schleicher and Schuell Inc., Keene, N. H.), and excess Nonidet P-40 was removed by adsorption with Bio-Beads SM-2 (Bio-Rad Laboratories). The preparations were sonicated for 5 s at a setting of 6 on a Branson Sonifier Cell Disrupter, model

350 (Branson Sonic Power Co., Danbury, Conn.), and were stored at $-70°C$. After thawing and before use in binding experiments, the preparations were again sonicated as above. Amounts of ^{125}I-C3b-OR are expressed as μg of complement protein, disregarding the sheep E membrane constituents to which they are bound.

Binding Assays. PMN were isolated from citrated blood by dextran sedimentation of E and centrifugation of the leukocytes on Ficoll-Hypaque cushions (Pharmacia Fine Chemicals; 30). Residual E were hypotonically lysed and the PMN were washed in calcium- and magnesium-free Hanks' balanced salt solution (Microbiological Associates, Walkersville, Md.) containing 2 mg bovine serum albumin (HBSS-BSA; Sigma Chemical Co.) per ml. Cell concentrations were determined with a model ZF Coulter Counter (Coulter Electronics, Hialeah, Fla.).

In standard antibody-binding assays, replicate samples of 5×10^6 PMN in 1 ml HBSS-BSA were preincubated for 20 min at $37°C$ with 100 μg of unlabeled nonimmune F(ab')$_2$ and anti-C3b receptor F(ab')$_2$, respectively. ^{125}I-F(ab')$_2$ or ^{125}I-Fab' anti-C3b receptor in 1 ml HBSS-BSA were then added to the replicate samples of cells and incubation continued for 20 min at 0 or $37°C$. The PMN were washed three times with 2 ml of ice-cold HBSS-BSA and resuspended with 200 μg of ^{131}I-IgG anti-F(ab')$_2$ in 2 ml HBSS-BSA. After incubation for 1 h at $0°C$, the cells were washed three times with ice-cold HBSS-BSA and assayed for bound ^{125}I and ^{131}I. Specific uptake of ^{125}I-F(ab')$_2$ or ^{125}I-Fab' was calculated by subtracting the amount of these antibodies bound to cells that had been preincubated with immune F(ab')$_2$ from that bound to cells preincubated with nonimmune F(ab')$_2$. Specifically bound ^{131}I-IgG anti-F(ab')$_2$ was calculated by subtracting the ^{131}I taken up by PMN previously incubated only with nonimmune F(ab')$_2$ from that bound to cells that had been incubated also with ^{125}I-F(ab')$_2$ or ^{125}I-Fab'.

Specific binding at equilibrium of ^{125}I-C3b-OR to PMN was assayed by incubating replicate samples of 2.5×10^6 PMN in 0.08 ml HBSS-BSA for 30 min at $0°C$ with 10 μg of anti-C3b receptor F(ab')$_2$ or nonimmune F(ab')$_2$, followed by the addition of incremental amounts of ^{125}I-C3b-OR in 0.02 ml HBSS-BSA. After incubation for 45 min at 0 or $30°C$, duplicate samples of 0.04 ml from each reaction mixture were layered on 0.3 ml of a mixture of 8 vol of dibutylphthalate and 2 vol of dinonylphthalate in polyethylene microfuge tubes (Brinkmann Instruments, Inc., Westbury, N. Y.). The tubes were centrifuged for 75 s at 8,000 g in a Beckman B microcentrifuge (Beckman Instruments Inc.) and cut just above the cell pellets, which were then assayed for ^{125}I.

Immunofluorescent Studies. For immunofluorescent studies, 5×10^6 PMN in 0.5 ml HBSS-BSA were incubated with 10 μg nonimmune F(ab')$_2$, 10 μg anti-C3b receptor F(ab')$_2$, 10 μg F(ab')$_2$ anti-C3, and 20 μg anti-C3b receptor Fab', respectively, for 20 min at either 0 or $37°C$. After the cells were washed twice with 2 ml ice-cold HBSS-BSA, they were resuspended with 0.5 ml HBSS-BSA containing 200 μg TRITC-IgG anti-F(ab')$_2$ that had been preadsorbed with 10^9 sheep E for 30 min at $0°C$, and incubated at $0°C$ for 60 min. The PMN were washed twice with HBSS-BSA and were fixed by resuspension in 0.2 ml HBSS containing 2.5% paraformaldehyde and further incubation for 30 min at $0°C$. The PMN were examined for fluorescence by placing a drop of cell suspensions on microscope slides, overlaying with coverslips, and observing at $\times 600$ magnification with a Leitz fluorescence microscope (E. Leitz, Inc., Rockleigh, N. Y.).

Results

Immunofluorescent Analysis of the Surface Distribution of C3b Receptors on PMN. PMN preincubated with nonimmune Fab' or F(ab')$_2$ at 0 or at $37°C$ bound no detectable TRITC-IgG. PMN that had taken up anti-C3b receptor Fab' at $0°C$ demonstrated a punctate, clustered distribution of fluorescence that was most apparent when the focal plane was at the upper or lower surfaces of the cells (Fig. 1); only peripheral rims of patchy fluorescence were observed with the focal plane at the middle of cells (Fig. 1). An identical distribution of fluorescence was observed on PMN that had been treated with 2.5% paraformaldehyde at $0°C$ for 20 min before binding of Fab' anti-C3b receptor. To determine whether this distribution of C3b receptors was induced by prior uptake of C3b by PMN either in vivo or during collection of the
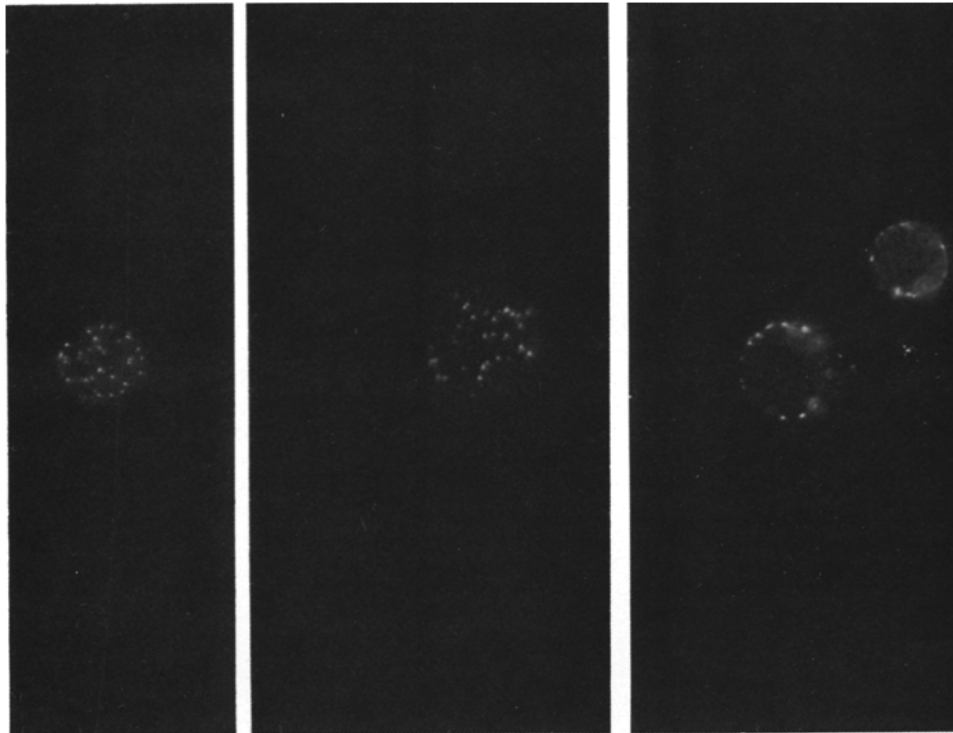
Fig. 1. Fluorescent photomicrographs of PMN that had been sequentially incubated at 0°C with Fab' anti-C3b receptor and TRITC-IgG anti-F(ab')₂. The plane of focus is at the top (left and middle panels) and at the middle of the cells (right panel).

PMN, cells were incubated with F(ab')₂ anti-C3 at 0°C, washed, and incubated with TRITC-IgG anti-F(ab')₂. No immunofluorescence was observed on any PMN, suggesting that the clustered appearance of C3b receptors as demonstrated by anti-C3b receptor represented their native distribution.

PMN incubated with F(ab')₂ at 0°C also demonstrated patchy fluorescence (Fig. 2a and b), whereas cells exposed to this bivalent ligand at 37°C had few fluorescence clusters, and some cells did not bind any detectable TRITC-IgG anti-F(ab')₂ (Fig. 2e and f). A replicate sample of the PMN-bearing F(ab')₂ anti-C3b receptor that had been taken up at 0°C was subsequently held at 37°C for 15 min, brought to 0°C, and was then incubated with TRITC-IgG anti-F(ab')₂ at 0°C. Fewer fluorescent clusters were visible on these cells (Fig. 2c and d) compared with the number present on the cells that were maintained at 0°C throughout the experiment (Fig. 2a and b). No capping of fluorescence was observed, although clusters on some PMN appeared to be distributed to one side of the cell. Thus, redistribution of the C3b receptor-F(ab')₂ complexes formed at 0°C to a cellular compartment not accessible to the fluorescent second antibody occurred during incubation of the cells at 37°C.

Adsorptive Endocytosis of F(ab')₂ Anti-C3b Receptor by PMN. The low uptake of TRITC-IgG anti-F(ab')₂ by PMN that had been incubated with F(ab')₂ anti-C3b receptor at 37 rather than at 0°C could have been secondary to impaired binding of the primary antibody or to its internalization by the PMN, rendering it inaccessible
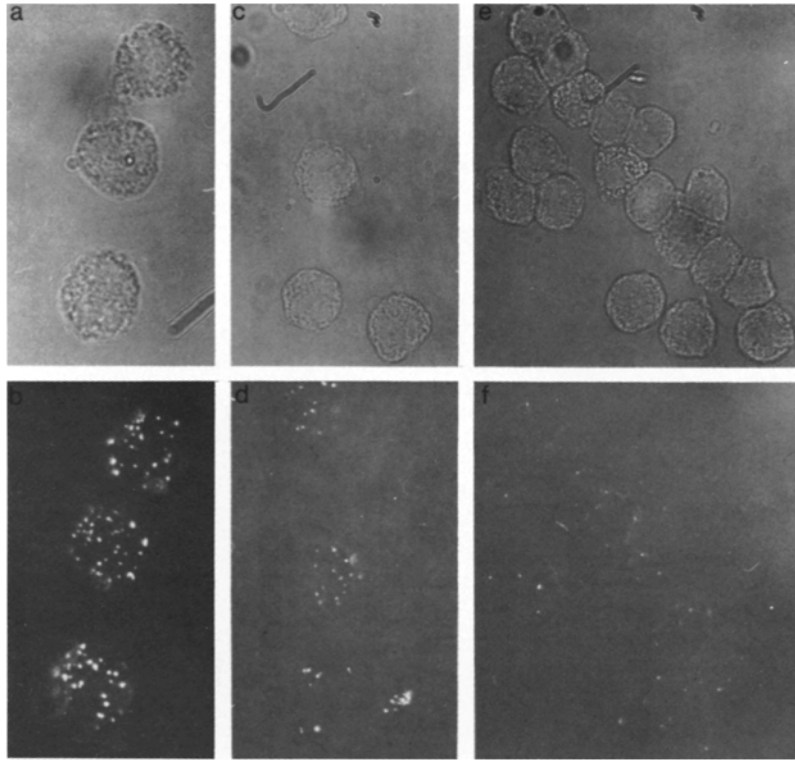
Fig. 2.   Light (a, c, and e) and corresponding fluorescent (b, d, and f) photomicrographs of PMN that had been sequentially incubated with F(ab')$_2$ anti-C3b receptor at 0°C, washed, and either held at that temperature (a and b) or incubated for 15 min at 37°C (c and d). PMN were also incubated for 20 min at 37°C with anti-C3b receptor (e and f). The PMN were then incubated with TRITC-IgG anti-F(ab')$_2$. The plane of focus was at the top of the cells and magnification is × ~600.

to the secondary antibody. To discriminate between these possible explanations, PMN that had been incubated with incremental amounts of ^{125}I-F(ab')$_2$ anti-C3b receptor at 0 and at 37°C, respectively, were compared for their capacity to take up ^{131}I-IgG anti-F(ab')$_2$ at 0°C. Binding of ^{125}I-F(ab')$_2$ anti-C3b receptor to the cells was saturable at both temperatures, and a larger fraction of the input was taken up at 37 rather than at 0°C (Fig. 3). The amounts of ^{131}I-IgG anti-F(ab')$_2$ that bound to PMN previously incubated with anti-C3b receptor at 0°C were such that an approximate 2:1 molar ratio of IgG to F(ab')$_2$ occurred over the entire dose range of cell-bound F(ab')$_2$ (Fig. 3). PMN that had taken up comparable amounts of ^{125}I-F(ab')$_2$ anti-C3b receptor at 37°C had relatively diminished capacities to bind ^{131}I-IgG anti-F(ab')$_2$, and there was an inverse relationship between the number of anti-C3b receptor molecules/PMN and the molar ratio of cell-associated IgG to F(ab')$_2$ (Fig. 3).

To examine further the temperature dependence of the inaccessibility of PMN-bound anti-C3b receptor, seven replicate samples of 5 × 10^6 PMN were each incubated for 20 min at 0°C with 10 μg ^{125}I-F(ab')$_2$ anti-C3b receptor and washed at 0°C. Six samples were brought to 37°C by the addition of 0.5 ml of pre-warmed HBSS-BSA and one sample was held on ice in this buffer. At timed intervals during incubation

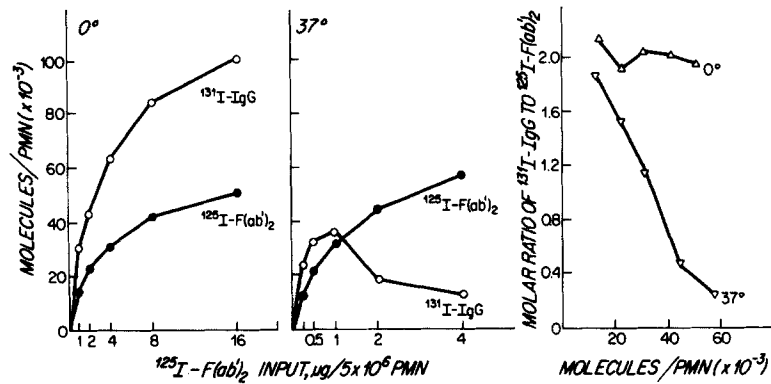FIG. 3. Comparison of the binding of ^{131}I-IgG anti-F(ab')₂ by PMN that had taken up variable amounts of ^{125}I-F(ab')₂ anti-C3b receptor at 0 and 37°C, respectively. Left panel: PMN were incubated with incremental concentrations of ^{125}I-F(ab')₂ anti-C3b receptor at 0°C, washed, incubated with excess ^{131}I-IgG anti-F(ab')₂ at 0°C, and assessed for cell-associated [125]I (●) and [131]I (○). Middle panel: the same as the experiment depicted in the left panel, except that the PMN were incubated with ^{125}I-F(ab')₂ anti-C3b receptor at 37°C. Right panel: dose-response relationship between the number of ^{125}I-F(ab')₂ molecules taken up by PMN at 0°C (△) and 37°C (▽), respectively, and the molar ratio of cell-associated ^{131}I-IgG to ^{125}I-F(ab')₂.
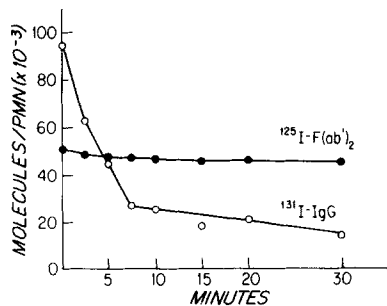

FIG. 4. Time course of the effect of incubation at 37°C on the capacity of PMN bearing ^{125}I-F(ab')₂ anti-C3b receptor (●) that had been taken up at 0°C to bind ^{131}I-IgG anti-F(ab')₂ (○) at 0°C.

at 37°C, individual samples of PMN were rapidly cooled by addition of 1.5 ml ice-cold HBSS-BSA, and held at 0°C. After 30 min, all samples of cells were centrifuged at 4°C, the supernatant fluids were removed, and the cells were assessed for their capacity to bind ^{131}I-IgG anti-F(ab')₂ at 0°C. PMN bearing 51,000 molecules of ^{125}I-F(ab')₂ anti-C3b receptor/cell and held at 0°C throughout the experiment bound 94,000 molecules of ^{131}I-IgG anti-F(ab')₂/cell, yielding a 1.84:1 molar ratio of IgG to F(ab')₂ (Fig. 4), which is similar to that observed in the experiment depicted in Fig. 3. PMN that were incubated at 37°C maintained their content of cell-associated anti-C3b receptor but rapidly and progressively lost much of their capacity to take up anti-F(ab')₂; the molar ratio of IgG to F(ab')₂ had decreased to 0.57:1 with PMN that had been incubated at 37°C for 7.5 min (Fig. 4).

The effect of the valency of the anti-C3b receptor on subsequent binding of anti-F(ab')₂ by PMN was assessed by incubating cells for 20 min at 37°C with incremental concentrations of ^{125}I-F(ab')₂ and ^{125}I-Fab' anti-C3b receptor, respectively. The PMN were washed and incubated for 1 h at 0°C with ^{131}I-IgG anti-F(ab')₂ and assessed for

cell-bound ^{125}I and ^{131}I. Uptake of bivalent and monovalent anti-C3b receptor by PMN were saturable reactions, although an input of 2.5 times more ^{125}I-Fab' was necessary to achieve binding that was equivalent to that observed with ^{125}I-F(ab')$_2$ (Fig. 5). The molar ratio of bound IgG to F(ab')$_2$ decreased almost fivefold over the dose range of 10,000–42,000 molecules of anti-C3b receptor/PMN (Fig. 5). However, binding of increasing amounts of mor ovalent anti-C3b receptor to PMN was not associated with impaired uptake of anti-F(ab')$_2$ and the molar ratio of cell-bound to IgG to Fab' remained in the range of 2:1 over a dose range of 22,000–74,000 ^{125}I-Fab'/PMN (Fig. 5).

The disparity in the accessibility to an extracellular ligand of PMN-bound monovalent and bivalent anti-C3b receptor was examined further by assessing the capacity of pronase to release these cell-associated antibody fragments. PMN were incubated at 37°C for 20 min with incremental concentrations of ^{125}I-F(ab')$_2$ and ^{125}I-Fab' anti-C3b receptor, respectively, and were washed and assayed for bound ^{125}I. The PMN were then resuspended in 1 ml HBSS-BSA containing 500 μg pronase/ml, incubated for an additional 15 min at 37°C and centrifuged, and the cell pellets were assayed for residual ^{125}I. At the highest input of ^{125}I-F(ab')$_2$, 75,000 molecules were bound per PMN and 79% remained cell-associated after treatment with pronase (Fig. 6). In contrast, only 17% of the 94,000 ^{125}I-Fab' molecules/PMN that were bound at the highest input of the monovalent anti-C3b receptor remained cell-associated after treatment of the cells with pronase (Fig. 6).

To assess the involvement of cytochalasin B-sensitive cytoskeletal elements in C3b receptor-mediated endocytosis, 5×10^6 PMN were incubated for 10 min at 37°C in 0.5 ml HBSS-BSA containing 5 μg cytochalasin B/ml and 0.5% dimethyl sulfoxide (DMSO), and a replicate sample of PMN was incubated with buffer containing only DMSO. 4 μg of ^{125}I-F(ab')$_2$ anti-C3b receptor was added to each reaction mixture and incubation was continued for 20 min at 37°C, after which the cells were washed
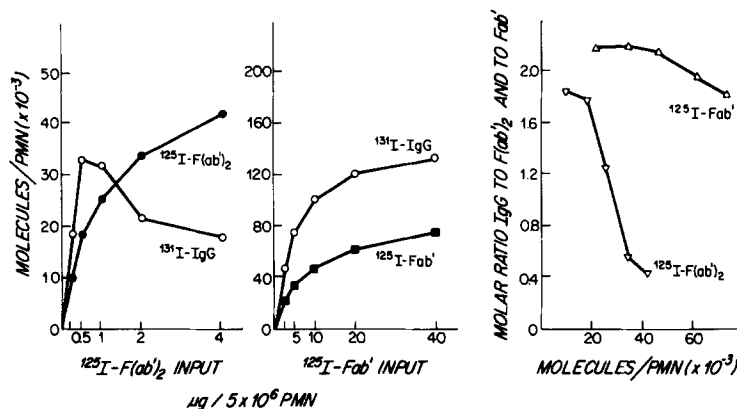

Fig. 5. Comparison of the binding of ^{131}I-IgG anti-F(ab)$_2$ by PMN that had taken up variable amounts of ^{125}I-F(ab')$_2$ and ^{125}I-Fab' anti-C3b receptor, respectively, at 37°C. Left panel: PMN were incubated with incremental concentrations of ^{125}I-F(ab')$_2$ anti-C3b receptor at 37°C, washed, incubated with excess ^{131}I-IgG at 0°C, and assessed for cell-associated ^{125}I (●) and ^{131}I (○). Middle panel: the same as the experiment depicted in the left panel, except that the PMN were incubated with incremental concentrations of ^{125}I-Fab' anti-C3b receptor. Right panel: dose-response relationship between the number of ^{125}I-labeled F(ab')$_2$ (∇) and Fab' (△) molecules, respectively, taken up by PMN and the molar ratio of cell-associated ^{131}I-IgG to the anti-C3b receptor antibody fragment.
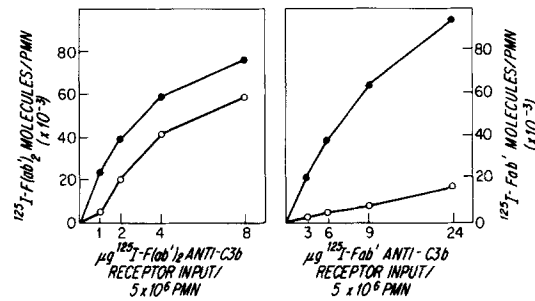
Fig. 6.   Dose-response relationship between input of ^{125}I-F(ab')$_2$ (left panel) and ^{125}I-Fab' (right panel) anti-C3b receptor and the number of antibody molecules initially taken up by PMN at 37°C (●) and remaining cell-associated after treatment of the PMN with pronase (○).
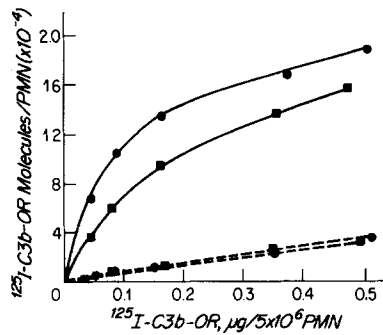

Fig. 7.   Binding of incremental inputs ^{125}I-C3b-OR to PMN at 0°C (■) and at 30°C (●) in the presence of non-immune F(ab')$_2$ (solid lines) or anti-C3b receptor F(ab')$_2$ (dashed lines).

and assessed for their capacity to bind ^{131}I-IgG anti-F(ab)$_2$ at 0°C. PMN preincubated with buffer containing only DMSO and bearing 51,000 F(ab')$_2$ molecules/cell subsequently bound 31,280 IgG molecules/cell, representing a 0.61:1 molar ratio of IgG to F(ab')$_2$. PMN that had taken up 45,300 molecules/cell of anti-C3b receptor in the presence of cytochalasin B bound 27,640 molecules/cell of anti-F(ab')$_2$, yielding an identical 0.61:1 molar ratio of IgG to F(ab')$_2$.

Adsorptive Endocytosis of ^{125}I-C3b-OR by PMN.   Incremental amounts of ^{125}I-C3b-OR that had been solubilized from sheep E membranes to which the complement protein had been affixed were incubated at 0 or at 30°C for 45 min with PMN in the presence of nonimmune or anti-C3b receptor F(ab')$_2$. Uptake of ^{125}I-C3b-OR by the cells in the presence of nonimmune F(ab')$_2$ was slightly greater at 37 than at 0°C, and the binding curves were concave to the abscissa throughout the analyses, indicating saturable reactions (Fig. 7). In contrast, the binding of ^{125}I-C3b-OR to PMN was greatly diminished by the presence of anti-C3b receptor, was a linear function of the input of the ligand, and thus, was nonsaturable or nonspecific (Fig. 7).

Internalization of ^{125}I-C3b-OR by PMN was assessed by incubating 5×10^6 PMN in 1 ml HBSS-BSA with incremental amounts of the ligand for 20 min at 37°C. The cells were washed twice with 2 ml HBSS-BSA and were assayed for bound ^{125}I-C3b-OR. The cells were then suspended in 1 ml HBSS-BSA containing 500 μg pronase and incubation was continued for 15 min at 37°C. After centrifugation and removal of the supernatant fluids, the cell pellets were assayed for residual-associated ^{125}I-C3b-

OR. Greater than 90% of the radioactivity initially bound by the PMN at each input of the ligand was susceptible to release by pronase, indicating that the C3b receptor-ligand complex had remained at the cell surface (Fig. 8).

Because bivalent, but not monovalent, anti-C3b receptor was internalized by C3b receptors, the effect of cross-linking ^{125}I-C3b-OR bound by PMN on the endocytosis of this ligand was examined. Replicate samples of 5×10^6 PMN in 1 ml HBSS-BSA were each incubated for 45 min at 0°C with 0.27 μg ^{125}I-C3b-OR, and the cells were washed twice with ice-cold buffer and resuspended in 1 ml HBSS-BSA alone or containing incremental concentrations of Fab' or F(ab')$_2$ anti-C3. After incubation for 20 min at 37°C, the PMN were washed twice at 0°C and assayed for cell-bound [125]I. The cells were then resuspended in 1 ml HBSS-BSA containing 500 μg pronase, incubated for 15 min at 37°C, centrifuged, and assayed for residual-associated ^{125}I after removal of the supernatant fluids. Incubation of PMN-bearing ^{125}I-C3b-OR with monovalent or bivalent anti-C3 did not alter the amount of cell-bound radiolabeled ligand before treatment with pronase (Fig. 9). Fab' anti-C3 had only a minimal effect on the percentage of ^{125}I-C3b-OR removed from the PMN by pronase, which was 91 and 85% with PMN that had been incubated with buffer and with the highest input of monovalent anti-C3, respectively. In contrast, F(ab')$_2$ anti-C3 induced a
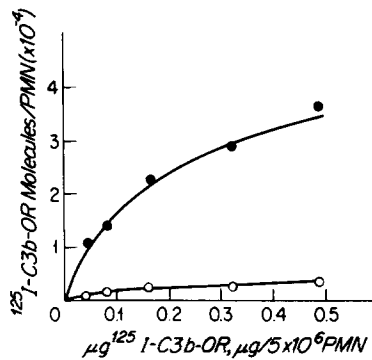
Fig. 8. Dose response relationship between input of ^{125}I-C3b-OR and the number of molecules taken up by PMN at 37°C (●) and remaining bound after treatment of the cells with pronase (○).
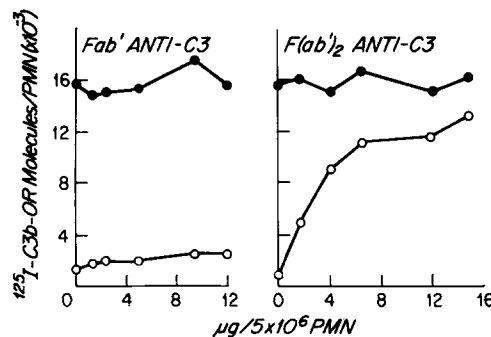
Fig. 9. Dose-response relationship between the amounts of Fab' (left panel) and F(ab')$_2$ (right panel) anti-C3 that were incubated for 20 min at 37°C with PMN-bearing ^{125}I-C3b-OR and the number of molecules of ^{125}I-C3b-OR per cell before (●) and after (○) treatment of the cells with pronase.

dose-related increase in the amount of ^{125}I-C3b-OR remaining cell-associated after pronase treatment, so that only 20–30% of the ligand was released by proteolytic treatment of cells that had been exposed to the highest concentrations of the bivalent anti-C3 (Fig. 9).

Discussion

The capacity of C3b receptors on PMN to mediate internalization at 37°C of ^{125}I-F(ab')$_2$ anti-C3b receptor was established by the relative inaccessibility of ligand taken up at 37°C to two extracellular probes, IgG anti-F(ab')$_2$ and pronase. The F(ab')$_2$ fragments were separated from residual intact IgG by gel filtration and adsorption with Sepharose-protein A, and no intact heavy chains were seen on polyacrylamide gel electrophoresis; thus, internalization of the antibody by Fc receptors was excluded. The clusters of C3b receptors that were observed by indirect immunofluorescence of PMN that had been bound anti-C3b receptor at 0°C were almost absent on cells that had been incubated with the antibody at 37°C, which suggests that endocytosis of the antibody had occurred at the higher temperature (Fig. 2). This possibility was confirmed and quantitated in dose-response experiments measuring the sequential uptake by PMN of ^{125}I-F(ab')$_2$ anti-C3b at 0 and 37°C, respectively, and of ^{131}I-IgG anti-F(ab')$_2$ at 0°C. Each molecule of anti-C3b receptor bound by PMN at 0°C subsequently took up an average two molecules of anti-F(ab')$_2$ over the entire dose-range of cell-associated ^{125}I-F(ab')$_2$, establishing a 2:1 molar ratio of cell-bound IgG to F(ab')$_2$ under conditions that excluded receptor-mediated endocytosis. In contrast, when increments of anti-C3b receptor were taken up by PMN at 37°C, there was a progressive decline in the capacity of the cell-associated antibody to bind IgG anti-F(ab')$_2$. A cell-bound molar ratio of only 0.2 IgG molecules/F(ab')$_2$ occurred with PMN that had taken up the highest number of anti-C3b receptor molecules at 37°C and this is taken to indicate that 90% of the ligand bound to C3b receptors had been endocytosed. Anti-C3b receptor bound to PMN at 0°C rapidly became inaccessible to the second antibody during incubation of the cells at 37°C, with a half-time for internalization of <5 min (Fig. 4), indicating that endocytosis was stimulated by the ligand, rather than being secondary to normal metabolic turnover of the receptor.

The increasing proportion of F(ab')$_2$ anti-C3b receptor that was rendered inaccessible to anti-F(ab')$_2$ when larger amounts of the primary antibody were taken up by PMN at 37°C (Fig. 3) suggested that multivalent interaction of this ligand with the receptor was required to stimulate internalization. Incubation of PMN with incremental amounts of monovalent ^{125}I-Fab' anti-C3b receptor at 37°C was not associated with impaired subsequent uptake of ^{131}I-IgG anti-F(ab')$_2$ as a relatively constant molar ratio of two IgG bound/Fab' was observed (Fig. 5). Moreover, treatment with pronase of PMN-bearing ^{125}I-Fab' anti-C3b receptor that had been taken up at 37°C removed >80% of the cell-bound ^{125}I, whereas, in a parallel experiment, pronase treatment of PMN that had bound 76,000 ^{125}I-F(ab')$_2$ anti-C3b receptor molecules/cell released only 25% of the cell-associated ^{125}I (Fig. 6). Thus, cross-linking of C3b receptors by ligand appears to be necessary for the pinocytotic reaction.

The capacity of C3b receptors on PMN to mediate internalization of the bivalent antibody fragment prompted an examination of their interaction with the natural ligand, C3b. This cleavage fragment of C3 may be present in its free form, C3b, in

which a carbonyl that is derived from an internal thioester (31) has reacted with H_2O, or in a state designated as C3b-OR, in which the carbonyl has formed an ester linkage to constituents of the immune complex bearing the C3 convertase (1). C3b-OR, rather than free C3b, was used in these studies because 15–80% of the former has been shown to be capable of binding to C3b receptors on human erythrocytes, whereas <1% of the latter had this activity.[2] ^{125}I-C3b-OR was bound by PMN in a saturable reaction that was abolished by preincubation of the cells with anti-C3b receptor F(ab')$_2$, indicating that both ligands interacted with the same cell surface structure (Fig. 7). Because treatment with pronase of PMN bearing incremental amounts of ^{125}I-C3b-OR that had been taken up during incubation at 37°C released >90% of the cell-associated ^{125}I, this monovalent receptor-ligand complex was not internalized (Fig. 8). Cross-linking of the bound ^{125}I-C3b-OR by introducing increasing amounts of F(ab')$_2$ anti-C3 to create multivalent ligands, however, induced a dose-related increase in the amount of the complement protein that was inaccessible to proteolytic stripping (Fig. 9). Thus, the natural ligand for C3b receptors on PMN elicited an endocytotic response when presented as a multivalent structure, as had antibody to the receptor.

The C3b receptor of PMN has been considered to participate only in the attachment phase of endocytosis because these cells bind, but do not internalize, sheep E bearing C3b (9, 10); phagocytosis occurs only when targets also carry IgG, which permits their interaction with Fc receptors on PMN. However, the finding that PMN internalized F(ab')$_2$ anti-C3b receptor and C3b-OR that had been cross-linked with anti-C3, indicates that C3b receptors are capable of mediating internalization of soluble, rather than particulate, ligand by adsorptive pinocytosis. A distinction between these two types of endocytotic reactions could reside in the cytoskeletal elements that are involved, because cytochalasin B, which impairs phagocytosis (32), had no inhibitory effects on internalization of F(ab')$_2$ anti-C3b receptor, which suggests that the latter reaction is not mediated by microfilaments. A cytoplasmic structural protein possibly involved in C3b receptor-mediated endocytosis is clathrin, because endocytotic receptors on other cell types accumulate in clathrin-coated pits (20, 33), which yield a clustered distribution of receptors similar to that observed for the C3b receptor (Figs. 1 and 2). Regardless of the mechanism, the demonstration of this functional capacity for C3b receptors indicates that they can directly mediate the clearance of C3b-bearing soluble complexes.

Summary

C3b receptors on human polymorphonuclear leukocytes (PMN) were nonrandomly distributed in small clusters on the plasma membranes of these cells when assessed by indirect immunofluorescence at 0°C using monospecific rabbit Fab' or F(ab')$_2$ anti-C3b receptor and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat IgG anti-F(ab')$_2$. When PMN were incubated with the bivalent anti-C3b receptor at 37 rather than at 0°C, almost no immunofluorescence was observed, which indicates that the C3b receptor-F(ab')$_2$ complexes had been rendered inaccessible to TRITC-IgG anti-F(ab')$_2$. Endocytosis of the anti-C3b receptor ligand was quantitated by

measuring the binding ^{131}I-IgG anti-F(ab')$_2$ by PMN that had previously taken up ^{125}I-F(ab')$_2$ anti-C3b receptor at 0 and at 37°C, respectively. There was a constant 2:1 molar ratio of anti-F(ab')$_2$ to anti-C3b receptor with PMN that had been incubated with the first antibody at 0°C. In contrast, when increments of F(ab')$_2$ anti-C3b receptor were taken up by the cells at 37°C, there was a dose-related decline in this molar ratio to a minimum of 0.2 molecules of anti-F(ab')$_2$ bound per molecule of PMN-associated anti-C3b receptor. ^{125}I-F(ab')$_2$ anti-C3b receptor taken up by PMN at 37°C was also inaccessible to release by proteolytic treatment of the cells with pronase. The rate of endocytosis of ^{125}I-F(ab')$_2$ anti-C3b receptor was rapid as the PMN-bound antibody fragment became inaccessible to ^{131}I-IgG anti-F(ab')$_2$ within 10 min during incubation of the cells at 37°C. In contrast to these findings, ^{125}I-Fab' anti-C3b receptor that was taken up by PMN at 37°C remained accessible to both ^{131}I-IgG anti-F(ab')$_2$ and to proteolytic release by pronase, which suggests that monovalent interaction of ligand with C3b receptors was not sufficient for induction of endocytosis. The requirement for multivalency was also demonstrated using the C3b-OR, the normal ligand for the C3b receptor. ^{125}I-C3b-OR was specifically bound by PMN but remained on the cell surface, as determined by its accessibility to pronase, unless it was cross-linked with F(ab')$_2$ anti-C3. Although C3b receptors on PMN do not mediate internalization of particulate ligand, their newly recognized capacity to function in receptor-mediated adsorptive pinocytosis of soluble ligand indicates their potential for the clearance of C3b-bearing immune complexes without recruitment of other cell surface receptors.

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