IgG BEARING COVALENTLY BOUND C3b HAS ENHANCED BACTERICIDAL ACTIVITY FOR ESCHERICHIA COLI 0111

BY K. A. JOINER, L. F. FRIES, M. A. SCHMETZ, AND M. M. FRANK

From the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205

Bactericidal antibody is required for direct complement-mediated killing of most pathogenic gram-negative bacteria. Although it has been implicitly assumed that bactericidal antibody functions by increasing complement activation and deposition on the bacterial surface, this assumption may not be valid. We have shown previously (1) that bactericidal IgG increases the bactericidal efficiency of C5b-9 for the serum-resistant Escherichia coli 0111 strain 12015, without increasing the extent of C5b-9 deposition. Bactericidal IgG markedly increased the percentage of the total C5b-9 bound to E. coli 0111 that was inserted into the outer membrane in a salt- and protease-resistant form (2). The effect of IgG was mediated at a step before C5 convertase formation since the addition of IgG after the C5 convertase was formed, but before the C5b-9 was deposited, did not kill bacteria (3).

These experiments suggested that bactericidal IgG was changing either the location of complement activation or the molecular conformation of C5b-9 on the bacterial surface. We have now extended these studies by showing that (a) nearly one-fifth of the total C3 that is bound in the presence of antibacterial IgG binds covalently to the antibody molecule and (b) complexes of C3b-IgG are three- to fourfold more effective than IgG in presensitizing strain 12015 for complement-mediated killing. Formation of C3b-IgG complexes may be critical for effective killing of some gram-negative bacteria by the serum-complement system.

Materials and Methods

Buffers. The following buffers were used in these experiments: Hanks' balanced salt solution (HBSS) containing 0.15 mM CaCl2 and 1.0 mM MgCl2 (HBSS++); low ionic strength (μ = 0.060) dextrose-gelatin veronal-buffered saline with 0.15 mM CaCl2 and 1.0 mM MgCl2 (DGVBS++); isotonic gelatin veronal-buffered saline with 0.15 mM CaCl2 and 1.0 mM MgCl2 (GVBS++).

Bacterial Strains. E. coli 0111B4 (ATCC 12015), E. coli J5 (kindly provided by Dr. Elizabeth Zeigler, University of California, San Diego, CA), and Salmonella minnesota Re 595 (provided by Dr. Jack Hawiger, Boston, MA) were the bacterial strains used. Characteristics of these strains are as outlined previously (3).

Abbreviations used in this paper: Abs PNHS, absorbed PNHS; avidin-Sep, avidin covalently coupled to Sepharose 4B; C8D, C8 deficient; HBSS, Hanks' balanced salt solution; HBSS++, HBSS containing CaCl2 and MgCl2; LPS, lipopolysaccharide; NPGB, p-nitrophenyl p-guanidinobenzoate; O-Ag, O antigen; PBS, phosphate-buffered saline; PNHS, pooled, normal human serum; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.
Bacteria were grown in trypticase soy broth at 37°C with continuous agitation to mid-log phase for use in experiments. Cells were washed in HBSS++ at room temperature or 4°C and adjusted to the desired concentration spectrophotometrically.

**Serum.** Serum was obtained from seven normal volunteers, pooled, and frozen in aliquots at -70°C (PNHS). Serum samples from a patient with a congenital absence of C8 (C8D) and from a patient with acquired hypogammaglobulinemia were also used. For some experiments, serum was absorbed with strain 12015 before use to remove natural antibody. $1 \times 10^8$ organisms were mixed with 4 ml of serum at 0°C for 30 min, and the bacterial pellet was removed by centrifugation at 20,000 g for 15 min at 0°C (1). Serum absorbed in this fashion (Abs PNHS) lost <15% of the initial C3 hemolytic activity.

**Antibody Preparation.** The IgG fraction of rabbit antiserum to the lipopolysaccharide and O antigen (O-Ag) capsule of 12015 (4) was prepared and characterized as described earlier (1). A portion of the IgG preparation was radiolabeled with $^{125}$I- Na (New England Nuclear, Boston, MA) using Iodobeads (Pierce Chemical Co., Rockford, IL) to a specific activity of $9.87 \times 10^5$ cpm/µg. Another portion of purified IgG was biotinylated with n-hydroxysuccinimide biotin (Calbiochem-Behring Corp., La Jolla, CA) with reaction conditions identical to those previously described for biotinylation of C3 (5). Biotinylated IgG contained an average of 1.8 biotin residues per molecule. Biotinylated IgG had agglutination and bactericidal titers for 12015 that were unchanged in comparison to native IgG.

Monospecific antiserum to C3 was raised in goats. This antiserum recognizes determinants on native C3 and on all cleavage fragments of C3: C3b, iC3b, C3c, and C3dg. The IgG fraction of goat anti-C3 was coupled by cyanogen bromide linkage to Sepharose 4B using standard techniques. Avidin (Calbiochem-Behring Corp.) was covalently coupled to Sepharose 4B (avidin-Seph) as previously described (5, 6).

**Complement Component Purification.** Human C3 was purified to homogeneity by minor modifications of the procedure of Hammer et al. (7); C3 was radiolabeled with $^{125}$I- Na (Amersham Corp., Arlington Heights, IL) using Iodobeads (Pierce Chemical Co.) to a specific radioactivity of $2.8 \times 10^5$ cpm/µg. Iodination resulted in <20% loss of hemolytic activity.

**Distribution of C3 and IgG on E. coli 12015.** The distribution of IgG on the O-Ag capsule compared with that on the outer membrane of 12015 was determined before and after serum incubation. Strain 12015 was suspended to an OD$_{600}$ of 1.5 in HBSS**, then incubated with $^{125}$I-IgG at 50 µg/ml for 20 min at room temperature. Organisms were washed twice. An aliquot of cells bearing $^{125}$I-IgG was removed, passed twice through a French press cell at 16,000 lb/in$^2$, and processed as described below. The remainder of the presensitized organisms were suspended to the original volume in HBSS**, then mixed vol/vol with 20% absorbed (Abs) C8D. The suspension was incubated for 60 min at 37°C, and the organisms were washed twice and passed through a French press cell as described above.

The French press lysates from samples before and after serum incubation were centrifuged at 113,000 g for 2 h to separate the outer membrane (sediment) from the O-Ag capsule (supernatant) as described (4, 6). Distribution of $^{125}$I-IgG in the outer membrane and O-Ag capsule fractions was determined with a gamma scintillation counter. In control experiments, we found that <5% of $^{125}$I-IgG centrifuged under identical conditions in the absence of a bacterial lysate was recovered at the bottom of the tube. Distribution of C3 on the O-Ag capsule compared with that on the outer membrane of 12015 was also determined. Strain 12015 at OD$_{600}$ 1.5 in HBSS** was presensitized with 50 µg/ml of unlabelled IgG for 20 min at room temperature, then incubated for 60 min at 37°C in a final concentration of 10% Abs C8D containing $^{125}$I-C3. The cells were washed, and the $^{125}$I-C3 that was associated with the outer membrane and O-Ag capsule was measured as described above.

**Determination of C3 Binding to Biotinylated IgG on 12105 During Serum Incubation.** We determined the extent of covalent attachment of C3 to biotinylated IgG on 12015 during incubation in serum. Strain 12015 was suspended to OD$_{600}$ 1.5 in HBSS**. Aliquots of 2 ml were incubated for 20 min at room temperature with 300 µg/ml of either biotinylated
or nonbiotinylated IgG. Cells were washed once in HBSS++, then resuspended to the original volume in HBSS++. Presensitized organisms were mixed with an equal volume of either 20% C8D or 20% Δ56°C C8D in HBSS++. Suspensions were incubated for 60 min at 37°C with agitation. Aliquots of 150 μl were removed, applied to microcentrifuge tubes containing 1 ml of cold HBSS++, and centrifuged for 5 min at 12,500 g. The supernatant was removed and the counts per minute of 125I within the bacterial pellet were determined with a gamma scintillation counter. The total number of molecules of C3 bound per colony-forming unit within the bacterial pellet was determined as indicated earlier (8). The remainder of the samples were washed twice in HBSS++, and the washed pellets were solubilized in 1 ml of 1% sodium dodecyl sulfate (SDS) at 100°C for 10 min. Insoluble material was removed by centrifugation for 5 min at 12,500 g. The supernatants were diluted with 2 vol of 0.01 M Tris buffer, pH 8.5, containing 0.25 M NaCl, 0.3% SDS, and 1% Triton X-100 (buffer A) and applied to 200 μl of packed avidin-Sepharose. Buffer A was shown in preliminary experiments to minimize nonspecific binding of 125I-C3 to avidin-Sepharose in comparison with buffers containing a lower concentration of NaCl or different ionic (deoxycholate) or zwitterionic [SB12, CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate)] detergents. Mixtures were incubated overnight at room temperature. Avidin-Sepharose was washed three times with buffer A and the 125I-C3 bound to the resin was determined by counting in a gamma scintillation counter.

Preparation of C3b-IgG. Covalent complexes of C3b-IgG were prepared with minor modifications of the procedure described by Fries et al. (9). To 1 ml of IgG at 45 mg/ml in phosphate-buffered saline (PBS) was added 370 μg of 125I-C3 (IgG-to-C3 ratio of 120:1). N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp., Freehold, NJ) was added to achieve a trypsin-to-C3 ratio of 0.02:1.0 by weight. The sample was incubated for 8 min at 37°C, followed by the addition of the protease inhibitor β-nitrophenyl p-guanidinobenzoate (NPGB) (Sigma Chemical Co., St. Louis, MO) to 25 μM. Solid NaCl was added to achieve a final concentration of 1 M, and one part of 2.0 M acetate buffer, pH 5.6, was added to 19 parts of the C3-IgG mixture to reach a final of 0.1 M acetate. The sample was applied to a 1.5 × 100 cm column containing Ultrogel AcA-34 (LKB Instruments, Inc., Gaithersburg, MD) and chromatographed in 1 M NaCl, 0.1 M acetate buffer, 25 μM NPGB, pH 7.0. The elution profile is shown in Fig. 1A. Pool 1 was concentrated in a diafiltration cell (Amicon Corp., Danvers, MA) using a PM 30 membrane, and dialyzed against 90 mM NaCl, 20 mM PO4, 25 μM NPGB, pH 7.0. The dialyzed sample was applied to QAE Sephadex A-50 in the same buffer containing 0.002% gelatin and 0.01 M EDTA, and the column was eluted with a linear salt gradient from 90 to 250 mM NaCl in 20 mM PO4, 25 μM NPGB, 0.002% gelatin, pH 7.0. Pools 1 and 2 were made as indicated (Fig. 1A), concentrated by diafiltration, dialyzed against PBS, and analyzed as described below.

Characterization of Pools from QAE Sephadex A-50. Pools from QAE Sephadex A-50 were tested by double-diffusion analysis in 1% agarose gels containing antisera to human C3 and to rabbit IgG (Cappel Laboratories, Cochranville, PA). Samples eluted from Ultrogel AcA-34 (Fig. 1A) and from QAE Sephadex A-50 (Fig. 1B) were analyzed by 4–8% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (9). Gels were dried and autoradiography was done with Kodak XAR-5 film and DuPont Cronex Lightning Plus intensifying screens.

Pools from QAE Sephadex A-50 were analyzed by sucrose density gradient ultracentrifugation. Before application to sucrose density gradients, samples of 125I-IgG and 125I-C3b-IgG were centrifuged at 178,000 g for 20 min at room temperature in an air-driven ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). The top 50% of the tube was removed, and 25 μl was applied to a linear 10–40% sucrose gradient in PBS in 2 × 0.5-in polyallomer tubes (Beckman Instruments). Samples were centrifuged for 18 h at 4°C in an SW 50.1 rotor at 35,000 rpm in a Beckman L8 ultracentrifuge. Three-drop fractions were collected using a gradient fractionator (Bio-Rad Laboratories, Richmond, CA). Radioactivity within gradient fractions was determined by counting in a gamma scintillation counter. Molecular weight markers were applied to separate gradient tubes.
and included \(^{125}\text{I}}\text{-C9 (4.5 S), }^{125}\text{I}}\text{-C3 (8.0 S), and }^{125}\text{I}}\text{-fibronectin (13.6 S), the latter kindly supplied by Dr. John Bohnsack, NIH, Bethesda, MD. Peak tubes within the }^{125}\text{I}}\text{-C3b-IgG and }^{125}\text{I}}\text{-IgG gradient peaks were dialyzed against PBS, and the remainder of the gradient peaks were pooled and dialyzed against PBS and stored at 4°C. Most subsequent experiments with }^{125}\text{I}}\text{-C3b-IgG and }^{125}\text{I}}\text{-IgG prepared from sucrose density gradients were performed within 3 d of collection of the pools to minimize reaggregation within monomer preparations.}

**Serum Bactericidal Assays with IgG and C3b-IgG.** Killing of 12015 was measured in Abs PNHS after presensitization with \(^{125}\text{I}}\text{-IgG or }^{125}\text{I}}\text{-C3b-IgG. To 100-μl aliquots of strain 12015 (at OD\textsubscript{600} 0.300 in HBSS ÷+) were added 20-μl samples containing either 36, 180, or 900 ng of \(^{125}\text{I}}\text{-IgG or 80, 400, or 2000 ng of }^{125}\text{I}}\text{-C3b-IgG, an equimolar amount. Before use for presensitization, both }^{125}\text{I}}\text{-IgG and }^{125}\text{I}}\text{-C3b-IgG were centrifuged for 20 min at 178,000 g in an air-driven ultracentrifuge. The top 50% of the sample was removed for subsequent presensitization, and the remainder of the sample was discarded. ~40% of the applied }^{125}\text{I}}\text{ counts were recovered in the top 50% of the centrifuge tube for both }^{125}\text{I}}\text{-IgG and }^{125}\text{I}}\text{-C3b-IgG.}

For some experiments, 12015 was presensitized with samples of \(^{125}\text{I}}\text{-IgG and }^{125}\text{I}}\text{-C3b-IgG from sucrose density gradients. When pooled samples from the sucrose density gradient profile were used, 12015 was presensitized with 30, 150, and 750 ng of \(^{125}\text{I}}\text{-IgG and with 55, 275, and 1,375 ng of }^{125}\text{I}}\text{-C3b-IgG. Alternatively, 12015 was presensitized with the same amounts of C3b-IgG and IgG from the peak tubes of the sucrose density gradient profile. Mixtures were incubated at room temperature for 40 min, then divided into two aliquots of 50 μl each. An equal volume of 20% Abs PNHS in HBSS ÷÷ was added to one aliquot and 50 μl of Δ56°C Abs PNHS in HBSS ÷÷ was added to the other aliquot. Samples were incubated at 37°C for 60 min, and viable bacterial colonies were quantitated as previously described.**

Quantitative binding studies were done to determine the amount of \(^{125}\text{I}}\text{-IgG and }^{125}\text{I}}\text{-C3b-IgG bound to 12015 during the presensitization step. 30 μl of the reaction mixture was removed, applied to microcentrifuge tubes containing 1 ml of HBSS ÷÷, and centrifuged for 5 min at 12,500 g. The supernatant was aspirated and the pellet was counted in a gamma scintillation counter. Nonspecific binding of \(^{125}\text{I}}\text{-IgG and }^{125}\text{I}}\text{-C3b-IgG was determined in tubes containing a 50-fold excess of unlabeled IgG or IgG and C3b.**

**Results**

**Distribution of C3 and IgG on 12015.** The distribution of IgG and C3 on 12015 was determined after serum incubation. 82% of the \(^{125}\text{I}}\text{-IgG that bound to 12015 was distributed with the supernatant (O-Ag) fraction of presensitized 12015 after lysis in a French press cell and separation of O-Ag capsule and outer membrane fractions (Table I). The distribution of \(^{125}\text{I}}\text{-C3b on the O-Ag capsule and outer membrane of 12015 was also determined. Total C3 deposition on 12015 was increased by 66% after presensitization with bactericidal IgG (Table I). The partitioning of C3 was similar to that of IgG. ~80% of the C3 deposited on 12015 was in the O-Ag capsule fraction, in both the presence and absence of bactericidal IgG. The remaining 20% of the C3 was deposited on the outer membrane. These results showed that bacterial IgG did not function by redirecting a significant fraction of total C3 deposition from the O-Ag capsule to the outer membrane.**

**Binding of C3 to IgG on E. coli 12015.** We next investigated the possibility that C3 was deposited on the IgG molecule itself during serum incubation. Results showed that nearly one-fifth of the \(^{125}\text{I}}\text{-C3 that deposited on presensitized 12015 attached covalently to antibody (Table II). Furthermore, this value may be an underestimate of the percentage of C3 attached to IgG: only 86% of \(^{125}\text{I}}\text{-
TABLE I

| Component   | Total No. of molecules per CFU (×10^4) preceding French press lysis | Distribution after French press lysis |
|-------------|--------------------------------------------------------------------|--------------------------------------|
|             |                                                                  | In pellet | In supernatant |
| ^125^I-IgG  | 2                                                                  | 3.8 ± 1.3* | 18.0 ± 3.4 82.0 ± 3.4 |
| ^125^I-C3  | Without IgG                                                       | 4.1 ± 0.7 | 21.6 ± 6.4 78.4 ± 6.4 |
|            | With IgG                                                          | 6.8 ± 0.3 | 18.7 ± 5.1 81.3 ± 5.1 |

* Mean ± SD. The molecules of IgG and C3 bound to serum-treated 12015 were measured under conditions described in Materials and Methods. The distribution of these molecules was determined after French press lysis of the cells.

TABLE II

| Antibody   | Serum         | Percent of ^125^I-C3 on 12015 binding to avidin-Seph |
|------------|---------------|-------------------------------------------------------|
| Biotinyl IgG | 10% C8D      | 18.4 ± 2.1*                                          |
| Biotinyl IgG | 10% Δ56°C C8D | 1.0 ± 0.3                                             |
| IgG         | 10% C8D      | 0.5 ± 0.3                                             |
| None        | 10% C8D      | 0.8 ± 0.4                                             |

* Mean ± SD of two experiments performed in duplicate. Bacteria were presensitized with the indicated antibody source, then incubated in C8D serum containing ^125^I-C3. Cells were solubilized in 1% SDS and applied to avidin-Seph, and the percentage of ^125^I-C3 bound was determined.

biotinyl-IgG on 12015 bound to avidin-Seph under similar conditions (data not shown), suggesting that recovery of IgG on avidin-Seph was incomplete. Furthermore, ~11% of the total ^125^I-C3 deposited on 12015 was bound nonspecifically (data not shown). Therefore, as much as 24.0% (18.4% × 100/86 × 100/89) of specifically bound C3 may attach to IgG. Since the enhanced C3 deposition on 12015 mediated by IgG accounts for 40% of the total C3 bound, these results imply that at least half of the additional C3 deposited on 12015 as a consequence of presensitization with IgG is covalently bound to IgG.

Preparation of C3b-IgG. The results from the above experiments suggested that formation of C3b-IgG complexes might be critical to the action of bactericidal antibody. To study this possibility, we prepared complexes of ^125^I-C3b-IgG. Details of the purification scheme are provided in Materials and Methods. The column elution profiles are shown in Fig. 1, along with a 4–8% SDS-PAGE autoradiogram of selected samples from the purification process. The final ^125^I-C3b-IgG preparation for use in experiments was pool 1 from QAE Sephadex A-50. Three major iodinated bands were apparent by SDS-PAGE analysis of pool
BACTERICIDAL ACTIVITY OF C3b-IgG

Figure 1. Preparation of C3b-IgG. (A) Elution profile of C3b-IgG preparation on Ultrogel AcA-34 in 1 M NaCl, 0.1 M acetate, and 25 μM NPGB, pH 5.6. Pool 1 was concentrated, dialyzed, and applied to QAE Sephadex. (B) Elution profile of pool 1 from Ultrogel AcA-34 on QAE Sephadex A-50 in 90 mM NaCl, 20 mM NaPO₄, and 25 μM NPGB, pH 7.0, containing 0.002% gelatin. A linear NaCl gradient from 90 to 250 mM was applied. Pools 1 and 2 were prepared as indicated and analyzed by SDS-PAGE. (C) 4–8% SDS-PAGE autoradiography of pools from Ultrogel AcA-34 and QAE Sephadex A-50.

1 (Fig. 1C). A band migrating at 110,000 daltons is compatible with the noncovalently linked, free α' chain of C3b, and a band migrating at 75,000 daltons is compatible with the β chain of both free C3b and the C3b within C3b-IgG complexes. The most prominent band in the Sephadex A-50 QAE pool 1 migrated at ~180,000 daltons. This location is most consistent with a covalent complex between the α' chain of C3b and the heavy chain of IgG, as previously reported by others (9–12). A less intense band of 140,000 daltons presumably represents covalent attachment of the α' chain of C3b to the light chain of IgG.
By densitometric scanning of a less intensively exposed autoradiogram, 83% of the total $\alpha'$ activity was contained in the C3b-heavy chain complex.

Three separate lines of evidence suggested that minimal unbound IgG contaminated pool 1. First, IgG did not bind to QAE Sephadex A-50 and separated broadly from pools 1 and 2 (data not shown). Second, the agglutination titers of C3b-IgG and IgG were identical when equivalent inputs of IgG were compared (data not shown). Third, as discussed below, binding of $^{125}$I-IgG and $^{125}$I-C3b-IgG to 12015 was equivalent at equimolar inputs of the iodinated moiety.

We have shown in other experiments (data not shown) that up to 60% incorporation of $^{125}$I-C3 into $^{125}$I-C3b-IgG can occur and that the specific radioactivity of $^{125}$I-C3b is not changed significantly (<=15%) in comparison to $^{125}$I-C3. Therefore, IgG content within $^{125}$I-C3b-IgG can be calculated based on the specific activity of $^{125}$I-C3b within the complex. This finding was independently confirmed by (a) total protein determination on isolated $^{125}$I-C3b, isolated IgG, and on an aliquot of the $^{125}$I-C3b-IgG used in our experiments, which was purified without the use of gelatin; and (b) radial immunodiffusion assays (for gelatin-containing samples) on $^{125}$I-C3b-IgG and purified $^{125}$I-C3b in plates detecting C3c antigen. Specific activity (cpm/µg) of C3c antigen in $^{125}$I-C3b-IgG and uncomplexed $^{125}$I-C3b was identical.

**Bactericidal Activity of $^{125}$I-IgG and $^{125}$I-C3b-IgG.** We compared the bactericidal activity of $^{125}$I-IgG and $^{125}$I-C3b-IgG for 12015 in 10% Abs PNHS. At equivalent molar inputs of IgG, presensitization with $^{125}$I-C3b-IgG led to more extensive killing of 12015 than that with $^{125}$I-IgG alone (Fig. 2). In three separate experiments, the concentration of IgG required for 0.8 log$_{10}$ killing was 8.2–9.6 times greater than the concentration of IgG within $^{125}$I-C3b-IgG required for 0.8 log$_{10}$ killing. These results suggested that IgG within C3b-IgG was markedly more effective than IgG alone in presensitizing 12015 for serum killing.

![Figure 2. Bactericidal activity of C3b-IgG and IgG for 12015 in 10% Abs PNHS. 12015 was presensitized with increasing and equimolar concentrations of either IgG or C3b-IgG before incubation in 10% Abs PNHS. Log$_{10}$ killing was determined as described in Materials and Methods.](image-url)
Bactericidal Activity of C3b-IgG

Table III

Binding of $^{125}$I-C3b-IgG and $^{125}$I-IgG to 12015

| IgG input | Molecules per CFU ($\times 10^3$) |
|-----------|----------------------------------|
|           | C3b-IgG                          | IgG               |
| 750       | 8.0 $\pm$ 2.2*                   | 10.5 $\pm$ 4.0    |
| 150       | 1.7 $\pm$ 0.5                    | 1.8 $\pm$ 0.8     |

* Mean $\pm$ SEM for three experiments performed in duplicate. The bacteria were sensitized with equimolar inputs of $^{125}$I-C3b-IgG or $^{125}$I-IgG, and the number of molecules of each that bound to the organisms was determined.

Binding of $^{125}$I-C3b-IgG and $^{125}$I-IgG to 12015. We tested the possibility that enhanced bactericidal activity of $^{125}$I-C3b-IgG relative to $^{125}$I-IgG represented more efficient binding of $^{125}$I-C3b-IgG to 12015 during the presensitization step. Direct measurements of $^{125}$I-C3b-IgG and $^{125}$I-IgG binding to 12015 were performed in conjunction with the serum killing assays. Equivalent numbers of $^{125}$I-C3b-IgG and $^{125}$I-IgG molecules were bound per colony-forming unit (CFU) at equivalent molar inputs of IgG (Table III). This observation was true for both the $^{125}$I-IgG and $^{125}$I-C3b-IgG monomer preparations (Table III) prepared as described below by sucrose density gradient ultracentrifugation, and for the preparations used in the experiment shown in Fig. 2 (data not shown). Nearly fivefold more molecules of $^{125}$I-C3b-IgG and $^{125}$I-IgG were bound with a fivefold increase in input (150 to 750 ng) of IgG. Accurate determination of $^{125}$I-C3b-IgG and $^{125}$I-IgG binding at inputs of 30 ng was not feasible.

Sucrose Density Gradient Analysis of $^{125}$I-C3b-IgG and $^{125}$I-IgG. Aggregate formation within the $^{125}$I-C3b-IgG preparation could account for the enhanced bactericidal activity of $^{125}$I-C3b-IgG in comparison with $^{125}$I-IgG. In the experiments described above, large aggregates were removed from both the $^{125}$I-C3b-IgG and $^{125}$I-IgG preparations by ultracentrifugation before use. However, smaller oligomeric aggregates might not have been removed. Therefore, isokinetic 10–40% sucrose density gradient ultracentrifugation of $^{125}$I-C3b-IgG and $^{125}$I-IgG was performed to prepare putative monomer pools (Fig. 3). In addition, an aliquot was removed from the peak tube of the $^{125}$I-C3b-IgG and $^{125}$I-IgG peaks before collection of the monomer pools. Only a small percentage (<3%) of the samples applied to the gradient contained aggregates large enough to sediment to the bottom of the gradient, making it unlikely that aggregates accounted for the enhanced bactericidal activity of $^{125}$I-C3b-IgG in comparison with IgG (Fig. 2). This presumption was directly tested in the next series of experiments.

Serum Bactericidal Assay with Sucrose Density Gradient Pools. Pools containing $^{125}$I-C3b-IgG and $^{125}$I-IgG monomers from sucrose density gradients were used to presensitize 12015 for serum bactericidal assays. At equivalent molar inputs of IgG, killing with $^{125}$I-C3b-IgG significantly exceeded killing with $^{125}$I-IgG (Fig. 4). At IgG inputs of 150 ng or less, $^{125}$I-C3b-IgG was three- to fourfold more efficient than $^{125}$I-IgG at mediating bactericidal activity. The difference in log$_{10}$
FIGURE 3. 10-40% sucrose density gradient analysis of IgG and C3b-IgG. Samples of $^{125}$I-IgG and $^{125}$I-C3b-IgG were pre-spun for 20 min at 178,000 g in an air-driven ultracentrifuge to remove large aggregates, then applied to 10-40% sucrose density gradients in polymallomer tubes. Samples were ultracentrifuged for 18 h at 4°C in an SW 50.1 rotor at 55,000 rpm, and three-drop fractions were collected. Pools of $^{125}$I-C3b-IgG and $^{125}$I-IgG were made as indicated. Molecular weight markers were $^{125}$I-C9 (4.5 S), $^{125}$I-C3 (8.0 S), and $^{125}$I-fibronectin (13.6 S). Pools were made as indicated for use in serum bactericidal assays. The shoulders on the up-slope of the $^{125}$I-C3b-IgG profile presumably represent $^{125}$I-C3b not covalently bound to IgG, as suggested by the SDS-PAGE autoradiogram in Fig. 1.

FIGURE 4. Bactericidal activity of $^{125}$I-C3b-IgG and $^{125}$I-IgG from sucrose density gradient pools. The experimental conditions were as outlined in the legend to Fig. 2, except that $^{125}$I-C3b-IgG and $^{125}$I-IgG from sucrose density gradient pools were used for presensitization. Results shown represent mean ± SD for three experiments performed in duplicate. Killing at an input of 750 ng was also statistically significant. These results indicate that monomeric $^{125}$I-C3b-IgG is more effective than monomeric $^{125}$I-IgG in presensitizing 12015 for serum killing.
Bactericidal IgG markedly enhances alternative complement pathway–mediated killing of *E. coli* 12015, although deposition of C3 is only modestly increased in the presence of the antibody. We show here that up to one quarter of deposited C3 binds covalently to the bactericidal antibody molecule. These findings raise the possibility that C3b-IgG is particularly effective at mediating the serum bactericidal reaction. To test this presumption, C3b-IgG complexes were prepared. These complexes were found to have markedly enhanced bactericidal activity, since three- to fourfold fewer C3b-IgG molecules than IgG molecules were required to bind to 12015 to render the strain serum sensitive. Since C3b deposits on intact IgG molecules on 12015 during serum incubation of antibody-coated bacteria, it is possible that only IgG-bearing C3b can mediate the serum bactericidal reaction for 12015.

Conceptually, the enhanced bactericidal activity of C3b-IgG may be due to a change in the number, localization, or form of deposited C3b-9. However, our previous experiments with this organism indicated that IgG enhanced the bactericidal efficiency of C5b-9 without increasing the number of bound C5b-9 complexes. The present data suggest that IgG does not redirect complement deposition to a different location on the bacterial surface, since the distribution of C3b between capsule and outer membranes was unchanged. It is more likely that IgG changes the organization of C5b-9 on the outer membrane. C3b-IgG complexes may serve as an efficient focus for C5 convertase formation (13, 14) since C3b bound to IgG is relatively protected from inactivation by factors H and I (9). Clustering of C5b-9 complexes around the site of IgG deposition could result, which could, in turn, influence the capacity of deposited C5b-9 to disrupt the rigid bacterial outer membrane. In fact, in the absence of antibody, the deposited C5b-9 on *E. coli* 0111 does not insert into hydrophobic domains of the outer membrane whereas, in the presence of antibody, such insertion does occur (2).

It is also possible that covalent complexes of C3b-IgG alter the form rather than the localization of the C5b-9 complex. The stoichiometry of C9 binding within C5b-9 alters the bactericidal efficiency of the complex. We have recently shown (15) that an average C9/C5b-8 ratio of at least 3.3:1 is required for killing of the rough *E. coli* strain J5, and maximal killing is not achieved until the C9/C5b-8 ratio exceeds 6:1. A C9/C5b-8 ratio of 1:1 does not result in bacterial death. Previous experiments (1) showed that the average C9/C5b-8 ratio on 12015 was the same (5.7–6.4 to 1) in the presence or absence of bactericidal IgG. However, a change in the C9/C5b-8 ratio of the complexes associated with C3b-IgG might have been undetectable since these complexes represent a minority of the total C5b-9 deposited on the cell.

The bactericidal activity of IgG for 12015 is mediated through the alternative complement pathway (1). Antibody-dependent, alternative pathway–mediated killing of gram-negative bacteria is well documented (16–19), but C3b deposition on antibody was not examined in previous experiments. Covalent binding of C3 to antibody has been demonstrated during alternative pathway activation by immune complexes (9, 10), and it is postulated but not proven that this C3 is critical for the resultant dissolution of the complex. Moore et al. (20) showed
that immune rabbit IgG enhanced alternative pathway activation by mouse erythrocytes by increasing the efficiency of C3b deposition and C5 convertase formation without changing the extent of C3 consumption. The same authors suggested that C3 may have been deposited on the antibody molecule (21), but this presumption was not directly tested. In contrast, Schenkein and Ruddy (22) could not demonstrate C3 binding to IgG on zymosan during antibody-mediated acceleration of alternative pathway activation.

Antibody appears to be a particularly good acceptor for C3b deposition (23). The site of C3b deposition on the IgG molecule has been examined. Gadd and Reid (10) demonstrated that during activation of the alternative pathway by immune aggregates containing rabbit IgG, the α' chain of C3b binds covalently to one or two sites in the Fd portion of the heavy chain of IgG. Similar results were reported by Takata et al. (11). In both of these systems, C3b deposition was mediated by the alternative complement pathway. Brown et al. (12) reported that C3b binds to both heavy and light chains of rabbit IgG on Streptococcus pneumoniae when complement is activated through the classical pathway using purified C1, C4, C2, and C3 (11). Arnaout and colleagues (24) demonstrated light chain uptake of C3b using trypsin cleavage of C3. We did not examine the immunoglobulin chains bearing C3b when C3b-IgG complexes were formed on 12015 during serum incubation. However, the highly efficient C3b-IgG complex used in these experiments contained C3b that attached predominantly to the heavy chain of the rabbit IgG. This result using the IgG fraction of bactericidal antiserum is in accord with the findings of Fries et al. (9), who prepared complexes of C3b and human IgG by trypsinization.

Isotypes of IgG that serve as good acceptors for C3b deposition may be more effective bactericidal and opsonic molecules than isotypes which are inefficient C3 acceptors. Similarly, monoclonal antibodies from various animal species, if used for passive protection, may vary in their bactericidal activity, depending not only on their antigenic specificity but also on their capacity to serve as acceptors for C3b deposition. These theoretical possibilities are important in the consideration of vaccine development. Moreover, the ability to prepare C3b-IgG complexes that have greatly increased cytocidal efficiency may have important clinical implications.

**Summary**

The mechanism was sought by which bactericidal IgG for *E. coli* 0111 (strain 12015) increases the bactericidal efficiency of C5b-9. IgG did not affect the distribution of C3 deposition on the O-Ag capsule and the outer membrane of 12015, suggesting that bactericidal IgG was not directing complement activation to different sites on the bacterial surface. However, one-fifth of the C3 that was deposited in the presence of IgG attached covalently to the antibody molecule. Covalent complexes between purified C3b and IgG were prepared in order to study the role of C3b-IgG in the bactericidal reaction. 8–10-fold less C3b-IgG than IgG was necessary to sensitize 12015 for serum killing. When aggregates were eliminated from the C3b-IgG and IgG preparations by sucrose density gradient ultracentrifugation, C3b-IgG remained three- to fourfold more effective than IgG on a molecule-for-molecule bound basis in mediating the serum
BACTERICIDAL ACTIVITY OF C3b-IgG

bactericidal reaction. These results suggest that formation of C3b-IgG during the serum bactericidal reaction is critical for killing, and have important implications for the development of effective bactericidal vaccines.

Received for publication 25 March 1985 and in revised form 15 May 1985.

References

1. Joiner, K. A., R. C. Goldman, C. H. Hammer, L. Leive, and M. M. Frank. 1983. Studies of the mechanism of bacterial resistance to complement-mediated killing. V. IgG and F(ab')2 mediate killing of E. coli 011B4 by the alternative complement pathway without increasing C5b-9 deposition. J. Immunol. 131:2563.

2. Joiner, K. A., M. A. Schmetz, R. C. Goldman, L. Leive, and M. M. Frank. 1984. Mechanism of bacterial resistance to complement-mediated killing: inserted C5b-9 correlates with killing for Escherichia coli 0111B4 varying in O-antigen capsule and O-polysaccharide coverage of lipid A core oligosaccharide. Infect. Immun. 45:113.

3. Joiner, K. A., R. C. Goldman, C. H. Hammer, L. Leive, and M. M. Frank. 1983. Studies on the mechanism of bacterial resistance to complement-mediated killing, VI. IgG increases the bactericidal efficiency of C5b-9 for E. coli 0111B4 by acting at a step before C5 cleavage. J. Immunol. 131:2570.

4. Goldman, R. C., D. White, and F. Orskov. 1982. A surface polysaccharide of Escherichia coli 0111 contains O-antigen and inhibits agglutination of cells by O-antiserum. J. Bacteriol. 151:1210.

5. Berger, M., T. A. Gaither, R. M. Cole, T. M. Chused, C. H. Hammer, and M. M. Frank. 1982. Biotinylation of human C3. Mol. Immunol. 19:857.

6. Joiner, K. A., R. Goldman, M. Schmetz, M. Berger, C. H. Hammer, M. M. Frank, and L. Leive. 1984. A quantitative analysis of C3 binding to O-antigen capsule, lipopolysaccharide, and outer membrane protein of E. coli 0111B4. J. Immunol. 132:369.

7. Hammer, C. H., G. H. Wirtz, L. Renfer, H. D. Gresham, and B. F. Tack. 1981. Large scale isolation of functionally active components of the human complement system. J. Biol. Chem. 256:3995.

8. Joiner, K. A., C. H. Hammer, E. J. Brown, R. J. Cole, and M. M. Frank. 1982. Studies on the mechanism of bacterial resistance to complement-mediated killing. I. Terminal complement components are deposited and released from Salmonella minnesota S218 without causing bacterial death. J. Exp. Med. 155:809.

9. Fries, L. F., T. A. Gaither, C. H. Hammer, and M. M. Frank. 1984. C3b covalently bound to IgG demonstrates a reduced rate of inactivation by factors H and I. J. Exp. Med. 160:1640.

10. Gadd, K. J., and K. B. M. Reid. 1981. The binding of complement component C3 to antibody-antigen aggregates after activation of the alternative pathway in human serum. Biochem. J. 195:471.

11. Takata, Y., N. Tamura, and T. Fugita. 1984. Interaction of C3 with antigen-antibody complexes in the process of solubilization of immune precipitates. J. Immunol. 132:2531.

12. Brown, E. J., M. Berger, K. A. Joiner, and M. M. Frank. 1983. Classical complement pathway activation by antipneumococcal antibodies leads to covalent binding of C3b to antibody molecules. Infect. Immun. 42:594.

13. Daha, M. R., D. T. Fearon, and K. F. Austen. 1976. C3 requirements for formation of alternative pathway C5 convertase. J. Immunol. 117:630.

14. Medicus, R. G., O. Götze, and H. J. Müller-Eberhard. 1976. Alternative pathway of
complement: recruitment of precursor properdin by the labile C3/C5 convertase and potentiation of the pathway. *J. Exp. Med.* **144**:1076.

15. Joiner, K. A., M. A. Schmetz, M. E. Sanders, T. G. Murray, C. H. Hammer, R. Dourmashkin, and M. M. Frank. Multimeric C9 is necessary for killing of *E. coli* J5 by C5b-9. *Proc. Natl. Acad. Sci. USA.* In press.

16. Hill, W., A. L. Shears, and K. G. Hibbit. 1978. The requirement of specific antibody for the killing of *E. coli* by the alternative complement pathway in bovine serum. *Immunology.* **34**:131.

17. Steele, E. J., and D. Rowley. 1977. The mechanism of complement fixation by rabbit F(ab')2: properties of the bactericidal reaction. *Immunochimistry.* **14**:319.

18. Tarr, P. I., S. W. Hosea, E. J. Brown, R. Schneerson, A. Sutton, and M. M. Frank. 1982. The requirement of specific antcapsular IgG for killing of *Haemophilus influenzae* by the alternative pathway of complement activation. *J. Immunol.* **128**:1772.

19. Steele, N. P., R. S. Munson, D. M. Granoff, J. E. Cummins, and R. P. Levine. 1984. Antibody-dependent alternative pathway killing of *Haemophilus influenzae* type b. *Infect. Immun.* **44**:452.

20. Moore, F. D., D. T. Fearon, and K. F. Austen. 1981. IgG on mouse erythrocytes augments activation of the human alternative complement pathway by enhancing deposition of C3b. *J. Immunol.* **126**:1805.

21. Moore, F. D., K. F. Austen, and D. T. Fearon. 1982. Antibody restores human alternative complement pathway activation by mouse erythrocytes rendered functionally deficient by pre-treatment with pronase. *J. Immunol.* **128**:1302.

22. Schenkein, H. A., and S. Ruddy. 1981. The role of immunoglobulins in alternative pathway activation by zymosan. II. The effect of IgG on the kinetics of the alternative pathway. *J. Immunol.* **126**:11.

23. Jacobs, R. J., and M. Reichlin. 1983. Generation of low M.W. C3-bearing immunoglobulin in human serum. *J. Immunol.* **130**:2775.

24. Arnaout, M. A., N. Dana, J. Melamed, R. Medicus, and H. R. Colten. 1983. Low ionic strength or chemical cross-linking of monomeric C3b increases its binding affinity to the human complement C3b receptor. *Immunology.* **48**:229.