IN VITRO ADHERENCE OF SOLUBLE IMMUNE COMPLEXES TO MACROPHAGES*

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(Received for publication 30 March 1972)

Cells of the mononuclear phagocytic system possess specific receptors for γG-globulin (γG)[1] (1) and for the third component of the complement system, C3 (2). The characteristics of these receptors have been studied in vitro with rosette formation of coated erythrocytes around macrophages (3). The in vitro adherence of antibody-coated erythrocytes to macrophages was inhibited by less than physiological amounts of free γG in the medium. This inhibition was overcome by adding complement, a fact which suggests that complement may be necessary in vivo to mediate the uptake of immune complexes. However, recent in vivo studies showed no differences in the uptake of soluble immune complexes by the fixed macrophages of the liver after infusion into either normal or complement-depleted rabbits (4). The in vitro adherence of soluble immune complexes to macrophages has not been characterized. Macrophages may interact in a different fashion with soluble complexes than with antibodies on particulate antigens; this is because actual lattice formation does not exist with particulate antigens sensitized by subagglutinating amounts of antibodies.

Soluble immune complexes and aggregated γG-globulin have been thought to possess equivalent biological properties on a weight basis. Complement fixation, fixation to skin, and the ability to induce an Arthus lesion were comparable when the same amounts of homologous γG were employed in soluble complexes or aggregates (5). Complement fixation by soluble complexes (6) and adherence of soluble complexes to macrophages (7) were attributed primarily to a summation of individual binding sites offered by many antibodies in the lattice work. The presence of the same biological properties in heat-aggregated γG was concluded to be due to an analogous increase in the number and proximity of binding sites (8).

The present studies were designed to characterize the in vitro adherence of soluble complexes to macrophages. The data supported the conclusion that sol-

* Supported in part by Research Grant AM 11476 from the National Institute of Arthritis and Metabolic Diseases.

‡ Recipient of a Research and Education Associateship from the Veterans Administration.

[1] *Abbreviations used in this paper:* anti-HSA, rabbit antibodies against HSA; CFA, complete Freund's adjuvant; CoF, cobra venom factor; γG, γG-globulin; HGG, human γG-globulin; HSA, human serum albumin; RGG, rabbit γG-globulin; SDG, sucrose density gradient; SGG, sheep γG-globulin.

uble immune complexes containing more than two molecules of antibody preferentially adhered to macrophages in the absence of complement. Furthermore, the inhibition of adherence by free γG was not significantly altered by the addition of complement. Soluble complexes and homologous aggregated γG of comparable size were compared with respect to their relative abilities to adhere to macrophages and to fix complement. The data suggested that the strength of binding of soluble immune complexes to macrophages and the efficiency in fixing complement were not determined solely by a random summation of individual binding sites. Possible mechanisms of these biological activities of soluble complexes are discussed.

Materials and Methods

Preparation of Immune Complexes.—New Zealand rabbits were hyperimmunized with human serum albumin (HSA, five times crystallized, Schwarz Bio Research Inc., Orangeburg, N.Y.) emulsified in complete Freund's adjuvant (CFA). Each rabbit was injected subcutaneously with 1–2 mg of HSA weekly for 4 wk. Booster injections were given at monthly intervals during which time weekly bleedings were obtained. Specific antibodies were isolated from the antisera by immunoadsorbent techniques previously described (9). Elution from adsorbent columns was performed with 2.5 M KI. All studies were subsequently carried out with proteins in sodium borate buffer (0.2 M sodium borate, 0.16 M NaCl, pH 8.0). The purified antibodies were radiolabeled with ^{125}I by the monochloride method (10) to yield 1–2 moles of iodine per mole of γG. Approximately 1 mCi of ^{125}I was used per 10 mg of protein; a specific activity of 30,000–60,000 cpm/μg was achieved. For some studies, HSA was similarly labeled with ^{131}I. Free isotope was removed by exhaustive dialysis against borate buffer. Reduction and alkylation of antibodies were carried out respectively with 0.1 M 2-mercaptoethanol and 0.11 M iodoacetamide in borate buffer. Monomeric antigen and antibody preparations were obtained by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), either after iodination or after reduction and alkylation. The point of equivalence was determined by quantitative precipitin curves. Soluble complexes were prepared at threefold antigen excess (by weight) by adding antibodies to antigen while stirring.

Preparation of Proteins for Inhibition.—Rabbit, human, or sheep γG-globulin (subsequently abbreviated RGG, HGG, or SGG, respectively; Schwarz Bio Research Inc.) were further purified by gel filtration on Sephadex G-200. The excluded and included peaks were concentrated and utilized for further studies as minimally aggregated or monomeric proteins, respectively. Double diffusion in agar with antibodies to whole serum, or to γG, showed no evidence of the presence of proteins other than γG-globulin in the aggregated or monomeric RGG or HGG preparations. Heavily aggregated RGG was obtained by heating the G-200-excluded preparation at 71°C for 20 min (11).

Macrophage Preparation.—For preliminary studies, rabbit peritoneal macrophages were obtained by irrigating the peritoneum with Hanks' solution (without Ca^{++}; containing 0.05 mg heparin/ml) 3–4 days after injection of 30 ml of heavy mineral oil (Muri-Lube Heavy, Invenex Pharmaceuticals, San Francisco, Calif.). For all studies not otherwise specified, stimulated rabbit alveolar macrophages were utilized. These were obtained by modifications of the methods described by Myrvik et al. (12). 2-kg New Zealand rabbits were injected intravenously with 0.1 ml of CFA (Difco Laboratories, Detroit, Mich.), with repeated injections given at 2–3-wk intervals. Most animals were utilized within 1–3 wk of injection of the last adjuvant dose. The animals were sacrificed by air embolism. The lungs were carefully removed and then irrigated with warm Hanks' solution, using 10 washings of 30 ml each. The cells were centrifuged at 100 *g* and at 4°C for 10 min, and washed two times with Hanks'. Any red blood

cells present were lysed by a brief exposure to hypotonic saline (0.2% NaCl for 20 sec). The washed cells were finally suspended in Medium 199 (Microbiological Associates, Inc., Bethesda, Md.) (containing 0.05 mg/ml heparin, 100 units/ml penicillin, and 100 μg/ml streptomycin) at a concentration of 4×10^7 cells/ml. Viability was assessed by trypan blue dye exclusion and was greater than 97% in every experiment. The cell preparations consisted of 85–90% macrophages and 10–15% other cells including lymphocytes, neutrophils, and eosinophiles. The cells were utilized for experiments within 4 hr of removal from the animal. All glassware was siliconized.

Immune Complex Adherence and Inhibition Studies.—These procedures were modified from the methods described by Phillips-Quagliata et al. (7). All solutions were made 1 g/100 ml in rabbit albumin (Cohn fraction V, Schwarz Bio Research Inc.; or crystallized rabbit albumin, Pentex Biochemical, Kankakee, Ill.) to prevent nonspecific adsorption of proteins to the siliconized test tubes (12 × 75 mm) or to the cells. The 1 g/100 ml rabbit albumin solutions contained less than 50 μg/ml of rabbit γG-globulin, as measured by radial immunodiffusion using specific sheep antibodies to rabbit γ-chains (13). 2×10^7 cells (in 0.5 ml of Medium 199) were placed into each test tube and a portion of the inhibiting protein in borate buffer was added. The volume was adjusted to 1 ml with borate buffer containing 1 g/100 ml rabbit albumin. The suspensions were incubated at room temperature for 10 min with frequent mixing. The cells were preincubated with the inhibiting protein before addition of the immune complexes in order to simulate in vivo conditions, in which the macrophages are presumably exposed to γG-globulins. A sample of the radiolabeled immune complexes was finally added (25 μl containing 2 μg of antibody protein). Thereafter, the cells were incubated at 37°C for 30 min with frequent mixing. Under these conditions the cells usually remained in suspension and rarely clumped. Preliminary experiments indicated that neither the order of mixing (i.e. preincubating the inhibiting protein with the labeled complexes, then adding the cells), nor the length of incubation (up to 1 hr) altered the observed results. The tubes were then centrifuged for 5 min at 1000 *g* and 4°C, and the supernatant was decanted. The cell buttons were washed three times with 1 ml of Hanks' solution. Preliminary studies showed that further washings contained insignificant amounts of radioactivity.

The cells, supernatants, and a sample of the washes were counted in a Packard automatic well-type gamma counter (Packard Instrument Co., Downers Grove, Ill.). The per cent of the radioactivity adherent to the cells was calculated. Control tubes with a packed volume of sheep red blood cells equal to the packed macrophage volume were included in every experiment. Even though the amount of complexes adsorbed to red cells was always only a fraction of 1%, this value was subtracted from the values obtained for the macrophages. The amount adherent in the presence of inhibiting protein was compared with the amount of complexes adherent to macrophages in daily control tubes containing no inhibiting protein. Assuming the latter to be 100% adherence, the per cent inhibition of adherence of complexes with each amount of inhibiting protein was calculated. Each experiment was run in duplicate and often repeated two or more times as indicated in the results.

Other Methods.—Distribution of soluble immune complexes or of aggregated proteins was determined by sucrose density gradient (SDG) ultracentrifugation, as previously described (9). 10–30% sucrose gradients were centrifuged at 37,000 rpm and 4°C for 16 hr. Anticomplementary cobra venom factor (CoF) was purified and assayed by described techniques (14). Complement fixation by soluble complexes and γG preparations was performed with serial dilutions of the proteins in Veronal buffer and 1.5 CH_{50} units of guinea pig complement. The complexes and complement were incubated at 4°C for 18 hr. The amount of remaining complement was determined by fixation by sensitized sheep red blood cells (15, 16). Trypsin, chymotrypsin (Worthington Biochemical Corp., Freehold, N.J.), or pronase (Calbiochem, Los Angeles, Calif.) were suspended in Hanks' solution (with Ca^{++}) before use. Protein concentrations were determined by absorbance at 280 mμ and extinction coefficients ($E_{1cm}^{1\%}$) of

14.4 for rabbit anti-HSA, 6.6 for HSA, and 13.7 for HGG or SGG. Protein concentrations in SDG fractions of unlabeled proteins were determined by the Folin method (17). In calculations of the molar ratio of antigen and antibody in immune complexes, the following molecular weights were used: 145,000 for RGG and 67,000 for HSA. The fresh rabbit serum was obtained and pooled from the same animals that provided the stimulated alveolar macrophages. This pool possessed a CH_{50} titer of 32 units.

RESULTS

Adherence of HSA-Anti-HSA Complexes.—Initial experiments showed that the >11S complexes selectively adhered to the macrophages in these in vitro studies. The 11S or smaller complexes containing two or one molecules of antibody failed to adhere in the presence of larger complexes. Complexes were prepared at threefold antigen excess to obtain sufficient >11S complexes in dilute solutions. In such preparations 20–25% of antibodies were in the >11S complexes (see Fig. 1). At these low concentrations of antibodies (2 μg of antibody protein in 1 ml), the percentage of >11S complexes, as determined by SDG ultracentrifugation, was lower than in our previous studies performed at concentrations 1000 times greater (4, 9). Experiments with radiolabeled monomeric HSA or anti-HSA indicated that less than 1% of free antigen or free antibodies adhered to the macrophages under the described experimental conditions. Studies performed with complexes in which both the antigen and antibodies were labeled with ^{131}I and ^{125}I, respectively, showed that the molar ratio of the protein adherent to the cells was identical to the molar ratio of the complexes greater than 11S in size, as determined by SDG. These >11S complexes contained more than two molecules of antibody per complex.[2]

The amount of complexes adherent to stimulated alveolar macrophages ranged from 5 to 23% of the total available radioactivity. The reasons for this variable binding of the >11S complexes remained unclear, but appeared to be related to the time elapsed from the last injection of CFA. The maximum adherence of complexes was greatest with macrophages obtained from animals injected 2–3 wk previously. The maximum binding was not related to the number of times the rabbit had been injected, or to the total yield of cells, which varied between 1.65×10^8 and 40.9×10^8 cells per rabbit.

Comparison of Adherence of Complexes to Peritoneal and Alveolar Macrophages.—Alveolar and peritoneal macrophages were not significantly different in their interaction with soluble immune complexes. Stimulated alveolar or peritoneal macrophages were preincubated with serially increasing amounts of fresh rabbit serum; the last set of tubes contained 1 ml of serum. The concentration of normal RGG in this pool was 9540 μg/ml, as measured by radial immunodiffusion. The per cent inhibition of adherence of complexes was calculated and plotted against the micrograms of γG present (Fig. 2). With the addition of

[2] Arend, W. P., D. C. Teller, and M. Mannik. Molecular composition and sedimentation characteristics of soluble antigen-antibody complexes. Submitted for publication.

increasing amounts of normal serum, considerable inhibition of binding occurred early (50% inhibition of uptake at 790 μg of γG in serum), and then inhibition gradually rose to a maximum of 95% by undiluted fresh serum. The stimulated alveolar macrophages behaved similarly to the stimulated peritoneal cells, both in maximum binding of complexes (15.5% for the peritoneal cells) and in characteristics of inhibition by serum. The stimulated alveolar cells were used in the remainder of these studies.

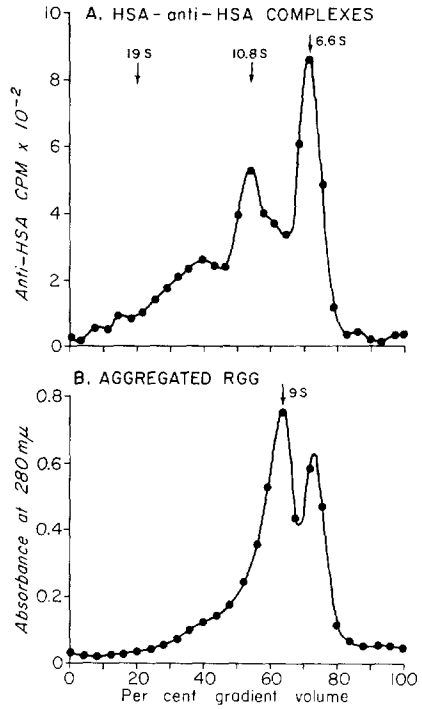

FIG. 1. Sucrose density gradient ultracentrifugation patterns. (A) HSA-anti-HSA complexes at threefold antigen excess; (B) minimally aggregated RGG. The top of each gradient is to the right (100%). In (A) the 10.8S peak represents primarily complexes containing two molecules of antibody and in (B) the 9S peak constitutes dimers of RGG. In each solution approximately the same proportion of material is present between the dimer peaks and 19S, with little protein present larger than 19S in size.

Role of Complement in the In Vitro Binding of Complexes.—These experiments were designed to investigate whether complement could overcome the inhibition of adherence of soluble complexes in the presence of free γG. A sample of the the fresh rabbit serum pool was decomplemented by heating at 56°C for 30 min. Another 1 ml sample was depleted of later acting complement components by incubation with 20 units/ml of purified CoF at 37°C for 60 min. (This is approximately 10 times the amount of CoF used in the in vivo studies [4].) The

sera depleted of complement by the two methods inhibited adherence of complexes in an identical manner; therefore the results were plotted together for graphic analysis.

The inhibition of adherence of complexes by decomplemented serum was the same as that produced by normal serum, when large amounts of γG were present. However, at less than 500 μg of γG/ml, the decomplemented sera inhibited

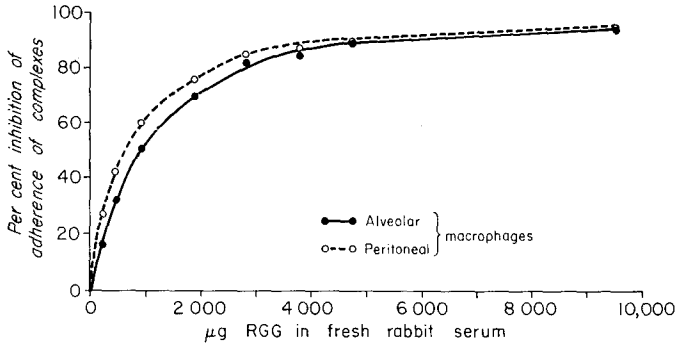

FIG. 2. Inhibition of adherence of immune complexes to stimulated peritoneal or alveolar cells by fresh rabbit serum. The last point on each curve represents inhibition by whole, undiluted serum. 2×10^7 macrophages were preincubated with the inhibiting protein at room temperature for 10 min, then 2 μg of labeled complexes added and incubated at 37°C for 30 min. The cells were washed three times. The amounts adherent to the macrophages were corrected by the amounts adherent to the same volume of packed red cells. Each point represents the average of duplicate determinations.

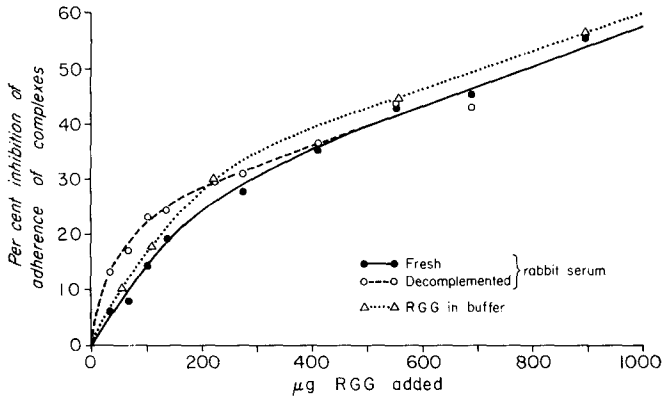

FIG. 3. Inhibition of adherence of complexes to macrophages by fresh rabbit serum, by decomplemented rabbit serum, or by monomeric RGG in borate buffer with 1 g/100 ml rabbit albumin. The serum was decomplemented by heating at 56°C for 30 min, or by treatment with purified cobra venom factor. Each point on every curve represents the average of four values. Note the change in scale of the abscissa from Fig. 2.

to a slightly higher degree (Fig. 3). The difference in inhibition between decomplemented and normal serum was not affected by the length of incubation through 30 min, but decreased with longer incubation. Furthermore, the difference was not altered by preincubating the serum with the complexes before the addition of cells. These observations suggested that the assistance of complement in adherence of soluble complexes to macrophages was not significant in the presence of free γG, when the concentration of the protein approached normal serum levels.

The inhibition curve of monomeric RGG in borate buffer (with 1 g/100 ml rabbit albumin) was not significantly different from that of γG present in whole serum (Fig. 3); 50% inhibition of adherence of complexes was given by 790 μg of RGG in fresh serum and 710 μg of RGG in buffer. Therefore, other serum proteins did not affect the adherence of soluble complexes to macrophages under the conditions of our experiments.

Inhibition of Adherence of Complexes by Aggregated or Monomeric γG from Various Species.—These studies were designed to determine the species specificity of the macrophage receptor for γG and the relative binding of aggregated and monomeric γG. Minimally aggregated and monomeric rabbit, human and sheep γG preparations were analyzed for size distributions by SDG ultracentrifugation. The monomeric preparations contained less than 5% of small aggregates. The minimally aggregated preparations consisted of approximately one-third each of monomers, dimers and larger aggregates with little material sedimenting faster than 19S (see Fig. 1).

The inhibition of adherence of complexes to macrophages observed with monomeric or with aggregated γG showed marked species differences (Figs. 4 and 5). With either monomeric or aggregated γG the inhibition observed was most effective with rabbit γG, followed by human and then sheep γG. Also, with proteins within each species the degree of inhibition was greater with the aggregated than with the monomeric material.

Effect of Reduction and Alkylation of γG on Inhibition of Adherence of Complexes.—The inhibition of binding of complexes by the reduced and alkylated rabbit γG solutions was less than that by the intact proteins (Fig. 6), although the aggregated γG still inhibited to a greater degree than the monomeric preparations. 13 times as much reduced and alkylated aggregated γG than intact aggregated γG was required to give 50% inhibition. By SDG ultracentrifugation the size distributions in these solutions were similar to those of the preparations with intact disulfide bonds (see Fig. 1).

Inhibition of Adherence of Complexes by Preincubation with Unlabeled Complexes Prepared with Intact or Reduced and Alkylated Antibodies.—The effect of reduction and alkylation of interchain disulfide bonds on the direct adherence of soluble complexes was first assessed by preparing complexes at threefold antigen excess using radiolabeled reduced and alkylated antibodies. These complexes showed a marked decrease in adherence to macrophages. An experiment

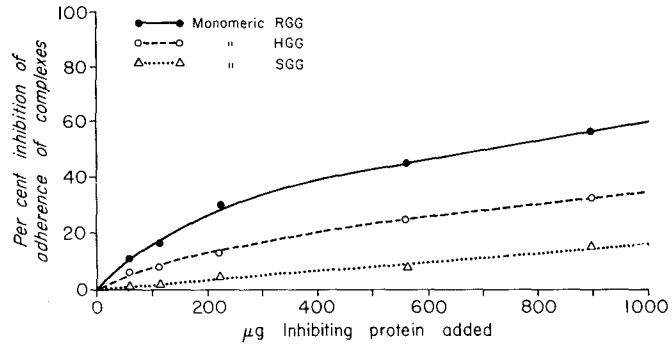

FIG. 4. Inhibition of adherence of complexes to macrophages by monomeric RGG, HGG, or SGG. Each experiment was carried out in duplicate.

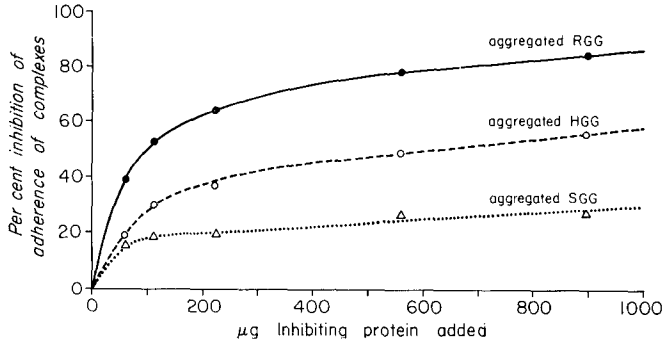

FIG. 5. Inhibition of adherence of complexes to macrophages by minimally aggregated RGG, HGG, or SGG, obtained by exclusion from G-200. The size distributions in each preparation were similar with approximately one-third each of 6.6S monomers, 9S dimers, and small aggregates between 10S and 19S. Each experiment was carried out in duplicate.

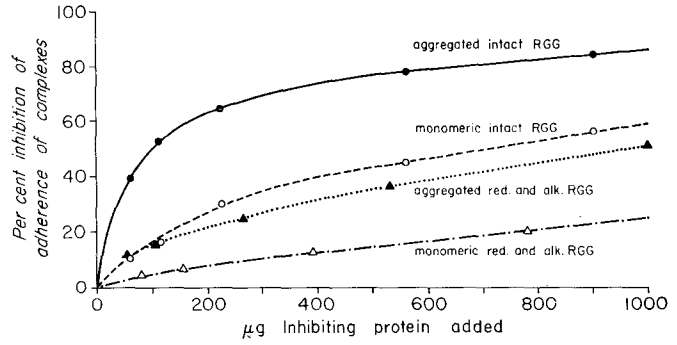

FIG. 6. Inhibition of adherence of complexes by minimally aggregated, or monomeric, RGG, either with intact or reduced and alkylated disulfide bonds. Each point represents the average of duplicate values. *red.*, reduced; *alk.*, alkylated.

performed on the same macrophage preparation showed only 1.89% adherence of complexes with reduced and alkylated antibodies, as opposed to 17.4% binding of complexes prepared with intact antibodies. The distribution of complex sizes was similar in the two preparations, as determined by SDG analysis.

The strength of binding of complexes containing intact or reduced and alkylated antibodies was further studied by using these unlabeled preparations as inhibitors of the subsequent adherence of intact labeled complexes. The unlabeled soluble immune complexes prepared with intact or reduced and alkylated antibodies had similar size distributions as the labeled complexes, i.e. 20–25% of >11S complexes. The completeness of reduction and alkylation of the antibodies was confirmed both by gel filtration under dissociating conditions and by complement fixation. The reduced and alkylated antibodies separated completely into heavy and light chains when chromotographed on a Sephadex G-100

TABLE I

Complement Fixation and Inhibition of Adherence of Complexes to Macrophages by Unlabeled Immune Complexes, Aggregated RGG, or Monomeric RGG

Test protein	50% inhibition of adherence*	50% complement fixation‡
	μg	μg
Immune complexes with intact antibodies	5.0	0.92
Immune complexes with reduced and alkylated antibodies	42	180
Minimally aggregated RGG	95	250
Heavily aggregated RGG	725	0.70
Monomeric RGG	710	415

* 2×10^7 stimulated alveolar macrophages were preincubated with serially increasing amounts of test protein and the number of micrograms giving 50% inhibition of the subsequent binding of 2 μg of labeled soluble immune complexes was determined.

‡ Micrograms of test protein fixing 50% of 1.5 CH_{50} units of guinea pig complement.

column equilibrated with 0.5 M propionic acid. Also, these complexes showed a 200-fold difference in the efficiency of complement fixation; 180 μg of reduced and alkylated antibodies in complexes gave 50% complement fixation as opposed to 0.92 μg of intact antibodies in complexes (see Table I). Macrophages were preincubated with these unlabeled complexes, and the subsequent adherence of 2 μg of radiolabeled complexes with intact antibodies was determined.

The complexes with intact disulfide bonds were eight times more effective than complexes with reduced and alkylated antibodies as inhibitors of subsequent complex binding. 50% inhibition of adherence was given by 5.0 μg of antibody in the former complexes as opposed to 42 μg of the latter (Fig. 7 and Table I). Furthermore, in the inhibition assay the complexes with reduced and alkylated antibodies were twice as efficient as intact aggregated RGG, and 30 times as efficient as reduced and alkylated aggregated RGG. However, the min-

imally aggregated intact rabbit γG was much less efficient in fixing complement than soluble complexes made with intact antibodies; the former required 250 μg to give 50% complement fixation (see Table I).

These experiments indicated that as inhibitory proteins in this in vitro system, homologous minimally aggregated γG was less firmly bound to the macrophages than were soluble immune complexes possessing antibodies with either intact or reduced and alkylated disulfide bonds. The weaker binding of the aggregated γG was further confirmed in an experiment using 2 μg of radiolabeled aggregated RGG as the test substance. 50% inhibition of binding of the aggregated RGG was achieved by preincubation of the cells with only 160 μg of unlabeled monomeric RGG, whereas five times that amount was required to give 50% inhibition of adherence of soluble immune complexes.

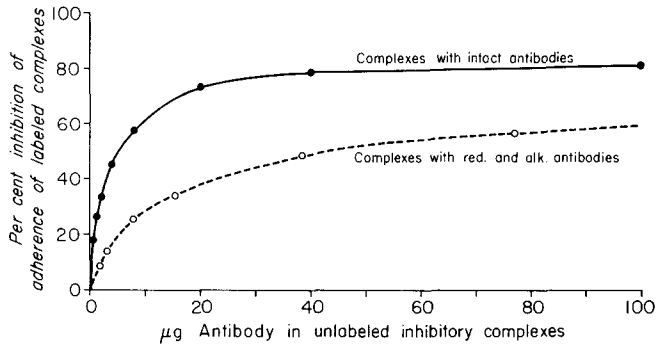

FIG. 7. Inhibition of adherence of labeled complexes by preincubation of the cells with unlabeled complexes prepared with intact antibodies or with reduced (*red.*) and alkylated (*alk.*) antibodies. The unlabeled complexes consisted of the same size distributions as the labeled test complexes. Each point is the average of four values. Note the change in scale of the abscissa from the preceding figures.

Inhibition of Adherence of Complexes by Aggregated Rabbit γG Possessing Complement-Fixing Ability Comparable with that of the Immune Complexes.—Because previous investigators concluded that soluble complexes and aggregated γG possessed similar biological properties on a weight basis (5), the inhibition of adherence of complexes by heat-aggregated RGG was studied. This preparation required only 0.70 μg to give 50% complement fixation (Table I), an amount equivalent to that of the immune complexes with intact antibodies. The heat-aggregated RGG contained primarily aggregates sedimenting faster than 20S, with little 9S dimers or small aggregates present, as determined by SDG ultracentrifugation. However, these large aggregates were ineffective as inhibitors of binding of immune complexes to macrophages. The large aggregates required 725 μg to give 50% inhibition; this is equivalent to the amount of monomeric RGG required to give a similar degree of inhibition.

Effect of Proteolytic Enyzmes on Macrophage Receptor for γG.—These exper-

iments were carried out to study the sensitivity of the macrophage receptor for γG to proteolytic enzymes. Portions of alveolar macrophages in Hanks' solution with Ca^{++} were preincubated with 10–100 μg/ml of trypsin, chymotrypsin, or pronase for 30 min at 37°C. The enzymes were then removed by four washings with Hanks' solution. The cells were resuspended in Medium 199, and the adherence of immune complexes was evaluated. SDG ultracentrifugation of the complexes in the supernatants after the experiments showed no evidence of degradation of the complexes, indicating that the proteolytic enzymes were completely removed by earlier washing. The extent of adherence was compared with that seen with control cells handled similarly but not exposed to enzymes.

TABLE II

Effect of Proteolytic Enzymes on Adherence of Soluble Complexes to Macrophages

Enzyme pretreatment		Per cent adherence*
	μg/ml	%
A. Trypsin	10	125
	50	142
	100	137
B. Chymotrypsin	10	101
	50	103
	100	113
C. Pronase	10	158
	50	146
	100	125

* Represents results from two separate experiments. The actual amounts of complexes adherent ranged from 5 to 15% of the total available radioactivity. These amounts were compared with the amounts of complexes adherent to normal cells in control tubes. The control values were designated 100% adherence.

An increase in immune complex binding over control levels was seen after pretreatment with each enzyme within the concentration range studied (Table II). Pronase was the most potent in increasing the adherence and chymotrypsin the least effective.

DISCUSSION

The presented evidence confirms the observation that soluble immune complexes possessing more than two molecules of antibody in their lattice work preferentially interact with the γG receptors on macrophages. However, the inhibition of adherence of soluble complexes by free γG in the medium was not altered by the presence of complement, contrary to the inhibition of rosette formation of sensitized erythrocytes around macrophages. Huber et al. (2) concluded that the macrophage receptors for γG and for C3 exerted a cooperative effect in the binding of antibody-coated erythrocytes in the presence of free γG. This inhibition of rosette formation by γG was related to the degree of red

cell sensitization, in that red cells sensitized with large amounts of antibody required more free γG to cause comparable degrees of inhibition (18). However, recent studies failed to confirm the presence of a distinct receptor for C3 on macrophages, and the suggestion was made that C3 potentiates phagocytosis, not adherence, through its peptidase activity (19). The experimental conditions in our studies were chosen to favor adherence, but not interiorization during the short preincubation of the inhibiting protein with the cells at room temperature. After the addition of the 2 μg of labeled antibodies in complexes, the incubation at 37°C for 30 min should have been sufficient for the occurrence of full pinocytosis of the soluble complexes (20, 21). Although engulfment of the soluble complexes was not examined in these in vitro studies, our earlier in vivo studies showed that both adherence and degradation of soluble immune complexes by hepatic macrophages were not altered by complement depletion (4, 9). It can be concluded that both adherence of soluble complexes to macrophages and subsequent interiorization can occur in the absence of complement, even though the soluble complexes are capable of fixing complement. In contrast, complement appears to play an important role in the interaction of sensitized erythrocytes with macrophages.

In our experiments, physiological amounts of γG in rabbit serum showed almost complete inhibition of adherence of the soluble complexes to macrophages. However, the same complexes in vivo were rapidly taken up by the liver, with a serum disappearance half-life of 15 min (4, 9). The reasons for these in vitro and in vivo differences have not been explained. It is possible that the macrophages in suspension in our in vitro experiments were not so active in their surface receptor functions as the Kupffer cells in vivo. It is of interest that glass-adherent peripheral monocytes underwent biochemical and metabolic changes and exhibited an enhancement in phagocytosis of particles (22). Similarly, alveolar macrophages exhibited 30 times the phagocytic capacity towards particles at 3–4 wk after culture on glass than at 1 day (23). The amount of complexes adherent to the macrophages in our studies was greatest when the donor animals had received their last dose of CFA 2–3 wk previously. Other workers described a similar maximum in phagocytic activity of liver homogenates towards radiolabeled endotoxin suspensions 2 wk after intravenous injection of killed Calmette-Guérin bacillus (24). However, it has not been shown whether this enhanced macrophage function is secondary to an increase in the numbers of macrophage receptors, or to a greater capacity of avidity of preexisting receptor sites. Thus, it is possible that a difference in macrophage receptor number or activity exists between the alveolar cells in our in vitro studies and the in vivo hepatic macrophages (4).

The amount of inhibiting protein required to give 50% inhibition of adherence of soluble complexes served as an index of the relative strength of binding of the protein to macrophages. These studies indicated that the soluble complexes were bound to macrophages more firmly than aggregates of homologous

γG, even though the latter had sedimentation coefficients similar to the complexes. We have shown that the 11S HSA-anti-HSA complexes contain two molecules of antibody,[2] and others have demonstrated that the 9–10S γG aggregates are dimers (25). Therefore, it appears reasonable to assume that the >11S complexes and >9S aggregates contain a comparable number of γG molecules. Not only did the immune complexes exhibit greater adherence to macrophages, they also were more efficient in complement fixation than were the small aggregates. These observations suggested that soluble immune complexes possessed structural characteristics not present in the small aggregates of γG, and that these characteristics contributed to the enhanced observed biological activities. Conformational changes in the antibodies in soluble immune complexes or a particular arrangement of the γG molecules in the lattice work of the soluble complexes may account for these differences.

Our studies demonstrated that reduction and alkylation of the antibodies in the complexes decreased their strength of binding to macrophages, both in direct adherence and in inhibition of binding of complexes with intact antibodies. Similarly, reduction and alkylation decreased the inhibiting capacity of aggregated or monomeric RGG preparations. Previous investigators have shown that the cytophilic properties of guinea pig γ1 antibodies were decreased by reduction and alkylation (20). Also, recent studies from our laboratory have shown that reduction and alkylation of γG antibodies markedly decreased the in vivo uptake of soluble complexes by fixed tissue macrophages of the liver (4). It is of interest that complexes prepared with reduced and alkylated antibodies were more strongly adherent to macrophages than was intact aggregated RGG. This observation suggested that the immune complexes still possessed structural characteristics which are important for binding to macrophages, even after rupture of the interchain disulfide bonds of the constituent antibody molecules. Furthermore, reduction and alkylation of antibodies decreased the ability of immune complexes to fix complement to a greater degree than it did their ability to adhere to macrophages (see Table I).

Other workers have concluded that similar molecular interactions occur upon formation of soluble complexes and aggregates of γG. The majority of heat-aggregated HGG has been shown to be linked through Fab fragments, by disulfide bonds and noncovalent interactions (8). In immune complexes the Fab regions of the antibodies are approximated by noncovalent bonds with the antigen. It is not known whether RGG is aggregated in a manner similar to HGG. Comparable changes in optical rotation have been observed in soluble immune complexes (26), in dimers of HGG (27), and in heat-aggregated HGG (28); this implies the presence of similar conformational changes in the γG molecule in both complexes and γG aggregates. In addition, immunological techniques have detected identical antigenic changes in the Fc fragments of the HGG antibodies in soluble complexes and in HGG aggregated by a variety of methods (29). However, between heat-aggregated γG and complexes of univalent hapten and

antibody there are differences in the susceptibility of the Fab fragment to degradation by proteolytic enzymes, which suggests that the molecular interactions are not entirely similar (8, 30).

Electron microscopy has revealed conformational changes in the γG molecule upon combination with antigen, although there are few data linking these changes to biological activities of the molecule (31). The combination of small haptens with antibodies induced minimal conformational alterations in the antibody as detected either by electron microscopy (31, 32) or by circular dichroism (33). On the other hand, large protein antigens appeared to induce greater changes in the conformation of antibodies than did small antigens, as detected by both electron microscopy (34) and optical techniques (28). These observations might explain some of the differences between the results reported here and the studies of Phillips-Quagliata et al. (7). These authors utilized a system of haptens of varying valency and antibodies prepared against a multivalent hapten. They noted greatest adherence of soluble complexes when prepared in antibody excess with polyvalent hapten, and they concluded that the binding of soluble complexes to macrophages was due to a summation of individual binding sites rather than to conformational changes in antibody molecules. However, because of their small size the haptens used as antigens may not have induced a significant conformational change. The observation that only 50 μg of free γG gave 50% inhibition of their complexes, as opposed to the 710 μg in our studies, may be partially explained by differences in experimental conditions, but is also implies that the HSA-anti-HSA complexes prepared in antigen excess in our studies adhered more strongly.

Our studies offer no direct evidence for the possible effect of conformational changes in the antibodies on increasing the biological activities of the soluble complexes. However, the recently published studies of Thrasher and Cohen (35) suggest that this change may be important. These authors contended that the greater sensitivity of the indirect cytophilic assay (sensitized erythrocytes added to macrophages), as opposed to the direct cytophilic assay (cytophilic antibody reacted with macrophages, unsensitized erythrocytes added later) (20), is not explained solely by a summation of individual binding sites. Furthermore, their studies suggested that the receptor sites for cytophilic antibody on macrophages were too widely spaced to offer the grouping of γG molecules necessary for complement fixation. This observation suggests that summation effects may not be so important as the increased binding offered by the individual antibody after reaction with the antigenic determinant on the erythrocyte.

An alternative explanation for the preferential binding of soluble complexes to macrophages might be a specificity offered by the lattice work. The γG antibodies in the lattice of soluble complexes might be oriented in a manner that leads to interaction and a potentiation of binding. It is possible that the arrangement of Fc fragments in the soluble complexes closely approximates the distribution of the receptor sites on the macrophage surface or on C1q. Although the

number of receptors for γG on alveolar macrophages has been estimated to be around 2 million per cell (7), their distribution is not known. This site density might be important in explaining some of the above observations. The failure of the large heat aggregates of γG to inhibit the binding of soluble complexes could be due to the smaller number of molecular aggregates per unit weight of γG, in that there would thus be enough receptors left free to interact with the complexes. In the small aggregates of γG the orientation of molecules may be different than in the immune complexes, a difference which could result in inefficient interaction of these aggregates with receptor sites. Similarly, in the studies of Phillips-Quagliata et al. (7), the lattice work in the complexes made in antibody excess might be different from that in our complexes made in antigen excess using a large multivalent antigen.

Pretreatment of macrophages with proteolytic enzymes increased the adherence of soluble complexes. Most studies have concluded that the macrophage receptor for γG, as opposed to the receptor for C3, is not trypsin sensitive. An enhanced binding of sensitized red cells, i.e. increased amounts, after pretreatment of the macrophages with proteolytic enzymes was recently described by LoBuglio and Rinehart (36); the results were similar to those of our studies using soluble immune complexes. These studies suggest that enzyme treatment may remove inhibiting material adjacent to the receptors for γG on the macrophages. They do not indicate whether more sites are exposed or the preexisting sites exhibit an enhanced binding capacity.

SUMMARY

The adherence of soluble immune complexes to stimulated alveolar macrophages was studied in vitro using HSA-anti-HSA complexes prepared in antigen excess. Those complexes containing more than two molecules of antibody preferentially adhered to macrophages in the absence of complement. Free γG in less than physiological concentrations inhibited the adherence of complexes, and the presence of complement did not significantly alter this inhibition. Complexes prepared with reduced and alkylated antibodies showed a decreased adherence. The strength of binding of soluble complexes to macrophages and their efficiency in fixing complement were greater than seen with small aggregates of homologous γG. These differences in biological properties were observed even though the immune complexes and aggregates contained comparable numbers of γG molecules. The γG receptor on rabbit macrophages exhibited species specificity. Pretreatment of macrophages with proteolytic enzymes led to adherence of larger amounts of soluble complexes.

These observations suggest that the strength of binding of soluble immune complexes to macrophages and their efficiency in fixing complement are not determined solely by a random summation of individual binding sites. It is proposed that conformational changes in the γG antibodies or a specific molecular

arrangement in the lattice work of complexes containing large protein antigens may influence the biological properties of the soluble complexes.

It is a pleasure to acknowledge the excellent technical assistance of Mr. Douglas Webster.

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