C3b COVALENTLY BOUND TO IgG DEMONSTRATES A REDUCED RATE OF INACTIVATION BY FACTORS H AND I

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Cleavage of C3 by the physiologic C3 convertases or by trypsin results in the generation of a metastable form of C3b bearing a reactive internal thioester (1). This thioester may undergo hydrolysis by solvent water molecules or may form ester or amide linkages with suitable acceptors. Immunoglobulin G (IgG) is reported to display a measurable affinity for uncleaved C3 (2) and has been shown to be an excellent acceptor of nascent C3b (3). In accord with these observations, it has been shown that complement activation by soluble, IgG-bearing immune complexes or IgG-sensitized bacteria results in substantial deposition of C3b onto antibody molecules (4–6). More recently, "innocent bystander" IgG in serum has been shown to bind C3b when complement is activated by exogenous immune complexes (7).

Several lines of evidence suggest that binding to IgG may alter the characteristics of C3b. It is well known that IgG enhances the rate and/or extent of alternative pathway activation in an Fc fragment–independent manner on a variety of activating surfaces (8–10). Further, IgG can confer alternative pathway–activating potential on nonactivating surfaces (11, 12). Sensitization with IgG dramatically augments complement-mediated killing of some serum-resistant bacteria. Recent studies by Joiner et al. (13) have shown that this effect is obtained only if IgG is present on the bacterial surface at the time of initial C3b deposition; addition of IgG subsequent to this step has little influence (13). This effect, which persists even when overall C3b uptake on the subject organisms is equalized, suggests an intimate interaction between C3b and IgG on the bacterial surface that is not replaced by random juxtaposition of the molecules. Using a fluid phase system, Medoff et al. (14) have shown that C3b which is incorporated into soluble, IgG-bearing immune complexes is largely insusceptible to the action of factors H (β1H) and I (C3b inactivator) at concentrations capable of quickly cleaving free C3b or C3b bound to "nonactivating" particles such as sheep erythrocytes.

In view of these data, we have undertaken studies of the influence of binding to IgG on the subsequent behavior of C3b. We report here that small, heterodimeric complexes of C3b and IgG are readily formed in vitro by trypsin cleavage

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of C3 in the presence of IgG. The majority of C3b α' chains in these complexes are covalently bound to IgG heavy chains by a hydroxylamine-sensitive bond. The rate of cleavage of heavy chain–bound α' chains by factors H and I is markedly slowed relative to free C3b α' chains. This relative resistance to factor H– and factor I–mediated cleavage results solely from a lowered affinity of the C3b covalently linked to IgG (C3b-IgG) for factor H. Substitution for IgG of ceruloplasmin, a glycoprotein of similar size but no known immunologic activity, results in hetero-dimers that display no retardation of α' chain cleavage relative to free C3b, suggesting that this may be a special property of IgG. On a weight basis, C3b complexed to IgG is shown to be significantly more effective in alternative pathway consumption of serum C3 than is free fluid-phase C3b.

Materials and Methods

Protein Preparations. C3 was prepared from normal human plasma by a modification of the method of Hammer et al. (15). C3 was functionally, immunochemically, and electrophoretically homogeneous. C3 was stored at 4°C in 0.15 M NaCl, 0.05 M phosphate buffer (pH 7.4), 0.01 M EDTA, and 25 μM *p*-nitrophenyl *p'*-guanidinobenzoate (NPGB)[1] (Sigma Chemical Co., St. Louis, MO). Freshly prepared C3 was used in trypsinization experiments after dialysis against phosphate-buffered isotonic saline, pH 7.4 (PBS). Stored C3 with diminished hemolytic activity was dialyzed against 0.1 M NaCl and 0.02 M phosphate buffer (pH 7.0) with 0.002 M EDTA and subjected to ion exchange chromatography on QAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ). As previously described (16), application of a linear salt gradient of 0.1–0.25 M NaCl allowed the separation of a fraction enriched for hemolytically active C3 eluting at conductivities of 11.0–12.5 mS/cm at 0°C. This material was concentrated, dialyzed against PBS, and used in the same manner as fresh C3.

Factor H was prepared from human plasma as previously described (17). Factor I was purchased from Cordis Laboratories Inc., Miami, FL, in a partially purified state and adsorbed twice with an anti–factor H immunoabsorbent to deplete all factor H activity (17).

Human IgG and ceruloplasmin were obtained as byproducts of C3 preparation (15). IgG was recovered from the drop-through fraction of plasma proteins applied to DEAE-Sephacel (Pharmacia Fine Chemicals) in 0.0032 M Na/K phosphate buffer (pH 7.4, 1.37 mS/cm at 0°C) containing protease inhibitors and EDTA as described (15). Application of a salt gradient to the above column resulted in the elution of ceruloplasmin at conductivities of 7.7–8.0 mS/cm at 0°C (15). Both IgG and ceruloplasmin were concentrated and exchanged into 0.15 M NaCl, 0.05 M phosphate buffer (pH 7.4), 0.01 M EDTA, and 0.02% sodium azide with the use of a Minitan ultrafiltration system (Millipore Corp., Bedford, MA). IgG produced a single line in immunoelectrophoresis against goat anti–whole human serum (Cappel Laboratories, Cochranville, PA), while ceruloplasmin demonstrated a trace contaminant (not seen on sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]), which proved to be C9 by double diffusion analysis. Before use in C3b-binding experiments, IgG and ceruloplasmin were concentrated as needed by vacuum dialysis, exhaustively dialyzed against PBS, and centrifuged for 30 min at 48,000 *g* to remove large aggregates.

Additional Reagents. Lyophilized human serum albumin (HSA), gelatin, and soybean

[1] *Abbreviations used in this paper:* CR_1, the C3b receptor; C3b-IgG, C3b covalently linked to immunoglobulin G; HSA, human serum albumin; iC3b, inactivated C3b, the major first cleavage product of C3b produced by the action of factors H and I; Mg/EGTA/NHS, 80% fresh normal human serum containing 0.002 M $MgCl_2$ and 0.01 M EGTA; NHS, normal human serum; NPGB, *p*-nitrophenyl *p'*-guanidinobenzoate; PBS, phosphate-buffered isotonic saline, pH 7.4; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; VBS, veronal-buffered isotonic saline, pH 7.4.

trypsin inhibitor were obtained from Sigma Chemical Co. Trypsin-TPCK was obtained from Worthington Biochemical Corp., Freehold, NJ. Goat anti-human C3 antiserum was produced in our laboratory as previously described (15). Goat anti-human ceruloplasmin and anti-HSA antisera were purchased from Atlantic Antibodies, Westbrook, ME, and staphylococcal protein A–Sepharose 4B was obtained from Pharmacia Fine Chemicals. For hemolytic titrations of C3, sheep erythrocytes were obtained from the National Institutes of Health animal facility. Guinea pig C1 was purchased from Cordis Laboratories, Inc.; functionally pure C2, C4, and C5 through C9 were prepared from human plasma by the methods of Hammer et al. (15).

Radiolabeling of C3. Purified human C3 was labeled with ^{125}I by either a modification of the method of Bolten and Hunter (18) or with *N*-chloro-benzenesulfonamide–derived polystyrene beads (Iodobeads; Pierce Chemical Co., Rockford, IL). In both cases, labeling was carried out at melting ice bath temperature. Labeled C3 retained 70–80% of its hemolytic activity and had specific activities of 0.136–0.329 μCi/μg C3.

Preparation of C3b-IgG and C3b-Ceruloplasmin Complexes. Human C3 labeled with ^{125}I (0.5–0.9 mg) was mixed with a 250-fold molar excess of IgG in 2–3 ml of PBS (final IgG concentration, 50–75 mg/ml) and warmed to 37°C. An amount of freshly dissolved trypsin-TPCK equivalent to 2% of C3 by weight was added, and the mixture was incubated for 8 min at 37°C (conditions were based on preliminary studies to determine optimal conditions for complete C3 conversion to C3b, without significant further fragmentation, in concentrated IgG). The cleavage was terminated by the addition of a fourfold weight excess of soybean trypsin inhibitor and a further 5 min incubation at 37°C. 2 M sodium acetate (pH 5.6) was then added to a final concentration of 0.1 M, and solid NaCl was added to achieve a concentration of 1 M. NPGB (0.05 M in dimethylformamide) was added to 25 μM, and the entire mixture was applied to a 1.5 × 100 cm column containing Ultrogel AcA-34 (LKB Instruments, Inc., Gaithersburg, MD) in 1 M NaCl and 0.1 M acetate (pH 5.6) with 25 μM NPGB and chromatographed in this buffer at a flow rate of 10 ml/h. Heavy and light peaks of ^{125}I radioactivity were pooled as shown in Fig. 1*A*. In initial experiments ^{125}I-C3b-IgG hetero-dimers were further enriched from the higher molecular weight pool by repeat AcA-34 chromatography, while the bulk of monomer IgG was removed from ^{125}I-C3b in the lower molecular weight pool by immunoadsorption with Sepharose-coupled goat anti-human IgG. In later experiments, both pools from the AcA-34 column were concentrated and dialyzed against 0.09 M NaCl, 0.02 M phosphate buffer (pH 7.0), 0.002% gelatin, and 25 μM NPGB. The pools were applied separately to 10–15-ml columns containing QAE-Sephadex A-50 in the same starting buffer. After being washed with two column volumes of starting buffer, the columns were developed with linear salt gradients of 0.09–0.26 M NaCl in 0.02 M phosphate (pH 7.0) with NPGB and gelatin. Elution was carried out at 0.5 column volumes/h, and 0.25 column volume fractions were collected. Fig. 1*B* depicts typical ion exchange profiles so obtained. Pool *C* represents the ^{125}I-C3b-IgG hetero-dimer, while pool *D* contains ^{125}I-C3b. SDS-PAGE analysis of the various pools, followed by autoradiography, is shown in Fig. 2. The purification of ^{125}I-C3b bound to ceruloplasmin was obtained by analogous methods, save that ceruloplasmin could be concentrated only to 30–40 mg/ml before significant precipitation occurred. ^{125}I-C3b-ceruloplasmin hetero-dimers were enriched from the initial reaction mixture by two sequential AcA-34 chromatography steps. ^{125}I-C3b and ^{125}I-C3b conjugates were concentrated by vacuum dialysis and stored in PBS with 0.02% sodium azide at 4°C.

Assay of Cleavage by Factors H and I of Free and Bound C3b α′ Chains. ^{125}I-C3b, which was either free, in covalent complex with IgG, or in complex with ceruloplasmin, was diluted to the desired concentration in PBS containing 0.01 M EDTA and 25 μM NPGB. A baseline sample was immediately removed, added to twice its volume of SDS-PAGE sample buffer containing 0.05 M Tris-HCl (pH 6.7), 5% SDS, 0.01% bromphenol blue, 25% sucrose, and 3% 2-mercaptoethanol, and heated at 95°C for 5 min. In serum cleavage studies, the experimental mixture was prewarmed to 37°C, and an appropriate amount of NHS pretreated with 0.01 M EDTA and 25 μM NPGB was added at time zero. Aliquots of the reaction mixture were removed at timed intervals and immediately

mixed with sample buffer and heated as above. In dose-response experiments with factors H and I, mixtures of the ^{125}I-C3b species, purified factor H, and factor I in the desired concentrations in PBS with 0.01 M EDTA and 25 μM NPGB were assembled in a melting ice bath and transferred to a 37°C bath at time zero. After the desired time, two volumes of the above sample buffer were added to each specimen and the mixture heated as above.

Specimens were subjected to SDS-PAGE by the method of Maizel (19) with 5, 7, or 4–8% gradient polyacrylamide slab gels in a model 220 vertical electrophoresis cell (Bio-Rad Laboratories, Richmond, CA). After being stained with Coomassie Blue, the gels were dried. Autoradiography was performed with the use of 18–24-h exposures of DuPont Cronex 6-Plus x-ray film at −60°C in cassettes containing DuPont Cronex Lightning Plus intensifying screens. Densitometric scanning of autoradiographs was carried out on a spectrophotometer (DU-8; Beckman Instruments, Inc., Fullerton, CA) with white light; peaks were integrated, and their area was expressed as a percentage of total for the lane. Cleavage of the various chains was monitored by plotting their diminution relative to the entire lane with time, with the percentage of the relevant chain taken at time zero as a 100% value.

Consumption of C3 from Serum by C3b Species. ^{125}I-C3b or ^{125}I-C3b-IgG were diluted to desired concentrations in ice-cold veronal-buffered isotonic saline (VBS), pH 7.4, containing 40 mg/ml of HSA and 2 mM $MgCl_2$. 4 vol of fresh normal human serum (NHS) were mixed with 1 vol of 0.01 M $MgCl_2$ and 0.05 M EGTA in VBS (Mg/EGTA-NHS). Sufficient Mg/EGTA-NHS to achieve a final serum dilution of 1:25 was then added on ice to tubes containing buffer alone or various dilutions of ^{125}I-C3b or ^{125}I-C3b-IgG. One buffer tube was immediately treated with an equal volume of ice-cold VBS containing 0.02 M EDTA and held on ice. All other tubes were placed simultaneously into a 37°C bath and incubated for 30 min. After 30 min, all tubes were returned to the ice bath and an equal volume of ice-cold VBS with 0.02 M EDTA was added to stop the reaction. In preliminary kinetic experiments, we found that C3 consumption by both C3b species had plateaued by 20 min at 37°C. Residual hemolytic C3 titrations were then carried out on all specimens by standard methods (15). C3 titers were based on the mean of four determinations on the linear portion of the dose-response curve. Results were expressed as percent consumption of hemolytic serum C3 relative to the buffer control not exposed to 37°C.

Results

Formation of ^{125}I-C3b-IgG. ^{125}I-C3b-IgG hetero-dimers were formed in small amounts by trypsinization of ^{125}I-C3 in the presence of IgG at serum concentrations. Increasing IgG input to 60–70 mg/ml and a 250-fold molar excess over ^{125}I-C3 resulted in routine incorporation of 50–60% of ^{125}I-C3 radioactivity into high molecular weight forms, the majority of which were bound by staphylococcal protein A–Sepharose (Figs. 1, *left*, and 2). ^{125}I-C3b-IgG was separated from ^{125}I-C3b by sequential sieve and anion exchange chromatography as described in Materials and Methods (Fig. 1, *left* and *right*). SDS-PAGE of ^{125}I-C3b revealed a single band with an apparent molecular weight of 190,000 under nonreducing conditions (Fig. 2). This band gave rise to α' and β chains upon reduction, with apparent molecular weights of 114,000 and 76,000, respectively. ^{125}I-C3b-IgG contained a major band of 330,000 mol wt and a minor band of the same size as ^{125}I-C3b on nonreduced gels. As shown in Fig. 2, reduction of this material with 2% 2-mercaptoethanol revealed α' and β chains, as in ^{125}I-C3b, as well as a major band at ~190,000 mol wt (consistent with α' bound to IgG heavy chains) and a minor band of 145,000 mol wt (consistent with α' bound to IgG light chains). These latter bands were identical to those adsorbed from the initial high molecular weight pool by staphylococcal protein A (Fig. 2). ^{125}I-C3b-IgG pools

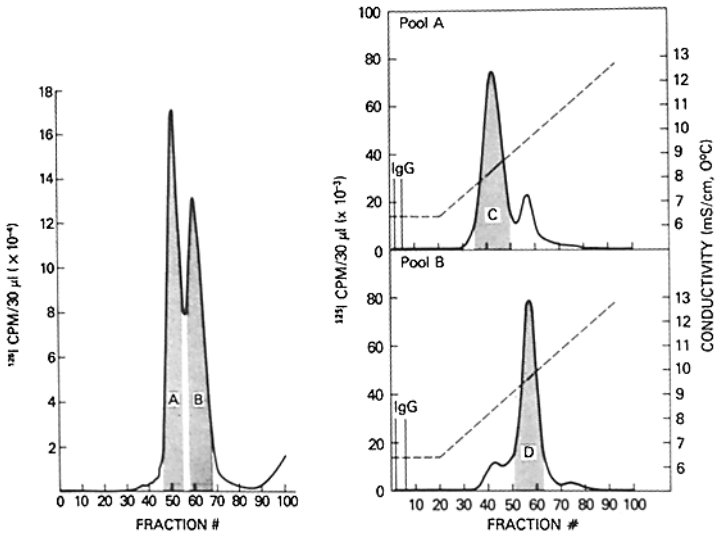

FIGURE 1. Chromatographic separation of C3b-IgG from C3b. (*Left*) Chromatographic profile of ^{125}I activity after AcA-34 sieve chromatography of the ^{125}I-C3b-IgG/^{125}I-C3b mixture resulting from tryptic cleavage of ^{125}I-C3 in the presence of a 250-fold molar excess IgG. The column dimensions were 1.5 × 100 cm, and chromatography was carried out in 1 M NaCl, 0.1 M acetate (pH 5.6), and 25 μM NPGB. The flow rate was 10 ml/h, and fractions were 0.01 column volumes. SDS-PAGE of pool *A* (^{125}I-C3b-IgG fraction) and pool *B* (^{125}I-C3b fraction) is shown in Fig. 2, *d* and *g*, respectively. (*Right*) Anion exchange chromatography of pools *A* (*upper* profile) and *B* (*lower* profile) on 10–15-ml columns containing QAE-450. Pools were applied in 0.09 M NaCl, 0.02 M phosphate (pH 7.0), 0.002% gelatin, and 25 μM NPGB to columns preequilibrated in the same buffer. Columns were washed with two column volumes of starting buffer and then developed with a linear 0.09–0.26 M NaCl gradient. Elution was carried out at 0.5 column volumes/h, collecting 0.25 column volume fractions. ^{125}I activity is shown by a solid line, conductivity (mS/cm, 0°C) by a broken line. Free IgG appeared in the drop-through fractions as shown. SDS-PAGE of pool *C* (^{125}I-C3b-IgG) and pool *D* (^{125}I-C3b) is shown in Fig. 2, *a*, *b*, *h*, and *i*.

demonstrated persistent contamination with 5–10% free ^{125}I-C3b; this contaminant may have arisen from noncovalent association of ^{125}I-C3b with IgG or spontaneous release of ^{125}I-C3b from the covalent complex. Both mechanisms have been previously described (2, 20). During prolonged storage at 4°C in PBS (pH 7.4), α′ chain was progressively released (without apparent proteolytic cleavage) from both heavy and light chain with an approximate half-life of 22 d (first-order rate constant of ~0.0013/h). The average ratio of α′-heavy chain to α′-light chain was 3.2:1 in four separate preparations. Treatment of ^{125}I-C3b-IgG with 1 M hydroxylamine and 1% SDS (pH 11.0) for 15 min at 37°C resulted in virtually complete release of radioactivity into two bands identical with the α′ and β of free ^{125}I-C3b on reduced SDS-PAGE (data not shown).

Inactivation of ^{125}I-C3b and ^{125}I-C3b-IgG by Serum. To assess the relative susceptibility of free C3b and C3b bound to IgG to inactivation by factors H and I, we studied the kinetics of cleavage of ^{125}I-labeled α′ chains and ^{125}I-labeled α′-heavy chains in the presence of dilute serum. 5 μg/ml of ^{125}I-C3b, which was free or bound to IgG, was treated with 1:50 NHS in PBS containing 0.01 M EDTA and 25 μM NPGB, as described in Materials and Methods. Aliquots were

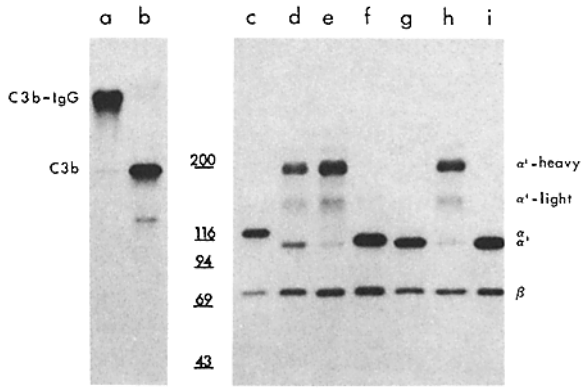

FIGURE 2. SDS-PAGE and autoradiography of ^{125}I-C3b-IgG and ^{125}I-C3b pools. SDS-PAGE on 4–8% polyacrylamide gradient gel. (*a*) ^{125}I-C3b-IgG (pool C), nonreduced. (*b*) ^{125}I-C3b (pool D), nonreduced. (*c*) ^{125}I-C3, reduced. (*d*) High molecular weight pool after AcA-34 (pool A), reduced. (*e*) Staphylococcal protein A–binding material eluted from protein A–Sepharose after immunoabsorption of pool A; reduced. (*f*) Nonadsorbed material after immunoabsorption of pool A with protein A–Sepharose; reduced. (*g*) Low molecular weight pool after AcA-34 (pool B); reduced. (*h*) ^{125}I-C3b-IgG (pool C), reduced. (*i*) ^{125}I-C3b (pool D), reduced. Standards are shown $\times 10^3$.

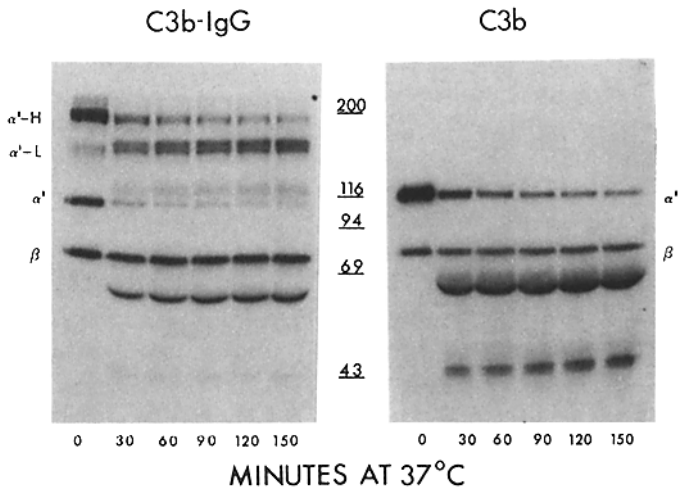

FIGURE 3. Serum inactivation of ^{125}I-C3b-IgG and ^{125}I-C3b. SDS-PAGE on 7% polyacrylamide gel and autoradiography of aliquots of ^{125}I-C3b-IgG (*left*) and ^{125}I-C3b (*right*) before (time 0), and at timed intervals during, incubation with 1:50 NHS in PBS (pH 7.4) with 0.01 M EDTA and 25 μM NPGB. Standards indicated are $\times 10^3$.

removed at time zero and after various time intervals and studied by SDS-PAGE and autoradiography. A typical autoradiogram is shown in Fig. 3. A progressive diminution in both α′-heavy chain and α′ chain was seen, with the latter being cleaved more rapidly. ^{125}I-C3b α′ cleavage gave rise to a fragment with a molecular weight of 43,000 (occasionally a doublet) and a broad band with an apparent molecular weight of 60,000. This latter band was distorted by serum albumin, for when SDS-PAGE of the cleavage products was performed after immunoprecipitation with anti-C3–Sepharose and washing away of serum pro-

teins, this fragment was shown to have an apparent molecular weight of 69,000 as previously reported by others (21). ^{125}I-C3b-IgG α' chain cleavage gave rise to a more complex pattern (Fig. 3). As with ^{125}I-C3b, a 43,000 mol wt fragment was produced. Cleavage of α'-heavy chain gave rise to a major fragment migrating just above or merging with uncleaved α'-light chain. The behavior of α'-light chain was difficult to assess because of this co-migrating fragment, but could be inferred from the appearance of a new fragment with an apparent molecular weight of ~120,000. Because the larger cleavage fragments of α'-heavy chain and α'-light chain behaved as somewhat heavier molecules than anticipated (150,000 vs. 124,000 and 120,000 vs. 94,000, respectively), we subjected serum-cleaved ^{125}I-C3b-IgG to treatment with 1 M hydroxylamine and 1% SDS, pH 11.0, for 15 min at 37°C. The fragments released were identical to the 69,000 and 43,000 mol wt fragments derived from ^{125}I-C3b (data not shown), suggesting that cleavage proceeded at the same site in free and IgG-bound C3b and that the 120,000 mol wt fragment arose from α'-light chain, not a second cleavage of the 150,000 mol wt α'-heavy chain fragment.

The relative rates of inactivation of C3b in the free state or bound to IgG were examined by densitometric scanning of autoradiograms of kinetic experiments as described in Materials and Methods. Fig. 4 depicts the cleavage kinetics of radiolabeled α'-heavy chain and free α' chain in six experiments in which we used three separate preparations of ^{125}I-C3b and ^{125}I-C3b-IgG. Cleavage of ^{125}I-labeled α'-heavy chain was uniformly retarded relative to that of ^{125}I-labeled α' chain. A statistically significant difference in the percentage of relevant α' chain

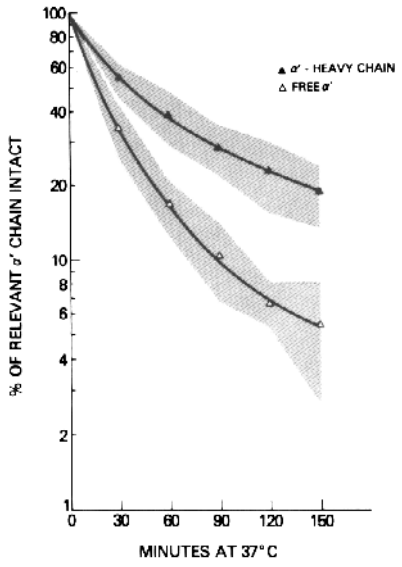

FIGURE 4. Kinetics of serum-mediated α' chain and α'-heavy chain cleavage. Cleavage of ^{125}I-labeled α' chain (open symbols) and ^{15}I-labeled α'-heavy chain (closed symbols) was followed by densitometric scanning of autoradiographs of kinetic inactivation experiments (Fig. 3). Cleavage of both ^{125}I-labeled α' chain species was assessed by diminution relative to the zero time point, which was assigned a value of 100%. Data shown represent mean values from six experiments; shaded areas denote 95% confidence intervals for the experimentally determined values.

intact was apparent at every time point examined. The kinetics of α'-light chain cleavage were not readily evaluable because of interference by the 150,000 mol wt cleavage fragment of α'-heavy chain.

Interaction of Factors H and I with ^{125}I-C3b and ^{125}I-C3b-IgG. Since the initial conversion of C3b to inactivated C3b (iC3b) is accomplished in serum by the concerted action of factors H and I, we reasoned that the interaction of one or both of these proteins with C3b may be altered when C3b is bound to IgG, thus producing a slowed rate of inactivation. To investigate this possibility, we studied the extent of cleavage of ^{125}I-labeled α' chain and ^{125}I-labeled α'-heavy chain under standardized conditions (30 min, 37°C, fixed factor I input) as functions of purified factor H concentration in the reaction mixture. Factor H concentrations of 0.715–174 μg/ml were studied in reaction systems containing 5–6 μg/ml of ^{125}I-C3b residues. As shown in Fig. 5, the dose-response curve for α'-heavy chain cleavage was significantly displaced from that of free α' chain. Over the majority of the range studied, approximately threefold more factor H was required for equivalent α'-heavy chain cleavage when compared with α' chain. These data suggested that binding to IgG significantly reduced the affinity of ^{125}I-C3b for factor H. To address the possibility that binding to IgG might also directly alter the interaction of C3b with factor I, two cubic polynomials describing the central portions of the curves in Fig. 5 were derived using a general polynomial least squares curve-fitting program. With the use of these expressions, factor H inputs of 17.7 μg/ml for ^{125}I-C3b and 51.9 μg/ml for ^{125}I-C3b-IgG were chosen as giving equivalent cleavage of 38% of the relevant α' chain under the standard conditions, and a dose-response experiment was performed over a range of factor I dilutions. As shown in Fig. 6, the dose-response curves generated were virtually identical. These results lead us to conclude that, given sufficient

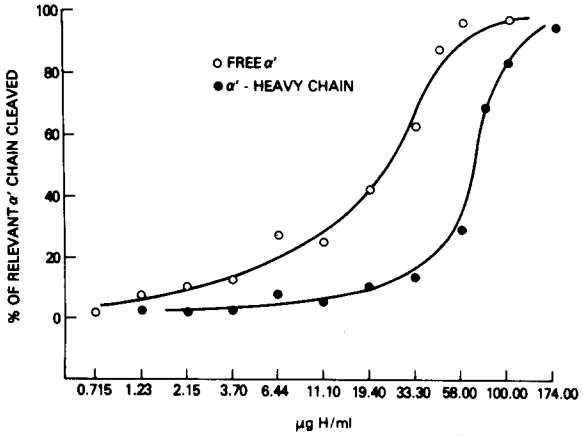

FIGURE 5. Cleavage of α' chain and α'-heavy chain as a function of factor H input. Cleavage of ^{125}I-labeled α' chains (open symbols) and ^{125}I-labeled α'-heavy chains (closed symbols) is plotted as a function of the final concentration of factor H in the reaction system. Cleavage was allowed to proceed for 30 min at 37°C in the presence of a constant dilution of factor I approximating 1:50 serum. Curves shown were generated from a cubic polynomial derived by application of a least-squares general polynomial curve-fitting program to the data points.

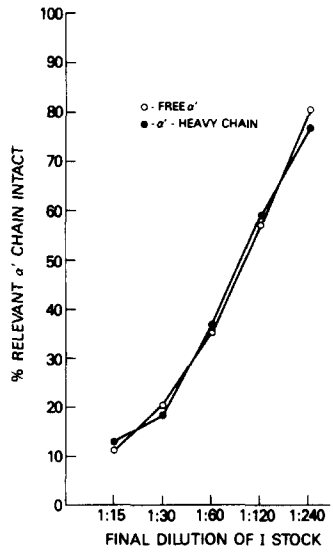

FIGURE 6. Cleavage of α' and α'-heavy chain as a function of factor I input. ^{125}I-C3b was mixed with 17.7 μg/ml of factor H and ^{125}I-C3b-IgG with 51.9 μg/ml factor H on the basis of data in Fig. 5, and cleavage was allowed to proceed at 37°C for 30 min in the presence of a range of dilutions of partially purified factor I. Cleavage of ^{125}I-labeled α' chain and ^{125}I-labeled α'-heavy chains was assessed as described in Materials and Methods.

factor H, the susceptibility to factor I activity was not intrinsically reduced for the IgG heavy chain–bound α' chain.

Cleavage of C3b Bound to a Non-IgG Glycoprotein. At a molecular weight of 150,000–160,000, IgG represents a bulky substituent when covalently linked to C3b. We questioned whether similar retardation of factor H– and I–mediated inactivation might occur when any glycoprotein of similar size was bound to C3b. We chose ceruloplasmin to test this hypothesis on the basis of its significant carbohydrate content (8.4%), similarity to IgG in molecular weight (134,000 vs. 150,000–160,000), and ready availability in good purity in our laboratory. We prepared ^{125}I-C3b-ceruloplasmin by methods analogous to those used in preparing ^{125}I-C3b-IgG and enriched the preparation for the hetero-dimer by two sequential sieve chromatography steps. As shown in Fig. 7, this preparation contained ~38% of total and 54% of α' chain–associated radioactivity in a broad band with an apparent molecular weight of 265,000–270,000 on reduced SDS-PAGE. This band could be immunoadsorbed by anti-ceruloplasmin–Sepharose, and radioactivity was specifically precipitated by anti-ceruloplasmin antisera in double diffusion (Fig. 7, inset). The cleavage of ^{125}I-labeled α'-ceruloplasmin in serum was studied in a manner analogous to that used for α' chain and α'-heavy chain. As shown in Fig. 8, ^{125}I-C3b α' chain bound to ceruloplasmin was cleaved by serum factors H and I with kinetics indistinguishable from those of free ^{125}I-C3b α' chain cleavage. These data suggested that the size of the substituent molecule bound to C3b may not be the sole determinant of C3b α' chain cleavage rate, and are consistent with the hypothesis that binding to IgG may mediate an enhancement of C3b survival not common to all C3b acceptors.

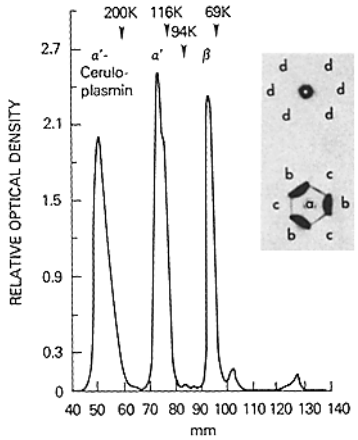

FIGURE 7. ^{125}I-C3b-ceruloplasmin. The ^{125}I-C3b-ceruloplasmin pool was subjected to SDS-PAGE under reducing conditions in a 5% slab gel. After staining and drying, autoradiography was performed as described in Materials and Methods, followed by densitometric scanning. The abscissa denotes the internal scale of gel-scanning apparatus and does not necessarily correspond to the absolute dimensions of the gel. The position of molecular weight marker proteins is noted above the scan profile. Inset demonstrates double diffusion analysis of ^{125}I-C3b-ceruloplasmin in 1% agarose. An antigen mixture of ^{125}I-C3b-ceruloplasmin (~10 μg/ml) with 100 μg/ml each of unlabeled ceruloplasmin, C3b, and HSA, was placed in central wells (a). Antisera in surrounding wells included anti-C3 (*b*), anti-ceruloplasmin (*c*), and anti-HSA (*d*). Note that ^{125}I (confined to C3b) was precipitated by anti-ceruloplasmin, but was not significantly trapped nonspecifically by HSA–anti-HSA precipitin lines.

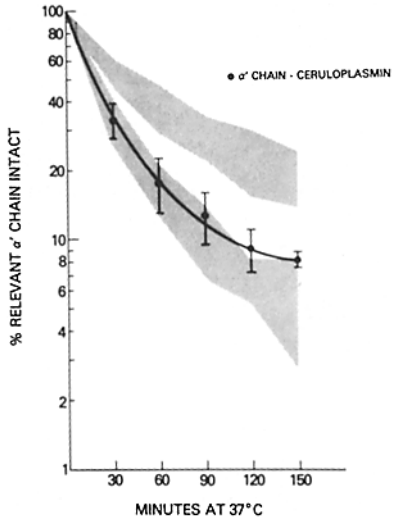

FIGURE 8. Kinetics of serum-mediated α′ chain–ceruloplasmin cleavage. Cleavage of ^{125}I-labeled α′ chain–ceruloplasmin by 1:50 serum was quantitated as previously described for α′ chain and α′-heavy chain. Data shown represent means and standard errors for three experiments and are superimposed on shaded areas denoting the 95% confidence intervals for α′ chain (*lower* band) and α′-heavy chain (*upper* band) cleavage kinetics.

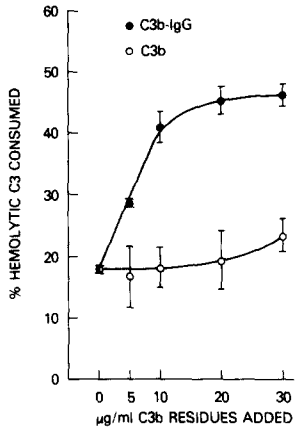

FIGURE 9. Consumption of C3 by C3b-IgG and C3b. Varying amounts of ^{125}I-C3b, free (open symbols) or covalently bound to IgG (≦10% free C3b, closed symbols), were added to 1:25 mg/EGTA-NHS, and the consumption of hemolytic C3 was assessed after 30 min at 37°C. Data shown represent means and standard errors from three experiments in which two different C3b-IgG preparations were used. 18.3% consumption at zero input represents the decline of C3 titer of 1:25 Mg/EGTA/NHS after 30 min at 37°C in buffer alone (relative to an aliquot of 1:25 Mg/EGTA-NHS held on ice in 0.01 M EDTA for 30 min).

Consumption of Serum C3 by C3b and C3b-IgG. We next studied the capacity of free ^{125}I-C3b and ^{125}I-C3b-IgG to mediate alternative pathway activation in serum. The two ^{125}I-C3b species were diluted in VBS (pH 7.4) with 2 mM $MgCl_2$ and 40 mg/ml HSA, and the capacity of equal amounts of free or IgG-bound ^{125}I-C3b to mediate hemolytic C3 consumption from 1:25 Mg/EGTA-NHS was studied over a range of ^{125}I-C3b residue inputs. As shown in Fig. 9, both C3b and C3b-IgG demonstrated a dose-dependent consumption of serum C3 activity. However, while free C3b inputs of >20 μg/ml were required for consistently observable consumption in excess of buffer controls, C3b-IgG produced significant consumption of C3 at inputs of 5 μg/ml. Markedly greater reductions in C3 titer were caused by C3b-IgG, compared with C3b, at all concentrations tested. Centrifugation of ^{125}I-C3b and ^{125}I-C3b-IgG at 178,000 *g* for 30 min to remove large aggregates failed to alter either consumption curve.

Discussion

Deposition of C3b at a site of complement activation is critical to the initiation of assembly of the late components (C5–C9) into the membrane-attack complex and also serves to engage the alternative, or amplification, pathway for further C3b production (21). These activities are abrogated when C3b is inactivated to form iC3b. In addition, C3b mediates attachment to specific receptors on phagocytic cells and primate erythrocytes (22). C3b dramatically enhances IgG-mediated phagocytosis of opsonized particles and, under appropriate circumstances, can itself precipitate phagocytosis (23). Inactivated C3b functions in some of these roles, but may not be equivalent to C3b in all cases (24).

The survival of C3b in an active state on an immune complex, soluble or particulate, may therefore be of considerable importance in determining the fate

of the complex. As we have previously noted, a variety of evidence suggests that C3b may exhibit altered behavior when bound to IgG. One interpretation of such evidence is that binding to IgG prolongs survival of C3b in the presence of the various control proteins, and the current experiments were undertaken to examine this possibility. Classically, C3b is inactivated in serum by the concerted action of factors H and I (25). More recently (14, 26), the role of the C3b receptor (CR_1) as a cofactor for factor I has been appreciated. While CR_1 may be central to the handling of large but soluble immune complexes in the blood, its role in C3b inactivation outside the bloodstream or on particulate complexes remains to be defined. Further, since CR_1 exhibits very low affinity for C3b monomer at normal ionic strength, the interaction of this receptor with complexes bearing only one C3b residue, or a low density of such residues, would be limited (3). Accordingly, we chose the well-characterized factor H and I system for our initial investigations of C3b-IgG inactivation.

In agreement with several previous reports (3, 27), we found monomeric IgG to be a ready acceptor of nascent C3b. C3b appeared capable of binding to both light and heavy chains of IgG, although heavy chain was the predominant acceptor, accounting for an average of 76% of bound α' chain. This finding agrees well with existing data from a variety of experimental systems (4, 5), wherein the major C3b acceptor site in IgG has been found to reside in the heavy chain and probably the Fd fragment. Light chain uptake of C3b has previously been reported by Brown et al. (6), who used a classical pathway system on bacteria, and by Arnaout and colleagues (3), who used trypsin cleavage of C3, whereas several other investigators (4, 5) have not noted such uptake in systems examining alternative pathway activation by model immune complexes. In our studies, virtually all bound α' chain was removed from both light and heavy chains of IgG by hydroxylamine treatment. This finding is at variance with those of Gadd and Reid (4) and Brown et al. (6), who found hydroxylamine sensitivities of 26 and 66%, respectively. More recently (5), however, Takata et al. have shown complete hydroxylamine release of C3b deposited via the alternative pathway on model immune complexes containing either intact IgG or $F(ab')_2$ fragments. They postulated that C3b may bind to IgG via an ester linkage to hydroxyl-bearing amino acids. This hypothesis is compatible with the findings reported here. The relative prevalence of ester and amide linkages, as well as the extent of light chain uptake of C3b, is probably a function of the mechanism of C3 activation and the state of the accepting IgG. It is reasonable to conclude, however, that C3b linked to a site within the heavy chain of IgG via an ester bond represents the major C3b-IgG species in most systems thus far reported (3–6). The cleavage of α'-heavy chain complexes that arise from such linkage is the subject of our kinetic data.

We observed that the α' chain of C3b is cleaved by serum factors H and I into fragments with molecular weights of 69,000 and 43,000, as has been demonstrated in a previous report (21). The lower molecular weight fragment frequently appeared as a 43,000 and 46,000 doublet, probably reflecting partial release of the 3,000 mol wt fragment C3f (21). The products of C3b-IgG α' chain cleavage included the same 43,000–46,000 mol wt fragments, as well as a major band of 150,000 and a minor band of 120,000. Hydroxylamine treatment

released a typical 69,000 mol wt α' chain fragment from both of these species, suggesting that they arise separately and not sequentially, and thus represent the products of α'-heavy chain and α'-light chain cleavage. The unexpectedly high apparent molecular weights of these C3b-IgG fragments presumably represents anomalous behaviour in SDS-PAGE; similar results have been reported by Takata et al. (5).

A consistent retardation of factor H– and I–mediated cleavage of heavy chain–linked C3b α' chain, relative to free C3b α' chain, was noted. The same extent of retardation was noted in C3b-IgG preparations containing from 5–30% contamination with free C3b, and thus is unlikely to represent competitive inhibition by free C3b. Additionally, in factor H dose-response experiments, large differences in the extent of α' and α'-heavy chain cleavage were readily observable even when the latter was exposed to factor H inputs >10-fold greater than total combined α' and α'-heavy chain input. Such dose-response experiments demonstrated that retarded inactivation of C3b-IgG is an exclusive result of diminished affinity of C3b-IgG for factor H. There was little or no observable alteration in the shape of the factor I dose-response curve for C3b-IgG inactivation relative to free C3b, provided that a sufficient level of factor H was supplied. This finding is similar to previous observations (28) which suggest that the major determinant of impaired C3b inactivation on alternative pathway–activating surfaces is reduced affinity for factor H.

The mechanism by which IgG interferes with the interaction of factor H and C3b is unclear. The simplest possibility would be a direct steric hindrance based on the size of the IgG molecule closely applied to C3b. Our experiments with ^{125}I-C3b-ceruloplasmin hetero-dimers demonstrated, however, that a glycoprotein similar in size to IgG could be bound to C3b with little apparent effect on the rate of serum-mediated α' chain cleavage. Additionally, in preliminary studies of the cleavage of low densities of C3b residues on sheep erythrocytes by 1:50 NHS at normal ionic strength, using the method of Gaither (17), we demonstrated kinetics quite similar to those we observed with fluid phase C3b. Taken together, these findings suggest that binding to IgG may render C3b relatively less susceptible to the action of factors H and I in a manner not common to all potential acceptors. It is possible to speculate that the portion(s) of IgG that mediate noncovalent binding to C3 and C3b (2) also provide a protective microenvironment for the covalently bound species. Alternatively, the relatively low sialic acid content of IgG compared with ceruloplasmin (and the majority of other human glycoproteins) may mediate protection of C3b by an as yet undefined mechanism (28).

C3b-IgG appears to be much more effective, on a molecule-for-molecule basis, than free C3b in mediating alternative pathway consumption of serum C3. This finding not only reinforces our observations regarding the resistance of C3b-IgG to inactivation, but suggests that IgG does not significantly interfere with the binding of factor B to C3b. In this manner also, C3b-IgG behaves similarly to C3b bound to an alternative pathway–activating surface (28).

The mechanism by which IgG exerts its effects on alternative pathway activation has not been delineated. Our data suggest that C3b which is covalently bound to IgG, even in small hetero-dimeric forms, demonstrates retarded inac-

tivation by factors H and I and behaves similarly to C3b bound to an alternative pathway–activating surface. Thus, it is possible to speculate that IgG confers alternative pathway–activating potential on nonactivating surfaces by providing a protected site for C3b deposition. On alternative pathway activators, IgG might act synergistically to further depress the action of the control proteins on deposited C3b. C3b covalently linked to IgG could, by virtue of its enhanced survival, participate more effectively in both C3 and C5 convertases with resultant increased C3b deposition and enhanced initiation of membrane-attack complex formation. Data suggesting the operation of both of these mechanisms have been obtained in bacterial killing and erythrocyte lysis systems (8–13). In addition, the long-lived C3b-IgG complex might be a potent signal for ingestion by phagocytic cells, since it presents closely linked ligands for two opsonically active cell surface receptors. In the case of C3b-bearing soluble immune complexes, our data may in part explain the observations of Medoff (14) demonstrating that erythrocyte CR_1 is required to support factor I inactivation of complex-bound C3b, although additional mechanisms must be operative to explain the apparent total insusceptibility of such C3b to factors H and I. Recent work by Jacobs and Reichlin (7) suggests that C3b-IgG hetero-dimers are formed from "innocent bystander" IgG in serum during complement activation. Since these small hetero-dimers would be expected to bind poorly, if at all, to CR_1 at normal ionic strength (3), retarded inactivation by factors H and I might contribute to prolonged circulation of these complexes as C3b-IgG, with as yet unpredictable effects on phagocytic cell receptors and, perhaps, immunoregulatory functions.

Summary

We have prepared C3b covalently linked to IgG via a hydroxylamine-sensitive bond between the C3b α' chain and sites predominantly, but not exclusively, located in the IgG heavy chain. This C3b species displays relative resistance to inactivation by factors H and I when compared with free C3b. This resistance appears to be due entirely to reduced affinity of C3b-IgG for factor H. Resistance to inactivation is not conferred on C3b by binding to another serum glycoprotein of similar size, ceruloplasmin, and may be a special property of IgG. C3b-IgG demonstrates an enhanced capacity to consume serum C3 relative to C3b. These alterations of the behavior of C3b when bound to IgG may in part explain the augmentation of alternative pathway activity by IgG. In addition, IgG-induced protection of C3b might influence both complement-mediated killing and phagocytosis of bacteria, as well as modify the in vivo handling of IgG-containing soluble immune complexes.

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