DEPOSITION OF C3b AND iC3b ONTO PARTICULATE ACTIVATORS OF THE HUMAN COMPLEMENT SYSTEM

Quantitation With Monoclonal Antibodies to Human C3

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Phagocytic cells (neutrophils, monocytes, and macrophages) possess at least two distinct receptors for the third component of complement, C3. Complement receptor type 1 (CR_1)[1] is specific for C3b, while complement receptor type 3 (CR_3) is specific for the C3b cleavage product iC3b (1). In addition, monocytes may also express a CR_2-like receptor that is specific for the d region of iC3b, C3d,g, and C3d (2, 3). CR_1 and CR_3 on neutrophils and monocytes promote phagocytosis of C3-coated particles mainly by promoting adherence of the particle to the phagocyte's surface (4, 5). In contrast, complement receptors (CR) on human and mouse macrophages may promote both binding and phagocytosis of C3-coated particles, depending on the in vitro or in vivo conditions from which the macrophages were obtained (6–10). The data on CR function has been derived from studies using sheep erythrocytes (E) coated with C3 using purified complement (C) components, mouse serum, or human serum. However, little is known about the actual C3 fragments that might be encountered by phagocytes in vivo.

Detailed information is available on the sequence of events that occurs after C3b has been fixed to a particle's surface through activation of the classical pathway (CP) or alternative pathway (AP) (reviewed in 11). The several components, C4-binding protein (12), factors H and I (13, 14), and erythrocyte (CR_1) (15, 16), play key roles in controlling the activation and propagation of both complement pathways, by their ability to convert bound, hemolytically active C3b into nonhemolytic fragments. In addition, it has become increasingly clear that the surface to which C3b becomes fixed plays a critical role in the susceptibility of C3b to these control proteins. Current data suggests that particles which

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[1] *Abbreviations used in this paper:* AP; alternative complement pathway; BDVEA, veronal buffer containing BSA, dextrose, EDTA, and sodium azide; BSA, bovine serum albumin; C, complement; CFU, colony-forming unit; CP, classical pathway; CR_1, complement receptor type 1; DTT, dithiothreitol; E, sheep erythrocyte; EGTA, ethyleneglycol-bis-(beta-amino-ethyl ether)*N,N'*-tetra acetic acid; EIgG, E coated with rabbit IgG anti-E antibody; EIgM, E coated with rabbit IgM anti-E antibody; E_R, rabbit erythrocytes; GVB^{++}, VBS^{++} containing gelatin; PAGE, polyacrylamide gel electrophoresis; PBSA, phosphate-buffered saline containing sodium azide; PHS, pooled human serum; SDS, sodium dodecyl sulfate; VBS^{++}, veronal-buffered saline containing $MgCl_2$ and $CaCl_2$; Y, yeast.

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can activate the AP do so because they provide a protected environment for bound C3b (17–20), whereas CP activators do not. This protective environment promotes AP activation by favoring the binding of factor B rather than factor H to C3b (21, 22). For example, sialic acid appears to be the critical membrane component that prevents sheep E from being AP activators (22, 23). The surface components on other particles, or, more importantly, on microorganisms, that play a role in determining whether or not the AP is activated have not yet been defined. In addition, these surface components could play an important role in determining how much C3b becomes inactivated to iC3b or C3d,g, and how much C3b remains intact on the particle's surface.

In the present study, two monoclonal antibodies, anti-C3c and anti-C3g, were used to quantitate the deposition by serum of C3b, iC3b, and C3d,g onto various particles and microorganisms. Antibody-coated sheep E, rabbit E (E_R), yeast (Y), and five different species of bacteria: *Escherichia coli, Staphylococcus aureus, Haemophilus influenzae* type b, *Streptococcus pneumoniae* type 3, and *Streptococcus pyogenes*, were examined. Three questions were addressed: (*a*) What is the number of C3 molecules that become fixed to bacteria and other particles opsonized in normal human serum; (*b*) what is the ratio of C3b to iC3b and/or C3d,g; and (*c*) what are the kinetics of C3b deposition and conversion to iC3b? The data show that the quantity of C3b fixed was dependent on the particle and the serum concentration, while the ratio of C3b to iC3b was mainly dependent on the particle or bacterium. iC3b was found to be the predominant form of bound C3 on Y and E, while C3b was the predominant form of C3 on bacteria. The importance of these observations as they relate to complement activation and to host defense is discussed.

Materials and Methods

Serum. Blood was obtained from six normal donors and allowed to clot in glass tubes for 45 min at 22°C. The clots were rimmed with wooden sticks, and then allowed to retract by incubation at 4°C for a further 45 min. The clotted blood was centrifuged at 8,000 *g* for 30 min to obtain serum. Serum from the six donors was pooled and aliquoted, and then was stored at −85°C until used. Serum from a patient with C7 deficiency (24) was processed and stored in an identical manner. Once thawed, serum was used immediately for one experiment, and any remainder was discarded. One batch of pooled human serum (PHS) or the C7-deficient serum was used for all experiments reported in these studies.

Particles. Sheep erythrocytes (E) stored in Alsever's solution were coated with IgG and IgM anti-sheep erythrocyte antibody (Cordis Laboratories, Miami, FL) according to the method of Kabat and Mayer (25). Antibodies were used at the recommended dilutions for optimal complement fixation (IgG, 1:200; IgM, 1:120). EIgM and EIgG were washed and stored in 3.5 mM veronal buffer, pH 7.2, containing 1% bovine serum albumin (BSA), 3.2% dextrose, 20 mM EDTA, and 0.2% sodium azide (BDVEA) (conductivity, 6 mS at 22°C). E_R were obtained from normal rabbits, washed three times in BDVEA, and were stored in BDVEA until used. Bakers yeast (Y) was processed as described by Lachmann and Hobart (26) and stored in 0.15 M saline.

Bacteria. Five species of bacteria were used in these experiments: *E. coli*, ATCC 25922; hemolytic *S. aureus*, ATCC 25923; a clinical throat isolate of group A, β hemolytic *S. pyogenes*; a clinical blood isolate of *S. pneumoniae* type III; and a clinical blood isolate of *H. influenzae* type b. All species were kindly provided by the Microbiology Laboratory of North Carolina Memorial Hospital at the University of North Carolina. Cultures were maintained on agar plates and subcultured once a week. *H. influenzae* was cultured on chocolate agar plates and the other strains of bacteria were cultured on sheep blood agar.

E. coli and *S. aureus* were grown in Tripticase soy broth and *S. pyogenes* and *S. pneumoniae* were grown in Todd-Hewitt broth (BBL Microbiological Systems, Becton, Dickinson & Co., Cockeysville MD); *H. influenzae* was grown in Mueller-Hinton broth (Difco Laboratories, Detroit, MI) containing 1% isovitalex (BBL Microbiological Systems) and 1% hemin. Before use, growth curves were determined on each bacterial species using standard procedures. Bacteria were obtained for experiments during the log phase of the growth curve. For some experiments, bacteria were obtained from log phase cultures and inactivated by heating at 65°C for 1h. *S. pneumoniae* were heat killed in 0.4% formalin. Heat-killed bacteria were washed twice in saline and then stored in 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.02% sodium azide (PBSA). Heat-killed bacteria were standardized by counting in a Pertroff Hauser chamber.

Opsonization. For opsonization, freshly thawed PHS or C7D serum (for erythrocytes) was diluted in veronal-buffered saline containing 0.5 mM Mg^{++} and 0.15 mM Ca^{++} (VBS^{++}). E_R and EIgM were washed and standardized to 1×10^8 E/ml in VBS^{++}, and EIgG were standardized in VBS^{++} containing 0.1% gelatin (GVB^{++}). (GVB^{++} was used for EIgG to minimize aggregation and spontaneous lysis of these cells.) Y cells were standardized in VBS^{++} at 1.5×10^9 Y/ml. Equal volumes of particles and diluted serum were mixed and then incubated for varying periods of time at 37°C. Control tubes contained particles plus buffer only. At the end of the incubation period, E were diluted in BDVEA, washed three times, and resuspended in BDVEA to $5–7 \times 10^8$ E/ml. Y were washed three times in cold PBS and then resuspended to 2×10^8 Y/ml in BDVEA.

Bacteria were grown to log phase, at which time a sample was removed, diluted, and plated on agar to determine the number of colony-forming units (CFU) per milliliter. The bacteria were aliquoted into several tubes and washed twice in saline. 1 ml of PHS (or saline for the control) was added to each pellet of bacteria, the bacteria were vigorously resuspended, and the tubes were then incubated at 37°C for varying periods of time. At the end of the incubation period, bacteria were washed twice in saline and resuspended to 2 ml in BDVEA. Heat-killed bacteria were opsonized in a similar manner.

Antibodies. Radiolabeled IgG anti-C3c and anti-C3g were used to assess the number of bound C3b and iC3b on the various particles and bacteria. Ascites fluid containing anti-C3c was obtained from Bethesda Research Laboratories, Gaithersburg, MD, and ascites fluid containing anti-C3g (clone 9) (27 and 28) was a generous gift of Dr. Peter Lachmann of the Medical Research Council, Cambridge, England. IgG was purified from ascites by sequential precipitation with caprylic acid and 50% ammonium sulfate as described previously (2). The purified antibodies appeared as single bands after electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels. ^{125}I-labeled IgG was prepared with Iodogen (Pierce Chemical Co., Rockford, IL) by the method of Fraker and Speck (29). For each labeling, 0.25 mCi of ^{125}I were used for 100 μg of IgG, to give a specific activity of $1–2 \times 10^6$ cpm/μg.

Radioimmunoassay. Opsonized and washed erythrocytes (E_R, EIgG, and EIgM) (1.25×10^7 E/tube in 50 μl of BDVEA) were mixed with saturating amounts of ^{125}I-anti-C3c or ^{125}I-anti-C3g and were incubated for 15 min at 22°C. After the incubation period, 5×10^6 E were layered onto 0.2 ml of a 4:1 (vol/vol) solution of dibutyl phthalate and dinonyl phthalate in a 0.5 ml microfuge tube. The tubes were centrifuged for 1 min in a Beckman Microfuge B (Beckman Instruments, Inc., Palo Alto, CA) and then frozen in an ethanol–dry ice bath. The cells in the pellet were clipped and then counted in a gamma counter (1185 series; Nuclear-Chicago, Des Plaines, IL).

50 μl of opsonized Y containing 1×10^7 cells were aliquoted into 1.5-ml microfuge tubes, saturating amounts of ^{125}I-anti-C3c or ^{125}I-anti-C3g were added, and the mixture was incubated for 15 min at 22°C. Y were washed four times in 1 ml of cold BDVEA to remove unbound antibody, and the yeast pellet was clipped into tubes for determination of bound radioactivity.

50 μl of opsonized bacteria were incubated with labeled antibodies in 15-ml conical centrifuge tubes. At the end of the 15 min incubation period, the bacteria were diluted with 10 ml of BDVEA. After mixing, 5 ml of bacteria were collected on 0.22 μm filters (Millipore/Continental Water Systems, New Bedford, MA). The filters were washed with 20 ml BDVEA and then placed into tubes for determination of bound radioactivity. The

number of bacteria per filter was calculated based on colony counts obtained from the sample removed at the beginning of each experiment.

Deposition of ^{125}I-C3 on Yeast and Bacteria. Purified human C3 (30) was labeled with ^{125}I in the same manner as the monoclonal antibodies. Yeast or heat-killed bacteria (5.5×10^8) were centrifuged, and the pellets were mixed with 36 μg of ^{125}I-C3 and 25% serum in VBS^{++} (final volume, 0.5 ml). Opsonization was allowed to proceed for 60 min at 37°C. At the end of the incubation period, the bacteria were washed in BDVEA, and the yeast were washed in VBS^{++}. The bacteria and yeast were resuspended to 0.5 ml in the wash buffer, and a 20 μl sample was removed to determine the amount of bound C3. Based on the counts per minute, an aliquot of yeast or bacteria was pelleted in a microfuge tube to produce a pellet containing $3–5 \times 10^5$ cpm. To determine the molecular weight of the bound ^{125}I-C3 fragments, the pellets were solubilized in 125 μl of 100 mM diethanolamine, pH 11.7, containing 100 mM NaCl, 1% SDS, and 8 M urea, for 1 h at 45°C to elute the membrane-bound ^{125}I-C3 fragments (31). The pH of the ^{125}I-C3 fragment mixture was neutralized to pH 7 with 1 N HCl, and dithiothreitol (DTT) was added to a final concentration of 100 mM. The mixture was then diluted with 65 μl of 25 mM Tris-HCl, pH 6.8 containing 2% SDS, 100 mM DTT, and 8 M urea, and boiled for 5 min; 75% glycerol containing 0.001% bromophenol blue was added so that the final volume was 300 μl. Similarly treated ^{125}I-C3 was used as a control. From each tube an aliquot representing 5×10^4 cpm was removed and electrophoresed in 9% polyacrylamide gel slabs (32). The gels were stained with Coomassie Blue, dried onto a sheet of chromatography paper (Whatman, Inc., Clifton, NJ), placed on preflashed (33) X-Omat AR film (Eastman Kodak Co., Rochester, NY), and incubated at −70°C for 6 h. After developing the x-ray film, the positions of the stained molecular weight markers were marked on the film, and the molecular weight of the ^{125}I-C3 fragments were calculated from their mobilities relative to the standards.

Data. In all experiments reported, data are expressed as the number of molecules of antibody bound per particle or CFU. In experiments with heat-killed bacteria, the data are expressed as antibody molecules bound per bacterium. Specific activity for the labeled antibodies was always corrected for decay of ^{125}I. Data represent the mean of two to three experiments.

Results

The specificity of monoclonal anti-C3c and anti-C3g is illustrated in Fig. 1. Anti-C3c binds to the C3c portion of fixed C3b. However, the antigenic determinant recognized by anti-C3g is not exposed in bound C3b (27, 28). When bound C3b is cleaved to iC3b, both of the monoclonal antibodies recognize and bind to their respective antigenic determinants on this fragment. If iC3b is

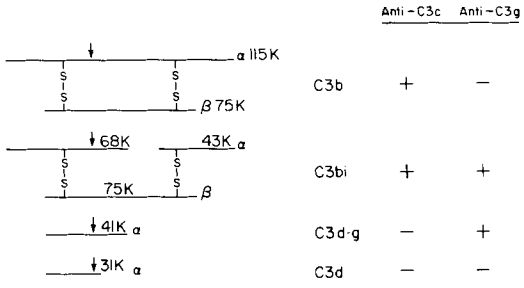

FIGURE 1. Binding of monoclonal antibodies anti-C3c and anti-C3g to particle-bound fragments of C3. Anti-C3c, but not anti-C3g, bound to particle-bound C3b. Conversion of C3b to iC3b resulted in equivalent binding by both antibodies. Conversion of iC3b to C3d,g resulted in the loss of C3c into the fluid phase. Thus, anti-C3g, but not anti-C3c, binds to this remaining fragment. Conversion of C3d,g to C3d resulted in the loss of binding by anti-C3g. The arrow over the alpha chain represents the position of the metastable binding site.

further cleaved to C3d,g, the binding of anti-C3c is abrogated due to the loss of C3c antigen, but anti-C3g binding remains constant. Final cleavage of C3d,g to C3d abrogates the binding of anti-C3g. Thus, radiolabeled anti-C3c and anti-C3g were used to quantitate the total number of bound C3 molecules and determine which C3 fragments were present.

CP Activators. EIgM and EIgG were used to assess deposition of C3b and conversion to iC3b, C3d,g or C3d by the classical pathway in human serum. Serum from a patient with C7D (24) was used to avoid lysis of the erythrocytes. The deficiency of C7 is the only defect detected in this patient's serum, and comparison of this serum with PHS in the opsonization of Y gave identical results. EIgM and EIgG were incubated in different concentrations of serum for 20 min at 37°C, and then incubated with the two monoclonal antibodies as described in Materials and Methods. In Figs. 2–8, the total number of C3 molecules bound is represented by anti-C3c. The number of bound iC3b molecules is represented by anti-C3g. The difference between the number of C3 molecules detected by anti-C3g and anti-C3c represents bound but unconverted C3b.

As shown in Fig. 2, the number of molecules of C3 bound to EIgM and EIgG was a function of the serum concentration. >100,000 molecules of C3 became bound to EIgM incubated in 5% PHS, while >80,000 molecules of C3 became bound to EIgG. For both EIgM and EIgG, there was complete conversion of bound C3b to iC3b at all serum concentrations tested. There was no evidence of cleavage of iC3b to C3d,g or C3d.

Serum deposition of C3b and conversion to iC3b on EIgM and EIgG was next examined as a function of time. Fig. 3 shows that maximum deposition of C3

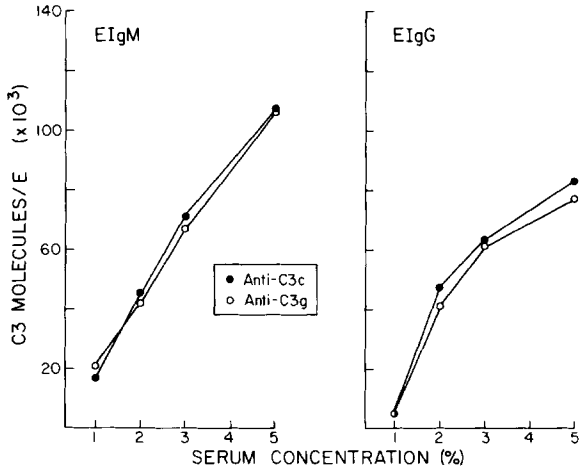

FIGURE 2. Quantitation of C3b and iC3b on EIgM and EIgG as a function of the serum concentration. 1 × 10^8 E/ml in VBS^{++} was mixed with an equal volume of different concentrations of C7-deficient serum diluted in VBS^{++}. The E were then incubated for 20 min at 37°C. Control tubes contained E plus buffer only. At the end of the incubation period, E were diluted in BDVEA, washed three times, and then resuspended in BDVEA to 5–7 × 10^8 E/ml. Bound C3b and iC3b were quantitated with the radioiodinated monoclonal antibodies anti-C3c and anti-C3g as described in Materials and Methods. Data are expressed as the number of molecules of antibody bound per E.

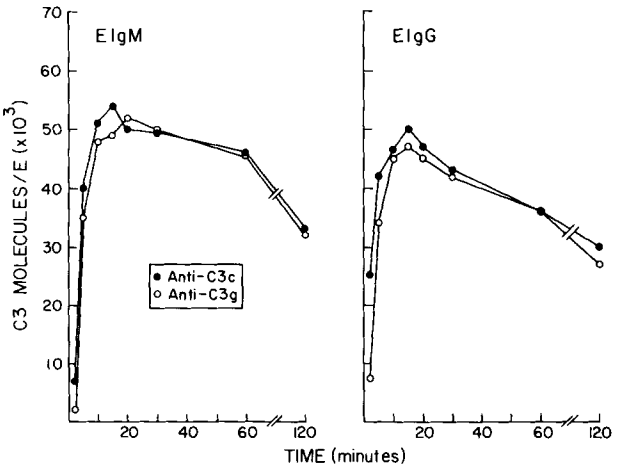

FIGURE 3. Quantitation of C3b and iC3b on EIgM and EIgG as a function of the time of incubation. The conditions of incubation were the same as in Fig. 2, except that the final serum concentration was 2%.

occurred at 15 min for both CP activators incubated in 2% serum. In addition, conversion of bound C3b to iC3b occurred immediately, even as more C3b was being bound to the erythrocytes. Again, there was no evidence of conversion of iC3b to C3d,g, even after 2 h of incubation in serum. Erythrocytes incubated in serum >30 min showed some decline in the number of C3 molecules that remained surface bound. This was due to the release of intact C3 and not C3 fragments, since the number of bound C3 molecules detected by both anti-C3c and anti-C3g decreased in parallel. This phenomenon has also been observed by other investigators (34, 35).

AP Activators. The number of C3b and iC3b bound to E_R in C7D serum was determined in the presence and absence of Mg-EGTA, due to the presence of "natural" antibodies in human serum to these cells. Mg-EGTA was used to prevent the activation of the CP by chelating Ca^{++}. This reagent still allows activation of the AP, which only requires Mg^{++} (36). Fig. 4 shows the results obtained when E_R were incubated in different concentrations of serum for 20 min at 37°C, with and without Mg-EGTA. In both cases, C3 deposition was dependent on serum concentration. At all serum concentrations tested, greater numbers of C3 molecules bound to E_R when both the CP and AP were activated (i.e., without Mg-EGTA). In the absence of Mg-EGTA, only 3% serum was required to bind 4–5 × 10^4 molecules of C3/E; in the presence of Mg-EGTA, 6% serum was required. The percent conversion of C3b to iC3b was also dependent on the serum concentration. As the serum concentration increased, so did the percent of bound iC3b.

The deposition of C3b and conversion to iC3b on E_R as a function of time is shown in Fig. 5. When both the CP and AP were operative (no Mg-EGTA, 2% serum), maximum C3 deposition occurred by 30 min. Conversion of C3b to iC3b was evident at 10 min and continued throughout the 2 h incubation period. At the end of 2 h, 79% of bound C3 was iC3b. When E_R were incubated in serum and used the AP only (Mg-EGTA, 6% serum), C3 deposition was complete by 30 min. Again, conversion of C3b to iC3b and deposition of more C3 were

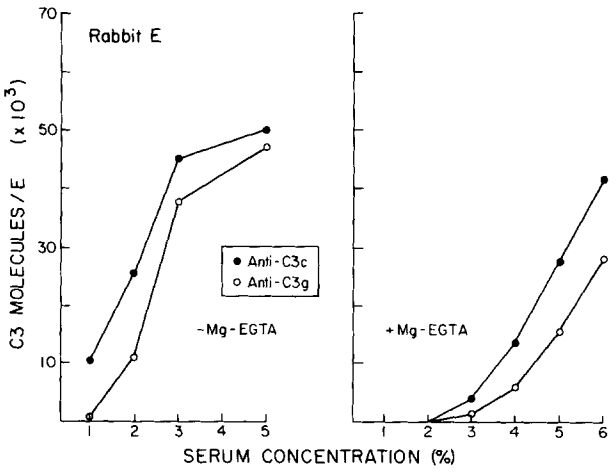

FIGURE 4. Quantitation of C3b and iC3b on E_R as a function of serum concentration. The conditions of incubation were the same as those described in Fig. 2. E_R were incubated in different concentrations of C7-deficient serum in the absence and presence of 0.1 mM $MgCl_2$ + 0.4 mM EGTA (Mg-EGTA).

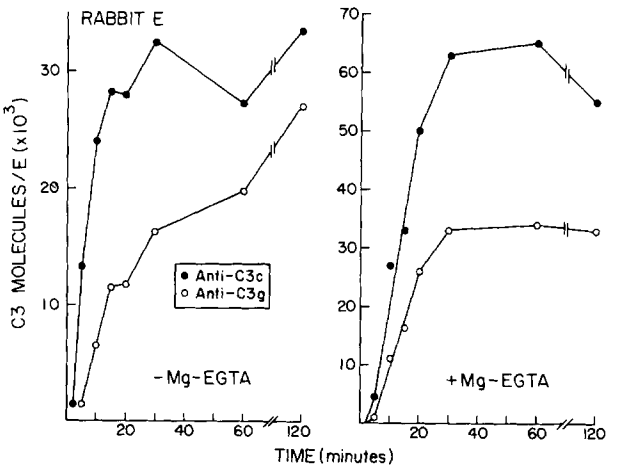

FIGURE 5. Quantitation of C3b and iC3b on E_R as a function of the time of incubation. The conditions of incubation were the same as in Fig. 2. A final concentration of 2% C7-deficient serum was used in the absence of Mg-EGTA; 6% serum was used in the presence of Mg-EGTA.

concurrent events. After 30 min, there was no further conversion of C3b to iC3b, and iC3b made up 52% of the total C3 bound.

Yeast (Y) was also studied as an AP activator. Fig. 6 shows the deposition and conversion of C3b onto Y as a function of serum concentration. At 50% serum concentration, maximal C3 deposition occurred with almost 9×10^4 molecules of C3 bound per Y. 84% of bound C3 was iC3b in 50% serum; 67% of bound C3 was iC3b in 25% serum. The examination of C3 deposition and conversion as a function of time in 50% serum is shown in Fig. 7. Maximal C3 deposition was rapid, and was essentially complete at 10 min. Conversion of bound C3b to

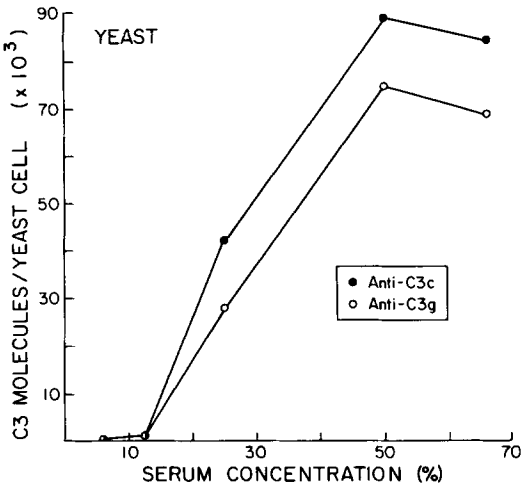

FIGURE 6. Quantitation of C3b and iC3b on Y as a function of the serum concentration. 1.5 × 10^9 Y/ml in PBSA was mixed with an equal volume of different concentrations of PHS diluted in VBS^{++}. The Y were then incubated for 20 min at 37°C. A control tube contained Y plus buffer only. At the end of the incubation period, the Y were washed three times in cold PBS, and then were resuspended to 2 × 10^8/ml in BDVEA. Bound C3b and iC3b were quantitated with the radioiodinated monoclonal antibodies anti-C3c and anti-C3g as described in Materials and Methods.

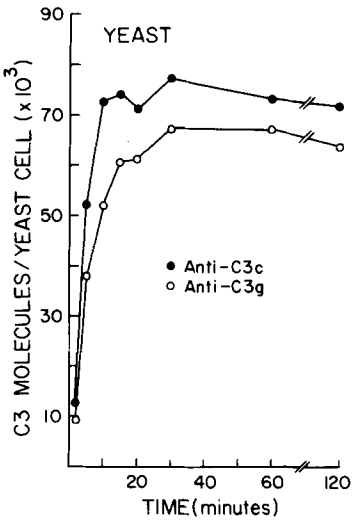

FIGURE 7. Quantitation of C3b and iC3b on Y as a function of the time of incubation. The conditions of incubation were the same as in Fig. 6. The final concentration of serum was 50%.

iC3b was also very rapid. 94% of bound C3 that was eventually converted to iC3b was completed within 15 min. At 30 min, 87% of bound C3 was iC3b. No significant changes in total bound C3 or the percent of iC3b occurred between 30 min and 2 h.

Table I is a comparison of the concentration of serum required to fix between

TABLE I

Concentration of Serum Required for Fixation of 4–5 × 10^4 C3 Molecules per Particle

Particle	Addition	Serum concentration
		%
EIgM	—	2
EIgG	—	2
E_R	—	3
E_R	Mg-EGTA	6
Y	—	25
Y	Mg-EGTA	25

Particles and serum were mixed and incubated for 20 min at 37°C. Particles were washed in buffer and the number of bound C3b molecules was determined as described in Materials and Methods. Mg-EGTA was added to the serum so that the final concentration in the incubation mixture was 1 mM Mg^{++} and 4 mM EGTA.

4 and 5 × 10^4 C3 molecules per particle. For EIgM, EIgG, and E_R (in the absence of Mg-EGTA), <5% serum was required, and only 6% serum was required for E_R in the presence of Mg-EGTA. Under the conditions of opsonization used for Y, these particles required 25% serum to achieve similar numbers of molecules. The presence or absence of Mg-EGTA did not effect the number of C3 molecules bound per Y, or the percent of bound iC3b.

Finally, we examined serum deposition of C3 and conversion to iC3b in five different species of bacteria. The species chosen were those that are pathogenic and require phagocytosis by professional phagocytes to be cleared from the host (37). Bacteria were grown to log phase, washed in buffer, and then incubated in neat PHS for varying periods up to 2 h. The number of molecules of C3b and iC3b bound to the bacteria (Fig. 8) was determined as described in Materials and Methods. The total number of bound C3 molecules per CFU on the different species of bacteria ranged from 3 × 10^4 molecules on *H. influenzae* type b to 6.6 × 10^4 molecules on *S. pneumoniae* type 3. Opsonization was usually complete after 60 min of incubation in serum, the main exception being *S. pneumoniae.* The most interesting aspect of these experiments was the noticeable lack of conversion of bound C3b to iC3b. At 60 min, the average percent conversion of C3b to iC3b was only 21% (range, 16–28%). In a separate set of experiments (Table II), heat-killed bacteria were used to assess the role of the AP in opsonization of these bacteria, and to determine what effect, if any, soluble, purified human erythrocyte CR_1 (38) might have on the ratio of C3b to iC3b (or to C3d,g). Bacteria were added to either PHS, PHS heated to 56°C for 60 min, PHS containing Mg-EGTA, or PHS containing 10 μg of CR_1, and were incubated for 20 min at 37°C. The bacteria were then washed, and bound C3b and iC3b was quantitated as before. Bacteria incubated in heat-inactivated serum had background counts <5% of that obtained with PHS. Except for *H. influenzae* and *S. pneumoniae*, the total number of bound C3 molecules per bacterium was the same whether the bacteria were viable or dead. Also, on four out of five bacterial strains, the percent of iC3b per bacterium was the same for viable and dead bacteria. *S. aureus* was the exception, with 58% iC3b on dead and 22%

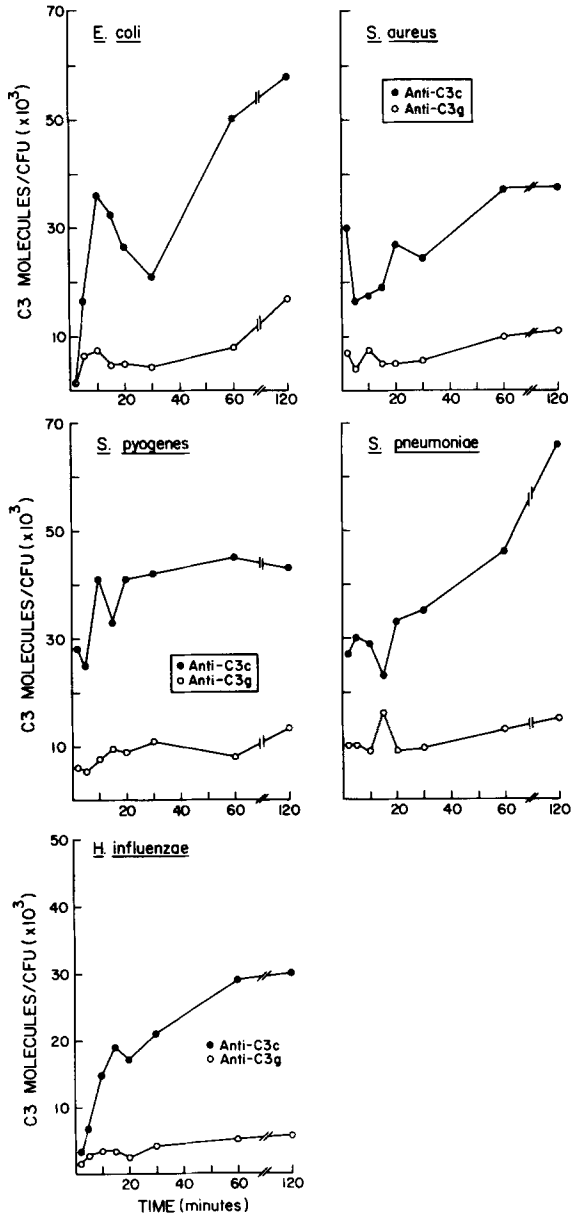

FIGURE 8. Quantitation of C3b and iC3b on five different strains of bacteria as a function of the time of incubation. Log phase bacteria were aliquoted into several tubes and washed twice in saline. 1 ml of PHS was added to each pellet of bacteria, the bacteria were vigorously resuspended, and the tubes were incubated at 37°C for various periods of time. At the end of the incubation period, bacteria were washed twice in saline and then resuspended to 2 ml in BDVEA. Bound C3b and iC3b were quantitated with the radioiodinated monoclonal antibodies anti-C3c and anti-C3g, as described in Materials and Methods.

iC3b on viable bacteria. Overall, the addition of Mg-EGTA to PHS did not have a significant effect on the total number of C3 molecules bound per bacterium or the percent of iC3b. However, opsonization of *S. pneumoniae* in the presence of

TABLE II

Quantitation of C3b and iC3b on Heat-killed Microorganisms

Microorganism	Addition*	Molecules per bacterium ($\times 10^4$)			Percent iC3b
		Total C3	C3b‡	iC3b	
E. coli	None	4.0	2.9	1.1	27
	Mg-EGTA	6.9	5.0	1.9	27
	CR_1	4.1	2.9	1.2	29
S. aureus	None	2.4	1.0	1.4	58
	Mg-EGTA	3.3	1.4	1.9	57
	CR_1	2.7	1.3	1.4	52
H. influenzae	None	5.1	3.6	1.5	29
	Mg-EGTA	5.2	3.4	1.8	35
	CR_1	4.6	3.0	1.6	35
S. pyogenes	None	4.0	3.0	1.0	25
	Mg-EGTA	3.3	2.3	1.0	30
	CR_1	4.8	3.9	0.9	19
S. pneumoniae	None	5.8	3.8	2.0	34
	Mg-EGTA	5.1	2.5	2.6	51
	CR_1	5.4	3.4	2.0	37

* Bacteria were incubated for 20 min at 37°C in neat PHS only, PHS containing 0.1 M $MgCl_2$/0.4 M EGTA, or PHS containing 10 μg of purified human erythrocyte CR_1.

‡ The number of bound C3b molecules was determined by subtracting the number of molecules of anti-C3g bound from that obtained with anti-C3c. (See Fig. 1 and first paragraph in Results).

Mg-EGTA appeared to result in a greater percent of bound iC3b than in the absence of Mg-EGTA. Addition of CR_1 to PHS did not significantly affect the total number of C3 molecules bound or the percent of iC3b, nor did it cause the degradation of iC3b to C3d,g.

In a previous study (30), we examined the molecular form of C3 on EC3b after treatment with various concentrations of factors H and I. In these experiments, the monoclonal antibody data revealed that iC3b but not C3d,g was formed under isotonic conditions. Conversion of C3b to C3d,g occurred only if the reaction with factors H and I took place in a buffer of low ionic strength or in the presence of purified CR_1. Conversion of EC3b to EC3d,g was accompanied by a decrease in binding of anti-C3c and was confirmed in experiments using ^{125}I-C3 to prepare EC3b, in which the C3 fragments formed were analyzed by SDS-PAGE and autoradiography.

To confirm that only C3b and iC3b become fixed to the surface of Y and bacteria opsonized in normal human serum, ^{125}I-C3 was mixed with Y and three of the strains of bacteria (*E. coli*, *S. aureus*, and *H. influenzae*) in 25% serum in VBS^{++}. Opsonization was allowed to proceed for 60 min at 37°C, and then the bacteria and Y were processed for SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography as described in Materials and Methods. Fig. 9*B* shows the α and β chains of ^{125}I-C3 run as a control. Fig. 9, *C–F* show the C3 fragments eluted from the *S. aureus*, *E. coli*, *H. influenzae*, and Y, respectively. The intact 115,000 kD α′ chain present on all four particles represents bound

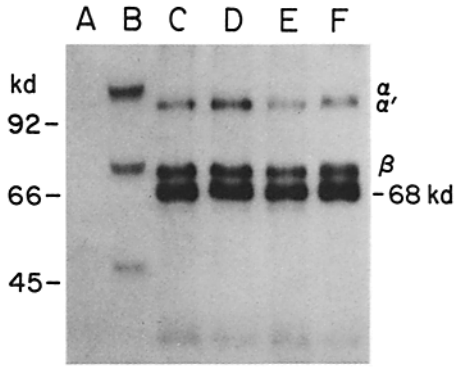

FIGURE 9. SDS-PAGE/autoradiography of ^{125}I-C3 fragments eluted from serum-opsonized Y and bacteria. (*A*) Position of the molecular weight markers. (*B*) Native ^{125}I-C3. (The extra band at 48,000 mol wt is a minor contaminant of the C3 preparation that did not show up on previous Coomassie Blue–stained gels. It did not appear in any of the other tracks, and therefore, did not bind to the Y or bacteria). (*C–F*) Eluted ^{125}I-C3 fragments from *S. aureus*, *E. coli*, *H. influenzae*, and Y, respectively. The bacteria and Y were opsonized in 25% serum for 60 min at 37°C in the presence of 36 μg of ^{125}I-C3. The bound ^{125}I-C3 fragments were eluted and then analyzed under reducing conditions on 9% polyacrylamide gels. The gels were stained and prepared for autoradiography as described in Materials and Methods. 5×10^4 cpm were run in each track.

C3b. The 68 kD fragment from the α' chain represents bound iC3b. The 43 kD band of the α' chain of iC3b is not seen in Fig. 9 because it is poorly labeled by the iodination procedure (14). However, the 43 kD band was observed on overexposed x-ray film (not shown). No bands were seen at 41 kD even after overexposure of the x-ray film, indicating that no C3d,g was formed.

Discussion

Two monoclonal antibodies, anti-C3c and anti-C3g, were used to examine serum deposition of C3b and its conversion to iC3b on particulate activators of the complement system. The particles studied activated both the CP and the AP. Human serum pooled from six normal donors (PHS) was used to study the opsonization of Y and bacteria, while serum from a patient with C7 deficiency (24) was used to study the opsonization of EIgM, EIgG, and E_R. This C7-deficient serum performed identically to PHS in the opsonization of Y. The validity of using anti-C3c and anti-C3g to define the ratio of C3b/C3bi on Y and bacteria was confirmed using ^{125}I-C3 and SDS-PAGE and autoradiography to show that no C3d,g was formed on these particles. Thus, the measurements obtained with anti-C3g represented only bound iC3b, and, therefore, the amount of bound C3b could be calculated by subtracting the values obtained with anti-C3g from the values obtained using anti-C3c. We have shown previously that this is also true for erythrocytes (30).

C3b deposition and conversion to iC3b on the CP activators EIgM and EIgG was a very rapid and almost simultaneous process. Bound C3b was converted to iC3b even as more C3b was being bound to the erythrocytes' surfaces. These data dramatically illustrate the total inability of the cell membrane of sheep E to provide any protection for bound C3b from serum factors H and I, and corroborate well with the original studies of Fearon (23).

In contrast, particles that activate the AP were quite varied in their ability to protect bound C3b from factors H and I. On Y, the total number of C3 molecules bound and the ratio of C3b to iC3b were the same in both the presence and absence of Mg-EGTA (82% iC3b). However, the presence of Mg-EGTA during the opsonization of E_R did effect the ratio of C3b to iC3b. After 1 h of incubation in 4% serum, 74% of bound C3 was iC3b in the absence of Mg-EGTA, compared with only 52% in the presence of Mg-EGTA. Thus, when both the CP and AP were operative with E_R, more C3b was converted to iC3b than when only the AP was activated. These differences may be due to C3b binding to different parts of the E_R membrane. C3b that bind via the CP may bind to the E_R membrane in an environment totally unprotected from factors H and I, while C3b that bind via the AP may adhere to a part of the membrane where it is partially protected. However, C3 deposition via the CP does not appear to be the main factor controlling the conversion of C3b to iC3b. Rather, the surface of the particle appears to be the key regardless of which C pathway is activated. Thus, pneumococci incubated in normal serum contain mainly C3b on their surface whether or not IgM anti-pneumococcal antibody was added to the serum (39).

From our experiments with EIgM, EIgG, E_R, and Y, four important conclusions can be made. First, the deposition of C3b and its conversion to iC3b on particulate activators of the complement system are concurrent events. C3b is cleaved to iC3b by factors H and I at the same time that more C3 is cleaved to C3b by the C3 convertase. Second, although activators of the AP can provide an environment for C3b that favors the binding of factor B over factor H (21, 22), 50–80% of bound C3b may still be converted to iC3b. Third, at least on the particles studied, and under the isotonic conditions used, iC3b is not cleaved further to C3d,g or C3d. C3d,g does appear to be the final C3 fragment on human erythrocytes from patients with chronic cold agglutinin disease (40). Finally, some bound C3b remains on activators of the AP, even after prolonged incubation in serum; this ratio of C3b to iC3b is mainly dependent on the particle.

This last conclusion is particularly significant with respect to the results obtained with the five different species of bacteria (*E. coli*, *S. pneumoniae* type III, *S. pyogenes*, *S. aureus*, and *H. influenzae* type b). These bacteria all activate the AP (41–45) and require phagocytosis for removal from the host (37). When they were incubated in serum for up to 2 h, only 16–28% of bound C3b was converted to iC3b, and no C3d,g was detected. The addition to the serum of Mg-EGTA (so that only the AP was activated) or purified human erythrocyte CR_1 (38), which has been shown to be a cofactor for factor I (15, 16), did not significantly affect the total number of C3 molecules bound or the ratio of C3b to iC3b, and did not cause the formation of C3d,g. This preparation of CR_1 had been shown previously (30) to promote the cleavage of sheep E–bound C3b to C3d,g in isotonic human serum. Thus, it would appear that the bacteria-bound C3b was resistant to factor I, even when CR_1 was provided as a cofactor. In experiments with *S. pneumoniae* (types 7 and 12, and unencapsulated R36A), Brown et al. (39) also found that only small amounts of C3b were converted to iC3b, as determined by the binding of ^{125}I-labeled bovine conglutinin to serum-opsonized bacteria. In addition, little iC3b was formed even when the bacteria

were coated with IgM anti-pneumococcal antibody before incubation in serum. This latter observation further emphasizes the importance of the particle's surface in determining the amount of C3b that is converted to iC3b, regardless of which complement pathway is activated.

In the experiments with bacteria we have not attempted to determine whether the C3b or iC3b molecules were bound to the bacterial cell wall or capsule, nor have we tried to determine if the ratio of C3b to iC3b varies with different strains within a species. However, in experiments with pneumococci, other investigators have reported that C3b becomes fixed to the pneumococcal cell wall during activation of the AP (46) and that, in the presence of anticapsular antibody, C3b becomes fixed to the pneumococcal capsule only via the CP (47). The predominant form of bound C3 on the pneumococci appears to be C3b, on both the cell wall and the capsule (39). Studies with *S. aureus* have also found that C3 binds to the cell wall of encapsulated organisms incubated in normal human serum (48), but that C3 becomes bound to the capsule in immune serum (49). In our experiments, most of the bound C3b is probably on the cell wall; this could be confirmed by using the monoclonal anti-C3c and anti-C3g in immunoelectron microscopy experiments. Finally, although different strains within a particular species have been reported to differ in their ability to activate C, it is likely that these differences affect the number of C3 molecules bound but not necessarily the ratio of C3b to iC3b. This is supported by recent studies (50) with three strains of *Salmonella* that differed from one another in the chemical structure of their *O*-polysaccharide. C3 deposition resembled C3 consumption in rate and extent, differing for surfaces with different *O*-polysaccharides. However, the ratio of bacteria-bound C3b to iC3b was the same for all three strains. In addition, Weis et al. (51) reported that both M^+ and M^- group A streptococci contained bound C3b and iC3b (but not C3d,g) when they were opsonized in normal human serum. Interestingly, the C3b and iC3b on the M^+ strain did not promote binding of the bacteria to erythrocyte CR_1 or to polymorphonuclear cells CR_1 or CR_3, suggesting that the M protein was most likely responsible for the resistance of these bacteria to phagocytosis.

As they pertain to the process of inflammation, these results suggest that C3b, and not iC3b, may be the predominant form of bound C3 encountered by neutrophils and monocyte/macrophages. At the very least, phagocytes may use both CR_1 (C3b) and CR_3 (iC3b), in addition to Fc receptors, to promote phagocytosis and the destruction of pathogenic microorganisms.

Summary

Monoclonal antibodies were used to determine the number and molecular form of C3 bound to particulate activators of the complement (C) system by human serum. Sheep erythrocytes (E) coated with IgM (EIgM) and IgG (EIgG) were used to study activation of the classical pathway (CP). Yeast (Y), rabbit erythrocytes (E_R), and five species of bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pneumoniae* type 3, *Streptococcus pyogenes*, and *Hemophilus influenzae* type b) were used to study activation of the alternative pathway (AP). The deposition of C3b onto EIgM and EIgG incubated in C7-deficient human serum was dependent on the serum concentration. At all serum concentrations tested, there was complete conversion of C3b to iC3b. Kinetic analysis of C3b

deposition and conversion to iC3b indicated that these events occurred almost simultaneously; the reaction was completed by 15 min. The deposition of C3 onto the AP activators E_R and Y was also dependent on serum concentration, and E_R, but not Y, required the presence of Mg-EGTA and thus the activation of only the AP. C3b deposition and conversion to iC3b on Y was complete in 15 min, with 82% of bound C3 converted to iC3b. For E_R, maximum C3 deposition required 30 min in both the presence and absence of Mg-EGTA. However, after 1 h of incubation, 74% of bound C3 was iC3b in the absence of Mg-EGTA, compared with only 52% in the presence of Mg-EGTA. Thus, even on AP activators, a large portion of C3b may be converted to iC3b, and this conversion is probably controlled by elements on the particle's surface.

Studies with the five species of bacteria yielded similar results. ~3–5 × 10^4 molecules of C3 were bound per microorganism, with opsonization being completed in 30 min. Remarkably, only 16–28% of bound C3 was in the form of iC3b, even after 2 h of incubation. The presence or absence of Mg-EGTA, or the addition of purified CR_1 to the reaction mixture, did not significantly effect the ratio of C3b to iC3b. Finally, SDS-PAGE and autoradiography of particle-bound ^{125}I-C3 fragments confirmed that there was no conversion of iC3b to C3d,g or C3d. The data obtained about the opsonization of bacteria suggest that the predominant form of C3 that is encountered by inflammatory phagocytes may be C3b.

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