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Complement-Fixing Activity of Rabbit Antibody Reconstructed from Half Molecules.* (29890)

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The effect of reduction and alkylation of antibody on its complement (C′) fixing properties has been studied in a number of laboratories with conflicting results. After rabbit anti-human γ globulin was reduced and alkylated, we found essentially no decrease in C′-fixing properties(1). Schur and Becker(2) observed a reduction in C′ fixation of less than 20% after treating rabbit anti-human albumin sera with cysteine, whereas Wiedermann *et al*(3,4) recently reported complete loss of the C′-fixing activity associated with human and rabbit antibodies after reduction in 0.1 M mercaptoethanol. As the exact condition for the reduction-alkylation treatment was different in each investigation, it is difficult to decide from these experiments which molecular structures are essential for the C′-fixing properties of antibody. Recently, Palmer *et al*(5) reported that rabbit γ globulin was split into half molecules by reduction in 0.1 M mercaptoethylamine followed by lowering the pH to 2.5, indicating that the inter-heavy (H) chain disulfide linkage(s) is reduced by the procedure. In view of these findings, the present experiments were undertaken to study the role of the inter-H chain disulfide linkages in rabbit antibody molecules on their C′-fixing properties. The results indicate that after irreversible reduction of the inter-H chain disulfide linkages, the half molecules of antibody can recombine in the presence of antigen to form antibody complexes which fix C′.

Materials and methods. Antigen and antibody. A crystalline bovine serum albumin (BSA) (Armour & Co.) rabbit anti-BSA system was employed in these experiments. Anti-BSA rabbit serum was obtained by immunization of rabbits for 4 weeks with an increasing amount of alum-precipitated BSA. The animals were bled 7 days after the last injection of antigen. Gamma globulin fraction of the antiserum was obtained by precipitation with ammonium sulfate at ⅓ saturation followed by DEAE cellulose chromatography(6).

Reduction of γ globulin. The γ globulin fraction of anti-BSA serum was reduced with mercaptoethylamine HCl (Nutritional Biochem. Co.) under N_2 by the method of Palmer *et al*(5). The fraction (1.5%) was incubated at pH 5.0 in 0.1 M mercaptoethylamine for 75 minutes at 37°C. Twice molar excess of iodoacetate was then added to the reaction mixture and the pH maintained at 8.0 by addition of 1 M Tris (Tris (hydroxymethyl) aminoemethane) solution. After one hour, the reduced-alkylated γ globulin was dialyzed against saline for 48 hours.

Complement fixation tests. Pooled guinea pig serum was used as the source of C′. The serum was purchased in a frozen state from Probio Inc., New York, and absorbed with 1/30 volume of packed sheep erythrocytes. The number of units of C′ inactivated by antigen-antibody complexes was determined

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TABLE I. Complement Fixation by BSA-Anti-BSA Precipitates.

Precipitates composed of	Acid exposure*	$C'F_{50}$†
		(μgN)
Untreated antibody	—	6.1
	+	6.3
Reduced-alkylated antibody	—	9.8
	+	8.3

* Precipitates were exposed at pH 2.5 in 0.1 M NaCl, then dialyzed against borate buffered saline (pH 8).

† Quantity (μgN) of antibody in precipitate required to fix 50 $C'H_{50}$ out of 100. In this calculation, anti-complementary activity of antibody alone was subtracted from the number of $C'H_{50}$ units fixed by precipitates (see method).

by the method of Mayer *et al*(7). An appropriate amount of complexes was added to 100 $C'H_{50}$ (50% hemolytic units) of C′ in a final volume of 10 ml. As a control, an aliquot of antibody preparation containing the same amount of γ globulin as the amount of antibody present in the complexes was incubated with C′. The number of $C'H_{50}$ units inactivated by the complexes was calculated by subtracting the number of units fixed by the antibody preparation from the number fixed by the complexes. The quantity of antibody in the complexes required to fix 50 out of 100 $C'H_{50}$ units, *i.e.*, $C'F_{50}$, was then calculated according to the method of Wallace *et al*(8).

Analytical ultracentrifugation. Analytical ultracentrifugation was carried out in a Spinco, model E ultracentrifuge at 20 ± 0.2°C, at 52,640 rpm. The relative apparent percentage of each component was determined from areas of the individual components. Corrections for the Johnson-Ogsten effect were not made.

Results. Precipitating antibody in the untreated and in the reduced-alkylated γ globulin fractions of anti-BSA serum was measured by quantitative precipitin reactions(9). In both preparations, 32% of total protein was precipitating antibody. Specifically, the total protein and the antibody concentrations were 3.0 mgN/ml and 0.97 mgN/ml for the untreated preparation, and 1.17 mgN/ml and 0.38 mgN/ml for the reduced-alkylated preparation.

On the basis of the quantitative precipitin curve, an equivalent amount of BSA was added to both the treated or untreated antibody preparations and the mixtures kept at 3°C for 24 hours. The specific precipitates were washed 3 times with cold saline and resuspended in saline. The C′-fixing capacity of the precipitates and anti-complementary activity of the antibody preparations were determined. The number of $C'H_{50}$ inactivated by 8 μgN of the antibody preparations was less than 6. The quantities of antibody in the precipitates required to fix 50 $C'H_{50}$ out of 100, *i.e.*, $C'F_{50}$ are shown in Table I. On a nitrogen basis, the C′-fixing capacity of antibody decreased to 62% after reduction and alkylation of antibody.

Experiments were undertaken to study whether inter-H chain disulfide linkages in the antibody molecules were irreversibly reduced by the reduction-alkylation treatments. Both treated and untreated γ globulin preparations were dialyzed at 3°C against 0.1 M NaCl at pH 2.5 for 48 hours and then analyzed in an analytical ultracentrifuge. Unreduced γ globulin was composed of a 5.8 S ($S_{20,w}$) component which comprised more than 90% of the total area and a smaller peak of higher sedimentation coefficient. The reduced-alkylated protein migrated almost entirely as a single peak with $S_{20,w} = 3.8$. Less than 10% of a faster-moving component ($S_{20,w} = 5.4$) was present. Sedimentation coefficients of the main components are slightly higher than those reported by Palmer *et al*(5); however, it is evident that 5.4 to 5.8 S and 3.8 S components correspond to whole molecule and half molecule, respectively. BSA showed a single peak with a sedimentation coefficient of 3.3 S at the same pH and salt concentration. After the reduced-alkylated γ globulin was neutralized by dialysis against borate buffered saline (pH 8.0), the major component of 6.9 S comprised about 55% of the total area. The preparation also contained a 5.1 S component (25%) and a 9.0 S component (20%).

Although the foregoing experiments indicate that reduction and alkylation results in irreversible reduction of the inter-H chain disulfide bonds, additional evidence was obtained with specific precipitates formed with either unreduced or reduced-alkylated anti-

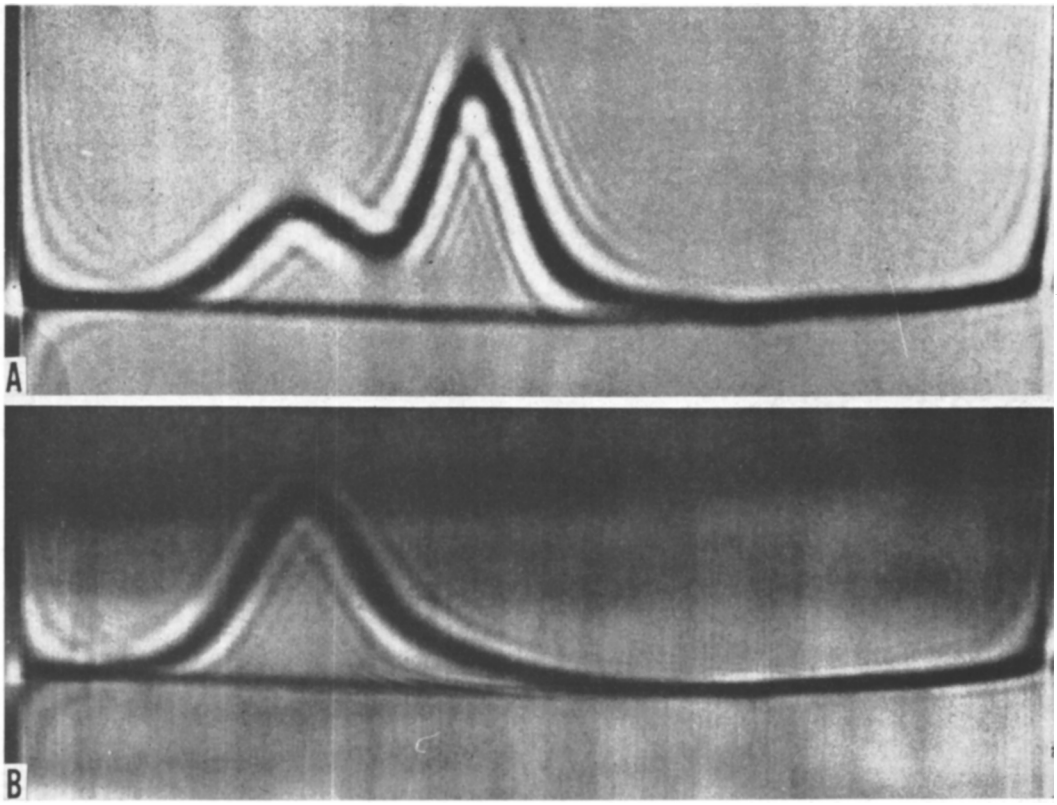

FIG. 1. Ultracentrifugal patterns of BSA - anti-BSA complexes in 0.1 M NaCl at pH 2.5. Sedimentation is from left to right. a) BSA- untreated anti-BSA. Photograph taken after 86 min at 52,640 rpm. b) BSA- reduced-alkylated anti-BSA. Photograph taken after 92 min at 52,640 rpm.

body at the equivalence zone. The precipitates were washed 3 times with cold saline, dissolved in 0.1 M NaCl at pH 2.5 and dialyzed against the same solution at 3°C. The preparation containing untreated antibody gave 2 peaks of which $S_{20,w}$ were 5.4 and 3.4. The relative area of the slower component (11%) was close to the percentage of BSA in the original specific precipitates in which the antigen/antibody weight ratio was 1:6. To identify the component as BSA, 3.1 mg of BSA was added to 13.8 mg of the specific precipitate and the mixture dialyzed against 0.1 M NaCl at pH 2.5. The relative area of the slower component in this preparation was 35% of the total area (Fig. 1a). Under the same condition, precipitates prepared from reduced-alkylated antibody showed a single peak with $S_{20,w} = 3.7$ (Fig. 1b). Lack of a 5-6 S component in this preparation indicates that reduced-alkylated antibody in the precipitates was almost entirely split to half molecules, and therefore reveals that inter-H chain disulfide linkages in antibody molecules were irreversibly reduced by the reduction-alkylation treatment.

An experiment was carried out to study whether the C′-fixing activity of antibody remains after acidification. Specific precipitates were formed at the equivalence point with either untreated or reduced-alkylated antibody. The precipitates were washed with saline, dissolved in and dialyzed against cold 0.1 M NaCl at pH 2.5 for 48 hours with several changes of saline. The preparations were then dialyzed against borate buffered saline (pH 8.0) at 3°C. In the preparation containing unreduced antibody, a precipitate formed within 24 hours, whereas longer dialysis was necessary in the case of the reduced-alkylated antibody. Dialysis was continued up to 72 hours and the C′-fixing activ-

ity of the suspensions was determined. As controls, reduced and unreduced globulin solutions containing antibody were treated by the same procedure and incubated with C′. The number of C′ units inactivated by 10 μgN of the preparations before and after the acid exposure was less than 8 $C'H_{50}$ out of 100. Assuming that antibody and normal γ globulin in the antibody preparations have the same anticomplementary activity, the number of $C'H_{50}$ units fixed by antigen-antibody reaction was calculated. The results shown in Table I indicate that the C′-fixing capacity of the antigen-antibody complexes did not change by the acid treatment.

Discussion. The data presented here clearly show that C′-fixing properties of BSA-rabbit anti-BSA complexes remained after reduction of the antibody in 0.1 M mercaptoethylamine followed by alkylation with iodoacetate. Sedimentation coefficient ($S_{20,w}$) of the reduced-alkylated γ globulin was 3.8 in 0.1 M NaCl at pH 2.5, whereas $S_{20,w}$ of untreated γ globulin was 5.8 at the same pH and salt concentration. Palmer *et al*(5) reported that $S_{20,w}$ of whole γ globulin and its half molecule were 5.0 and 3.4, respectively. It seems that the 5.4-5.8 S component and the 3.8 S component observed in the present experiments correspond to whole and half molecules. Reasons for the slightly higher values in our experiments are unknown. When specific precipitates were formed with either untreated or reduced-alkylated antibody and then analyzed by ultracentrifugation at pH 2.5, the complex preparation composed of untreated antibody showed two components, one of which corresponded to BSA ($S_{20,w} = 3.4$) and the other to γ globulin ($S_{20,w} = 5.4$), whereas the complexes composed of reduced-alkylated antibody showed a single peak ($S_{20,w} = 3.7$). Since the antigen-antibody combination completely dissociates at pH lower than 3.0(10), both preparations should be mixtures of free antigen and antibody at the pH studied. Lack of a 5-6 S component in the BSA-reduced-alkylated antibody mixture indicates that essentially all antibody molecules dissociated to half molecules at pH 2.5 and, therefore, that inter-H chain disulfide linkages in almost all antibody molecules had been reduced irreversibly by the reduction-alkylation treatment. Since the C′-fixing capacity of the specific precipitates of the reduced-alkylated antibody was 62% of that of untreated antibody on a weight basis, it seems that inter-H chain disulfide linkages in the antibody molecules are not essential for induction of C′-fixation. Palmer *et al*(5) reported that 8.4 SH groups per molecule of rabbit γ globulin are liberated by the reduction in 0.1 M mercaptoethylamine. Porter (11) proposed 3 inter-H chain disulfide linkages in a rabbit γ globulin molecule; however, recent studies by Palmer and Nisonoff(12) indicated the presence of a single disulfide bond between two H chains. The data of these investigators(5,12) suggest the possibility that some intra-chain disulfide linkages may also be susceptible to reduction in 0.1 M mercaptoethylamine. It is likely that the 38% decrease in C′-fixing activity of antibody by reduction and alkylation in the present experiments is due to some changes other than cleavage of inter-H chain disulfide bond(s), *per se*.

The present experiments also showed that C′-fixing capacity of antigen-antibody precipitates did not change after exposure of the precipitates at pH 2.5 in 0.1 M NaCl. Since the specific precipitates composed of the reduced-alkylated antibody and antigen split to half molecules of antibody and free antigen in the medium, formation of precipitates by increasing pH and ionic strength would involve two processes, *i.e.*, reconstruction of bivalent antibody from the half molecules and combination of antigen to antibody combining sites. Palmer *et al*(5) reported that tests of specific precipitability on reconstructed antibody were not reproducible. In the present experiments, however, the specific precipitates were always obtained upon raising the pH, although formation of the precipitates with the reduced-alkylated antibody was slower than that with unreduced antibody. It may be that the presence of an equivalent amount of antigen during dialysis of the half molecules of antibody aids the process of reconstruction. In any case, the present data indicate that the antibody reconstructed from half molecules in the pres-

ence of antigen fixes C′. These findings are different from the observations by Weil *et al* (13) that anti-pneumococcal rabbit serum lost C′-fixing properties by acid treatment. Disappearance of the properties in their experiment may have been due to complex formation of antibody with other serum protein.

The question arose that the C′ fixation by the mixture of reconstructed antibody with antigen might be due to denaturation of antibody by the acid exposure rather than antigen-antibody reaction. However, the anti-complementary activity of the reduced-alkylated antibody preparation was not changed by the acid exposure. The $C'F_{50}$ of the preparation was 300 μgN. Furthermore, the C′-fixing activity of the reconstructed antibody was observed in preliminary experiments. Half molecules of anti-BSA rabbit antibody were obtained by gel filtration of reduced-alkylated antibody through a Sephadex G-200 column equilibrated with 0.025 M NaCl, pH 2.4(12). The antibody reconstructed from the half molecules by raising the pH fixed C′ when antigen was added.

The problem remains as to whether the complexes composed of the reconstructed antibody fix all components of C′. More recent experiments have shown that the minimum amount of the antigen-antibody precipitate required for induction of the immune adherence reaction did not change either by the reduction and alkylation of antibody and/or by acid treatment of the precipitates. Nelson(14) has shown that C'_1, C'_4, C'_2 and C'_{3c} are essential for induction of immune adherence reaction. It is therefore probable that the complexes composed of the reconstructed antibody combine 4 or more components of C′. More detailed studies on the fixation of each component of C′ are planned.

Summary. Rabbit anti-BSA antibody was reduced in 0.1 M mercaptoethylamine (pH 5.0) followed by alkylation with iodoacetate. Specific precipitates formed with the treated antibody and antigen fixed C′. The C′-fixing activity of the specific precipitates did not change by the treatment at pH 2.5 in 0.1 M NaCl, which results in dissociation of the precipitates into BSA and half molecules of antibody. The results indicate that the antibody reconstructed from the half molecules fixes C′ with antigen and that inter-H chain disulfide linkages in original antibody molecules are not essential for C′ fixation.

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