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Effect of Mercaptoethanol on Complement Binding Ability of Human 7 S Gammaglobulin.* (28440)

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It is well known that the $beta_{2M}$ or 19 S γ globulin-antibodies are split into smaller 7-8S units by breaking disulfide bonds with a variety of sulfhydryl reagents with concomitant loss of all antibody activity(1). In contrast, such treatment does not split 7 S gamma immune globulins and does not destroy their antigen combining capacity. This difference in reactivity has been widely used as a presumptive test to distinguish these two classes of antibodies(2). While the ability of 7 S gammaglobulin antibodies to react with antigen is unaffected by such treatment, no data are available regarding the effect on secondary reactions reflecting the interaction between antigen and antibody, such as complement fixation. This question is of importance in view of the suggestion by Grabar (3) and Becker and his collaborators(4) that sulfhydryl structures may be involved in complement fixation, and also because of the wide use of reductive cleavage as a screening test for 19 S antibodies.

Data are reported here showing that mercaptoethanol treatment of human 7 S gammaglobulin and 7 S gammaglobulin antibodies abolishes their capacity to bind complement when they react with antigen or after heat-aggregation, a procedure known to make 7 S gammaglobulins anticomplementary (5).

Materials and methods. Sera from patients suffering from systemic lupus erythematosus (SLE) and from infectious hepatitis, which contained complement binding antibodies against cytoplasmic particulate constituents (mitochondria, lysosomes, microsomes), were used in this study. The 7 S fractions of these sera were obtained by

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ultracentrifugation in a saline gradient using 21%, 14% and 7% saline(6). A 1% solution of human plasma fraction II (Lederle #C755), purified by chromatography on DEAE-cellulose(7), was heat aggregrated in a water bath at 60°C for various time periods and subsequently assayed for complement fixation and for its reactivity with a potent rheumatoid factor. The immunoelectrophoretic examination of this plasma fraction, using goat and rabbit antisera to human gammaglobulin, showed one single precipitation arc; no $beta_{2A}$- or $beta_{2M}$-lines were detectable.

Mercaptoethanol was added to serum or to a 1% solution of human 7 S gammaglobulin to a final concentration of 0.1 M. The samples were incubated at room temperature for 2 hours and subsequently dialyzed against iodoacetamide (0.02 M in saline) or against 0.15 M NaCl. Such treatment is known completely to inactivate 19 S gammaglobulin antibodies. Mercaptoethanol treatment of pooled 7 S gammaglobulin was performed either before or after heating of the protein solution. For complement fixation the solution was diluted to a final concentration of 0.8 mg/ml.

Complement fixation tests with SLE and hepatitis sera were performed according to the method of Deicher *et al.*(8) in plastic trays. The incubation of serum, antigen, and complement was carried out at 37°C for 2 hours. After addition of sensitized cells the trays were shaken carefully for 5 minutes and immediately thereupon incubated at 37°C for 45 minutes. During the incubation the trays were repeatedly shaken to prevent settling of the erythrocytes.

Quantitative complement fixation was performed according to the method of Mayer *et al.*(9,10) using 110-140 C'50 units/10 ml.

Cytoplasmic fractions of fresh rat liver were prepared by differential centrifugation of the homogenized tissue in 0.25 M sucrose (400 g: nuclei; 3200 g: mitochondria-rich fraction; 22000 g: lysosome-rich fraction; 100,000 g: microsome-rich fraction; and the supernatant containing soluble fractions).

The antiglobulin consumption test was done according to the method of Steffen(11). One ml of a mixture of mitochondria, lysosomes and microsomes was incubated with 2 ml of the patient's serum and 2 ml of saline for 30 minutes at 37°C and subsequently for 60 minutes at 4°C. After this incubation the antigen was centrifuged at the speed used for sedimenting microsomes and washed 3 times with a mixture of equal amounts of .25 M sucrose and saline and once with saline alone. The washed antigen was then suspended in 0.5 ml of a 2% saline solution at 4°C for 10 minutes and subsequently at 60°C (waterbath) for 20 minutes. At 5 minute intervals the tubes were shaken. Finally, the tubes were transferred into a preheated centrifuge container and centrifuged at high speed. The eluate, thus obtained, was tested according to the dilution-consumption method. The eluate was divided equally among various dilutions of a high titer antihuman globulin serum. After 3 minutes the Coombs test was carried out with all serum dilutions and the reduction in titer was compared to a control eluate prepared with normal serum.

The reactivity of heat aggregated human plasma fraction II with rheumatoid factor was assayed by the capillary precipitation method at a concentration of 10 mg/ml. One serum with known strong rheumatoid factor potency and a latex fixation titer of 40,000 was used for all determinations and the amount of precipitate with the undiluted serum was graded from 1 + to 3 +.

Results. A. Effect of mercaptoethanol treatment of human anti tissue antibodies on their complement fixing capacity. Sera from 3 patients with SLE, and 2 patients with infectious hepatitis, which fixed complement with cytoplasmic fractions from rat liver at high serum titers were used for this purpose. All 5 sera lost the complement fixing activity after treatment with mercaptoethanol. The serum with the highest titer was also tested by the antiglobulin consumption method. With a mixture of all cytoplasmic particulate fractions a 2 titer antiglobulin consumption was obtained using a 2-fold dilution series of antihuman globulin serum. This reactivity was not lost after mercaptoethanol treatment of the serum thus

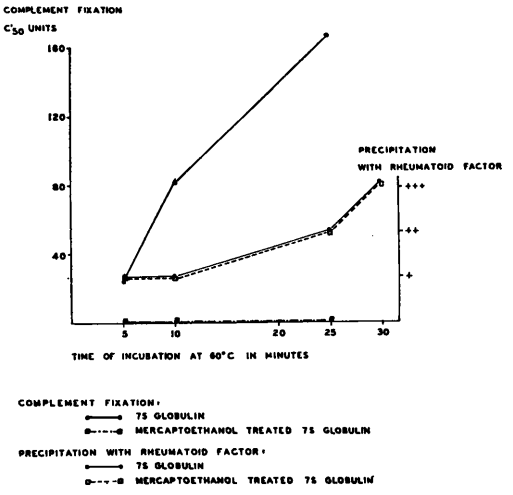

FIG. 1. Effect of mercaptoethanol on ability of human gammaglobulin to react with complement and with rheumatoid factor after heat aggregation.

showing that the antibody was still able to react and combine with the cytoplasmic fractions.

These findings suggested the presence of 2 types of antibodies; one, a 7 S gammaglobulin antibody, which did not fix complement; the other, a 19 S gammaglobulin complement fixing antibody, which was inactivated by the sulfhydryl reagent. Therefore, to determine the sedimentation constant of the serum proteins reacting with the cytoplasmic fractions and responsible for complement fixation, 3 sera were submitted to ultracentrifugation in a saline gradient. In all instances the 19 S layer exhibited no serological activity by complement fixation. With one serum a doubtful result was obtained with the intermediate layer. All sera gave a positive complement fixation with the layer that contained only 7 S gammaglobulin and was free of high molecular weight protein. The immunoelectrophoretic analysis of the fractions of the 7 S layer showed one gammaglobulin line. Some of these 7 S fractions and the fractions of the intermediate layer exhibited a $beta_{2A}$ line. These findings indicated that complement fixing antibody was also associated with the 7 S fraction but that the ability to fix complement was destroyed by mercaptoethanol.

B. Influence of mercaptoethanol on the complement binding capacity of 7 S gammaglobulin induced by heat aggregation. In preliminary studies a concentration of 0.8 mg human plasma fraction II was found most suitable for quantitative complement fixation. No complement was fixed by the unheated preparation. Five minutes heating at 60°C resulted in the fixation of 25 units, 10 minutes heating in the fixation of 80 units, and heating for 25 minutes made the gammaglobulin so anticomplementary that only 0.5 ml aliquots could be used rather than one ml. This resulted in the fixation of 83 units (= 166 units/ml) (Fig. 1).

When native unheated 7 S gammaglobulin was treated with mercaptoethanol, dialyzed against iodoacetamide, and heat aggregated at 60°C for 5-25 minutes, the degree of turbidity was similar to native 7 S gammaglobulin so heated. However, these preparations no longer fixed significant amounts of complement (Fig. 1).

When the mercaptoethanol treated samples were dialyzed against saline instead of iodoacetamide, small amounts of complement were fixed after heat aggregation of these preparations (10 units with a 10 minute heat aggregated sample).

Iodoacetamide treatment of gammaglobulin without mercaptoethanol did not diminish its complement binding capacity after heat aggregation.

The precipitability with rheumatoid factor of these heated fractions was also examined. Maximum precipitation was obtained with the gammaglobulin preparations which had been heat aggregated for 30 minutes. Gammaglobulin treated with mercaptoethanol and then heated for 30 minutes at 60°C also served as an effective reagent for rheumatoid factor since it did not lose its ability to be precipitated by the rheumatoid factor. Though difficult to quantitate accurately, the precipitation reactions of the heat aggregated native and mercaptoethanol treated samples appeared to be comparable.

Aggregation of gammaglobulin by heating followed by treatment with mercaptoethanol and dialysis against iodoacetamide resulted in a considerable but not complete loss of complement fixing ability.

Discussion. The mechanism by which 7 S

γ globulin triggers complement fixation has been studied by a number of procedures. Digestion of this immune globulin with papain and cysteine gives rise to 3.5 S fragments; the antibody combining sites are associated with one part of the molecule (fragment I and II in the rabbit, corresponding to fraction A and C in man) which does not bind complement. Complement fixation was found to be dependent on rabbit fragment III or human fragment B. Complement is not only fixed upon reaction of 7 S antibody with antigen, but also if the 7 S molecules are unspecifically aggregated by heat(12,13, 14). Whereas heat aggregated antigen-combining fragments obtained by papain digestion are not anticomplementary, heat aggregated rabbit fragment III and human fragment B fix complement, proving that these fragments are essential for complement fixation.

Our results with human gammaglobulin (human plasma fraction II) and with human 7 S immune globulins have confirmed that treatment with a sulfhydryl reagent does not alter the ability of the immune globulin to combine with antigen, as measured by the antiglobulin consumption test, nor to prevent gammaglobulin from aggregating as indicated by its opalescence and ability to react with the rheumatoid factor. However, 7 S gammaglobulin and certain 7 S gammaglobulin antibodies so treated can no longer fix complement after combining with antigen or artificial heat aggregation. In view of the specificity of action of sulfhydryl reagents and the partial restoration of activity upon dialysis against saline as compared to the permanent loss of activity upon dialysis with iodoacetamide, it would appear that the ability of the aggregated fraction B to fix complement is related to the integrity of the disulfide bonds in the molecule. The loss of complement fixing ability after exposure to mercaptoethanol appears not to be limited to human 7 S gammaglobulin antibodies, since independent studies by Taranta(15) and by Spiegelberg(16) suggest that 7 S rabbit antibodies to sheep and rat erythrocytes also lose their capacity to fix complement upon treatment with sulfhydryl reagents.

The fact that mercaptoethanol treated gammaglobulin still aggregates after heating and subsequently reacts with the rheumatoid factor is important in view of the mechanism of the latter reaction. This result suggests that the reactive state of 7 S gammaglobulin for fixing complement is different from that necessary to react with the rheumatoid factor.

The finding of selective loss of the ability of some human 7 S antibodies to fix complement upon exposure to a sulfhydryl reagent is also of practical importance in view of the wide use of mercaptoethanol inactivation as a screening test for 19 S antibodies. The result of this procedure is probably only valid in distinguishing between 7 S and 19 S antibodies if the assay procedure measures the ability of the treated immune globulin to react directly with its corresponding antigen and not if secondary effect such as complement fixation is used. Studies of large numbers of classical human 7 S antibodies are needed to establish this point with certainty.

Summary. The effect of mercaptoethanol on the ability of human 7 S-antibodies and human 7S gammaglobulins (γ_2) to react with antigen, complement and rheumatoid factor was studied. 1. Human 7 S-immune globulin continued to combine with antigen after mercaptoethanol treatment. 2. Human gammaglobulin, treated with mercaptoethanol, could be aggregated by heat as measured by the ability to precipitate with a potent rheumatoid factor. 3. Mercaptoethanol treatment of 7 S-antibodies and gammaglobulin resulted in a loss of ability to fix complement after reacting with antigen or after nonspecific heat aggregation respectively. These results stress the importance of disulfide bonds in the complement fixation reaction.

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In vivo Incorporation of Iodinated Anti-Rat Brain Globulin and Normal Globulin into Rat Organs. (28441)

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The use of antiorgan sera to produce damage to the corresponding organ has been reported frequently: for kidney, Masugi(1) and Baxter(2); pancreas, Armin(3); colon, LeVeen(4); thyroid, Witebsky(5); but not for brain. Kolb and Bolton(6) failed to produce changes in rats after intravenous injections of anti-rat brain serum. Williams(7,8) produced a transient encephalopathy in dogs after intrathecal injections of anti-dog brain serum (on 2 occasions after i.v. injections), and there were deviations from the normal brain histology. When anti-rat brain serum was injected intravenously or intrathecally into rats no such reaction occurred.

Since it was felt that perhaps changes in rat brain after injecting anti-rat brain serum were not sufficiently marked to be observable grossly, labelled anti-brain serum was used to determine whether the antibody was localizing in the brain or not.

The results of studies of intravenous and intrathecal injections of I^{131} and I^{125} labelled anti-rat brain rabbit globulin and normal rabbit globulin into rats sacrificed at varying intervals after injection are reported here.

Materials and methods. Production of antiserum. The antigen used for antiserum production was a saline homogenate prepared by grinding fresh rat brain in saline to make a 10% suspension (10 g per 90 cc saline).

White male rabbits weighing approximately 7 kg were injected intraperitoneally twice a week starting with 1 cc of suspension and increasing to 4 cc until the serum obtained from a trial bleeding gave a high precipitin titer against the supernate from the rat brain homogenate, usually 1-800 to 1-1600. The complement-fixation titer averaged 1-160.

Blood was obtained from several rabbits by cardiac puncture, kept overnight in the refrigerator, the sera were removed, pooled and stored at −14°C until used. *Preparation and labelling of gamma globulin.* Jager and Nickerson's method(9) of precipitation with saturated ammonium sulfate at pH 7 was used to separate the antibody-containing gamma globulin from the rabbit serum. Normal rabbit serum globulin was also prepared. Ten cc of the resulting globulin solution was dialyzed overnight against 1 liter of borate buffer (0.16 M NaCl and 0.20 M H_3BO_3 brought to pH 8 with NaOH). The contents of the dialysis bag were concentrated by suspending the bag in front of a stream of air until the globulin content was 8-10 mg per cc as measured by the Greenberg method.

Iodination of the globulin was performed according to the method of Goodland *et al.* (10) with the modification that the globulin-