INVESTIGATION OF THE ACTION OF A REDUCING AGENT ON THE EFFECTOR FUNCTIONS OF RABBIT γG ANTIBODIES

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Reduction of the sole disulfide bond in the hinge region of the rabbit γG antibody molecule has a significant effect on the effector functions of the antibodies during their interaction with antigen. The activity of the antibodies in the complement fixation test is reduced by 40% and in the direct and reversed passive cutaneous anaphylaxis tests by 60 and 70% respectively. No change in the antigen-binding properties of the reduced antibodies in the passive hemagglutination test was observed.

During the investigation of the structure and function of immunoglobulins and their subunits, conclusive proof was obtained that the antigen-binding sites of the γG antibodies are located in the Fab-fragments, whereas sites responsible for the more important effector functions of these proteins (the property of fixing complement, of adherence to cells, of passing through physiological barriers) are located in the region of the Fc-part of the molecule [7]. Although the Fab- and Fc-fragments of the γG-globulin molecule are characterized by marked structural autonomy, their interaction with antigen is essential for the antibodies to perform their effector functions.

In the light of this fact it was postulated that activation of the effector centers of γG globulin responsible for these properties is connected with the modification of the tertiary structure of the molecule, based on changes in the mutual arrangement of its Fab- and Fc-fragments, rendered possible because of the existence of a flexible hinge region in the molecule [2].

One way of testing this hypothesis is by studying the effector properties of antibodies after selective injury to the structure of the hinge region of the molecule; this can be done by cleavage of the interchain disulfide bond located in this region, which is highly sensitive to the action of reducing agents.

EXPERIMENTAL METHOD

Rabbit γG globulin from antiserum to egg albumin, purified on DEAE-Sephadex, was used.

Reduction of the disulfide bonds of the protein was carried out with 2-mercaptoethanol (2-ME) at pH 7.2 and 20°C for 2 h. The number of reduced disulfide bonds was determined by spectrophotometric titration of the protein with p-chloromercuribenzoate at 255 nm in 0.3 M acetate buffer, pH 4.6, after removing the 2-ME from the titrated sample on a column with Sephadex G-25 equilibrated with 0.15 M NaCl, pH 3.5. The reduced SH groups of the protein were blocked with Na monoiodoacetate or with iodoacetamide at pH 7.2 (20 min at 20°C).

To determine the position of the ruptured disulfide bond the γG globulin was carboxymethylated with C^{14}-labeled Na monoiodoacetate and hydrolyzed with pepsin [8], and the Fab'-fragment was isolated from the digest on CM-cellulose in 0.01 M acetate buffer, pH 5.8.

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TABLE 1. Liberation of Sulfhydryl Groups by Reduction of Rabbit γG Globulin with Various Concentrations of 2-ME

Concentration of 2-ME	Mean number of SH groups in γG-globulin molecule	Number of ruptured disulfide bonds per mole protein
—	0	0
0,005 M	2	1
0,01 M	2,5	—
0,025 M	4,2	2
0,05 M	6,1	3
0,1 M	8,0	4

The complement fixation test was carried out in the quantitative modification [3]. Complement-fixing activity was expressed as the number of 50% hemolytic units of complement fixed by 0.025 μg of the test preparation with the equivalent dose of antigen.

The direct and reversed passive cutaneous anaphylaxis tests were carried out by Ovary's method [9]. As the reacting agent egg albumin was used in the direct test and donkey serum against rabbit γG globulin in the reversed test.

The antigen-binding activity of native and reduced antibodies was determined by the indirect hemagglutination test [5].

The double diffusion tests in agar and immunoelectrophoresis were carried out in micromodifications [1, 4].

Sedimentation analysis was carried out in the Spinco Model E ultracentrifuge at 20°C, using 0.15 M NaCl as the solvent. The speed of the centrifuge for the standard cell was 59,780 rpm.

EXPERIMENTAL RESULTS

Before the effector functions of the γG globulin could be studied it was necessary to establish whether the γG globulin in the preparations for study is in the aggregated form, for if so it would have the property of binding complement nonspecifically and of inducing allergic reactions of the Prausnitz–Kuestner type if injected intradermally [2].

Analysis of the γG globulin, purified on DEAE-Sephadex, by gel-filtration on Sephadex G-200 showed that, besides the monomeric form of γG globulin, it also contained small quantities of aggregated material. To remove this material the γG globulin preparations obtained on DEAE-Sephadex were further fractionated on a column with Sephadex G-200. The preparations of γG globulin, freed from aggregates, used in all subsequent experiments were homogeneous in the double diffusion test in agar and on immunoelectrophoresis when developed with donkey serum against rabbit immunoglobulins. In the ultracentrifuge they were sedimented as one peak with a sedimentation constant of 6.65.

In order to choose the conditions necessary for rupture of only the interchain disulfide bond in the hinge region of the molecule, the rabbit γG globulin was reduced with various concentrations of 2-ME. As will be clear from Table 1, cleavage of a single disulfide bond under the experimental conditions chosen took place in the presence of 0.005 M 2-ME. The localization of this disulfide bond in the molecule was determined by studying the distribution of radioactive Na monoiodoacetate, used to block the SH groups liberated as a result of reduction, in the various fragments of the γG globulin. The results showed that 90% of the radioactivity of the reduced alkylated γG-globulin was contained in its pepsin Fab'-fragments. The sedimentation constant of the Fab-fragment obtained from the γG globulin with one disulfide bond ruptured in its molecule, as a result of hydrolysis with pepsin, was 3.5S, corresponding to the size of the univalent Fab-fragment. It will be evident from the facts described above and the classical ideas of the structure of γG globulin [6] that treatment with 0.005 M 2-ME leads to rupture of the disulfide bond in the hinge region of the γG globulin molecule.

With a tenfold increase in the concentration of the reducing agent another two disulfide bonds (probably linking heavy and light chains in the Fab-fragments) were ruptured.

Preparations of γG globulin from rabbit antiserum against egg albumin, reduced with 0.005 M and 0.05 M 2-ME, were treated with Na monoiodoacetate or iodoacetamide in order to block the liberated SH groups and were studied in the quantitative complement fixation test, the direct and reversed passive cutaneous anaphylaxis tests, and the indirect hemagglutination test.

The results illustrated in Fig. 1 show that rupture neither of one nor of three interchain disulfide bonds led to changes in the antigen-binding capacity of the γG antibodies in the indirect hemagglutination test. Meanwhile, after reduction of one disulfide bond in the hinge region of the molecule the complement-fixing ability of the γG globulin was reduced by 40%. The activity of the same preparations in the direct and reversed passive cutaneous anaphylaxis test was reduced by 60 and 70%, respectively. The degree of inactivation of the complement-fixing properties of the antibodies and of their ability to react in the passive cutaneous anaphylaxis test after the rupture of two further interchain disulfide bonds in the γG globulin

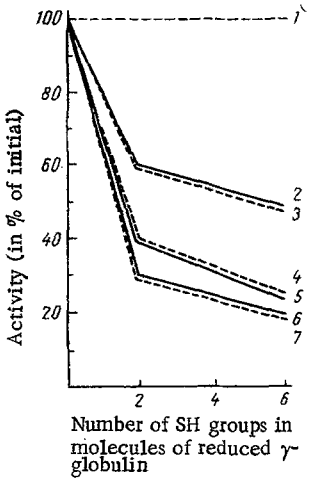

Fig. 1

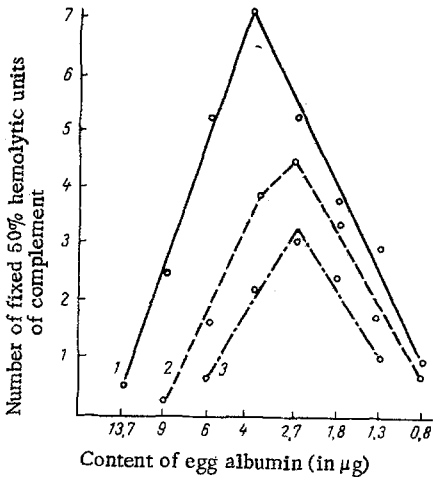

Fig. 2

Fig. 1. Effect of rupture of interchain disulfide bonds in rabbit γG antibody molecule on their activity in complement fixation, indirect hemagglutination, and passive cutaneous anaphylaxis tests: 1) indirect hemagglutination test; 2, 3) complement fixation test; 4, 5) direct passive cutaneous anaphylaxis test; 6, 7) reversed passive cutaneous anaphylaxis test. 1, 2, 4, 6) SH groups blocked by Na monoiodoacetate; 3, 5, 7) SH groups blocked with iodoacetamide.

Fig. 2. Quantitative determination of complement fixation by native and reduced rabbit γG antibodies in the presence of the specific antigen: 1) native γG globulin, samples of 0.025 μg; 2) γG globulin with one reduced disulfide bond, 0.025 μg; 3) γG globulin with three reduced disulfide bonds, 0.025 μg.

molecule was increased only very slightly. The closely similar values of the decrease in activity of the reduced γG globulin in the direct and reversed passive cutaneous anaphylaxis reactions unquestionably indicate loss of its ability to undergo fixation in the skin. The degree of inactivation of the antibodies in the above tests, it must be emphasized, was independent of the nature of the agent used to block the SH groups (Fig. 1).

Examination of the complement fixation curves for a wide range of antigen concentrations shows that rupture of interchain disulfide bonds in the γG globulin molecule not only leads to a decrease in the quantity of complement fixed at the point of equivalence, but also to narrowing of the fixation zone, especially in the region of excess antigen (Fig. 2). When the same preparations of reduced, alkylated γG globulins were tested in the quantitative precipitation test, narrowing of the precipitation zone also was revealed, especially in the region of excess antigen.

Taken as a whole the results indicate that the decrease in the complement-fixing activity and cytotropic properties of γG globulin is not directly dependent on the number of reduced interchain disulfide bonds, but that rupture of the disulfide bond in the hinge region of the molecule gives rise to the greatest changes in these properties.

Since there is every reason to suppose that the complement-fixing site and the site responsible for the ability of γG globulin to undergo fixation to the skin are located in the Fc-fragment of the molecule, rupture of the disulfide bond in the hinge region cannot directly affect the structure of these centers. Consequently, as a result of reduction indirect activation of these sites takes place, and this can be connected only with a change in the general conformation of the γG globulin molecule, expressed as a change in the mutual arrangement of the Fab- and Fc-fragments, both in the free form and during interaction of the antibody molecule with the antigen.

LITERATURE CITED

1. A. I. Gusev, in: Immunochemical Analysis [in Russian], Moscow (1968), p. 99.
2. A. Ya. Kul'berg, Uspekhi Sovr. Biol., 76, No. 1, 110 (1973).
3. I. A. Tarkhanova, in: Immunochemical Analysis [in Russian], Moscow (1968), p. 79.

4. V. S. Tsvetkov, in: Immunochemical Analysis [in Russian], Moscow (1968), p. 120.
5. S. V. Boyden, J. Exp. Med., 93, 107 (1951).
6. D. Givol and F. De Lorenzo, J. Biol. Chem., 243, 1886 (1968).
7. G. M. Edelman and W. E. Gall, Ann. Rev. Biochem., 38, 415 (1969).
8. A. Nisonoff, F. C. Wissler, and D. N. Woernley, Arch. Biochem., 89, 230 (1960).
9. Z. Ovary, Progr. Allergy, 5, 459 (1958).