PROPERTIES OF GUINEA PIG 7S ANTIBODIES

V. INHIBITION BY GUINEA PIG γ_1 ANTIBODIES OF PASSIVE IMMUNE LYSIS PROVOKED BY γ_2 ANTIBODIES*

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Several recent studies have characterized two types of guinea pig 7S antibodies capable of reacting with the same antigen but differing in their electrophoretic mobility (1–3), binding to DEAE cellulose columns (4), and biological properties (5, 6). Guinea pig $7S\gamma_1$ antibodies were shown to mediate passive systemic and cutaneous anaphylaxis in the guinea pig; $7S\gamma_2$ antibodies did not mediate these activities. Guinea pig $7S\gamma_2$, but not γ_1 antibodies, fixed complement *in vitro* in the presence of antigen, under the conditions tested, and lysed antigen-coated erythrocytes in the presence of complement. Both antibodies were able to agglutinate such cells and yielded an identical precipitin line with the antigen.

Guinea pig gamma-2 antibodies specifically inhibited passive cutaneous anaphylactic reactions provoked by their gamma-1 counterpart, presumably by competing for antigen. Gamma-2 antibodies, lacking the property involved in fixation to guinea pig skin, were unable to inhibit passive cutaneous sensitization by a heterologous antibody system (5). The present report deals with the *in vitro* inhibition by guinea pig $7S\gamma_1$ antibodies of passive immune lysis provoked by $7S\gamma_2$ antibodies directed against the same hapten antigen.

Materials and Methods

Procedures used in the preparation of hapten-conjugate antigens, immunization of guinea pigs, purification of antibodies, separation of protein by starch block electrophoresis, immunoelectrophoresis, and serologic characterization of guinea pig γ_1 and γ_2 antibodies, have been previously described (1, 5, 6).

Antigens.—

2,4-dinitrophenyl–bovine gamma globulin (DNP-BGG) and 2,4-dinitrophenyl–bovine serum albumin (DNP-BSA) were prepared as in reference 4. *p*-Aminosuccinanilic acid was

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conjugated to BGG (SAn-BGG) or to BSA (SAn-BSA) by diazotization (7). These preparations were all highly conjugated.

Antibodies.—

Antisera, prepared as described (1), contained primarily 7S antibodies.

I. Preparation of γ_1 and γ_2 Globulin Fractions by Electrophoretic Separation of Guinea Pig Antihapten Antisera.—Sera obtained from guinea pigs hyperimmunized with DNP-BGG or SAn-BGG emulsified in complete Freund's adjuvant, were subjected to starch block electrophoresis as previously described (1). Antibody fractions were eluted from ½ inch cuts, made in the gamma globulin region, dialyzed against distilled water containing 0.005 M phosphate buffer, pH 7.5, and concentrated approximately 15-fold by lyophilization and resuspension in 1.0 ml saline. The protein content of the eluates was determined by the Folin method (8). The content of hemolytic and guinea pig skin-sensitizing antibodies in individual fractions was assayed by passive hemolysis and PCA tests. With guinea pig anti-DNP–BGG antisera, it was possible to test the inhibiting capacity of individual fractions obtained from the "fast" gamma globulin region of the starch block. However, the low antibody content of eluates obtained by electrophoretic separation of the weaker guinea pig anti-SAn–BGG antisera precluded testing individual fractions. Therefore, all slow migrating hemolytic fractions (without demonstrable skin-sensitizing activity) were combined and concentrated by lyophilization, "pool S anti-SAn–BGG," as were all fast migrating skin-sensitizing fractions (without hemolytic activity) "pool F anti-SAn–BGG."

II. Preparation of γ_1 and γ_2 Antibody Fractions by Electrophoretic Separation of Purified Guinea Pig Antihapten Antibodies.—Purified guinea pig anti-DNP–BGG antibodies were isolated from two pools of guinea pig antisera, each prepared with serum from 4 animals. The final preparations contained some non-dialyzable hapten (1).

Purified antibody preparations were separated by starch block electrophoresis, eluates prepared and treated as described above. Reconstituted fractions were characterized in immunoelectrophoresis developed with rabbit serum R2 (anti-guinea pig serum, reference 9). Fast migrating fractions yielding a single precipitin line in the γ_1 region were combined as "pool I (or II) purified γ_1 anti-DNP–BGG antibody." Slow migrating fractions yielding a single precipitin line in the γ_2 region on immunoelectrophoresis were combined as pool I (or II) purified γ_2 anti-DNP–BGG antibody. Identification of γ_1 or γ_2 antibodies by immunoelectrophoresis led to satisfactory separation; skin-sensitizing antibodies were not detected by PCA in the purified γ_2 anti-DNP–BGG antibody preparation (upon intradermal injection of 10 μg of antibody) nor was passive hemolytic activity detected, under experimental conditions described below, in the purified γ_1 anti-DNP–BGG antibody preparation, using 300 μg γ_1 antibody per ml, to sensitize antigen-coated cells.

III. Preparation of γ_1-Globulin by Electrophoretic Separation of Normal Guinea Pig Serum.—Two pools of normal serum were separated by starch block electrophoresis and fractions eluted from the zone of migration of γ_1 globulins were combined and lyophilized. This preparation, called "normal guinea pig γ_1 globulin" contained mostly γ_1 globulins, and small amounts of γ_2 globulin and of faster migrating proteins on immunoelectrophoresis.

Passive Hemagglutination and Hemolytic Procedures.—

I. Preparation of Sensitized Cells.—Tannic acid–treated sheep red blood cells (SRBC) were prepared by the method of Stavitsky (10) and were immediately coated with highly conjugated hapten-protein antigens. 0.5 ml of packed tanned SRBC was suspended in a mixture of 5 ml of buffered saline (pH 6.4) and 0.2 ml of a DNP-BSA solution (10 mg protein per ml, 36 haptenic groups per mole), held at room temperature 30 minutes, washed twice in phosphate-buffered saline (pH 7.2), and resuspended in this buffer as a 1 per cent suspension. Tanned SRBC were similarly coated with SAn-BSA by using 0.6 ml of a SAn-BSA prepara-

tion (3.94 μg protein per ml, 20 groups per mole). Coated cells were used within 48 hours. Prior to use, cells were resuspended in fresh buffered saline to avoid any inhibitory effects of dissociated antigen. Although different cell preparations yielded variable hemagglutination titers under these conditions, the hemolytic titer obtained with a fixed amount of antibody was almost constant. Nevertheless, a series of experiments was always performed on the same day, using one antigen-coated cell suspension.

II. Passive Hemagglutination Procedure.—Antibody solutions were inactivated at 56°C for 30 minutes and twice absorbed with an equal volume of packed SRBC at 37°C for 30 minutes and at 0°C for 15 minutes. Dilutions were made in veronal buffer (8) containing 1 per cent normal rabbit serum (inactivated, thoroughly absorbed with SRBC). To serial 2-fold dilutions of antibody in 0.5 ml volume, 0.1 ml of 1 per cent antigen-coated cell suspension was added; patterns of agglutination (10) were read after 18 hours at room temperature.

III. Passive Hemolysis Procedure.—All experiments were performed with absorbed antibodies, and veronal buffer (8) as diluent.

A. Titration of complement: The scaled-down procedure of complement titration was employed (8). Reconstituted lyophilized guinea pig complement (Certified Blood Donor Service, Jamaica, New York) was titrated in 1.5 ml total reaction volume with 0.2 ml of optimally sensitized standard sheep erythrocyte suspension. The $C'H_{50}$ unit was defined as lysing 50 per cent of the cells added; *i.e.*, 0.5×10^8 cells. Under these conditions, absorbed complement had 644 $C'H_{50}$ units per ml and unabsorbed complement had 781 $C'H_{50}$ units per ml. In all experiments involving passive hemolysis, complement was previously absorbed with sheep erythrocytes (8).

B. Hemolysin titrations: Hemolysin titrations were performed in 0.7 ml volume. To 0.5 ml of the antibody dilution was added 0.1 ml of 1 per cent antigen-coated cell suspension. After 30 minutes of incubation at 37°C, the tubes were cooled, and 0.1 ml of a dilution of complement titrating 43 $C'H_{50}$ per ml was added. (The amount of complement added represents more than 5 times the amount necessary to lyse 100 per cent of the sensitized, tanned SRBC in this system.) The tubes were reincubated at 37°C for 7 or 10 minutes. Degree of lysis was estimated visually and the final dilution showing 100 per cent lysis was considered end-point of the solution tested. The highest antibody dilution yielding total lysis in this system was carefully determined and defined as containing one H_{100} unit of lytic γ_2 antibody. This amount of antibody was used in the inhibition experiments.

C. Inhibition of lysis:

1. Simultaneous incubation of non-lytic and lytic antihapten antibodies: Serial 2-fold dilutions of non-lytic γ_1 antibodies were prepared in 0.25 ml aliquots. To each tube was added 0.25 ml of a lytic γ_2 antibody dilution containing one H_{100} unit followed by 0.1 ml of 1 per cent antigen-coated cell suspension. The mixture was incubated at 37°C for 30 minutes. Lysis was performed as described above, followed by the addition of 1.5 ml, ice-cold stop diluent (8). After centrifugation, degree of lysis was estimated visually or by spectrophotometer measurement at 413 mμ (11).

2. Sequential incubation of hapten conjugate–coated erythrocytes with non-lytic followed by lytic antihapten antibodies: Serial 2-fold dilutions of non-lytic γ_1 antibodies were prepared in 0.5 ml aliquots, 0.1 ml of 1 per cent cell suspension was added, mixed, and incubated at 37°C for 30 minutes. Tubes were then centrifuged, supernates decanted, and cells washed in 1 ml veronal buffer and recentrifuged. The cells were resuspended in 0.5 ml of a dilution of lytic antibody containing one H_{100} unit and incubated at 37°C for 30 minutes. Thereafter, lysis was performed as above.

Passive cutaneous anaphylaxis (PCA) was performed as described (5). Guinea pigs were challenged intravenously with 250 μg DNP-BSA or 400 μg SAn-BSA.

Complement fixation with the anti-DNP antibodies was performed as described in (6). 0.5 ml of antibody dilution (in veronal buffer) was combined with 0.5 ml of complement dilution (containing 3.5 $C'H_{50}$ units) and 0.5 ml of antigen dilution. Reactants were held at 0°C for 18 hours followed by the addition of 0.2 ml of sensitized SRBC and incubation at 37°C for 30 minutes. Degree of lysis in supernates was estimated visually.

RESULTS

I. Inhibition Experiments with Purified Antihapten Antibodies.—

A. Inhibition of lysis by simultaneous incubation of hapten conjugate–coated erythrocytes with non-lytic γ_1 and lytic γ_2 antihapten antibodies: In a first series of experiments, DNP-BSA–coated, tannic acid–treated erythrocytes were exposed to the lytic activity of purified γ_2 anti-DNP–BGG antibody in the presence of varying concentrations of non-lytic γ_1 anti-DNP–BGG antibodies (both prepared from anti-DNP–BGG antiserum, pool I). The characteristics of the reac-

TABLE I

Characteristics of Purified Guinea Pig γ_1 and γ_2 Anti-DNP–BGG Antibodies (Pool I)

Pool I	Passive hemagglutination	Passive hemolysis	PCA
	μg/ml	*μg/ml*	*μg/ml*
γ_2 Anti-DNP–BGG	0.3	3.0	None
γ_1 Anti-DNP–BGG	0.3	None	0.5

Titers are expressed as the lowest antibody concentration in μg/ml capable of producing significant agglutination, total lysis, and PCA reaction of 10 mm.

tants in this system are presented in Table I. Both purified γ_1 and γ_2 antibodies produced significant agglutination (2+ reaction) of DNP-BSA–coated erythrocytes at a concentration of 0.3 μg antibody/ml. Complete lysis of these cells was produced by 3.0 μg γ_2 antibody per ml; γ_1 antibodies failed to produce lysis at a concentration of 300 μg per ml under same conditions. γ_2 Antibody did not sensitize guinea pig skin for PCA when tested at a concentration of 100 μg/ml; γ_1 antibodies yielded significant PCA reaction in guinea pig skin at 0.5 μg per ml. No anticomplementary effects were observed with the γ_1 preparation at a concentration of less than 200 μg per ml (titrated after incubation at 0°C for 18 hours).

Fig. 1 *A* illustrates results obtained on incubation of varying amounts of γ_1 antibody in 0.25 ml volume with a fixed amount of γ_2 antibody; *i.e.*, one H_{100} unit in 0.25 ml volume. Under these conditions, γ_1 antibody at a final concentration of 100 μg per ml completely inhibited lysis, whereas concentrations ranging from 100 to 10 μg per ml produced partial inhibition of lysis; less than 10 μg antibody per ml failed to affect lysis.

Similar results were obtained with pool II γ_1 and γ_2 anti-DNP–BGG antibodies.

Normal guinea pig gamma-1 globulin at a concentration of 500 μg and 1 mg per ml failed to inhibit lysis provoked by one H_{100} unit of γ_2 anti-DNP–BGG antibodies.

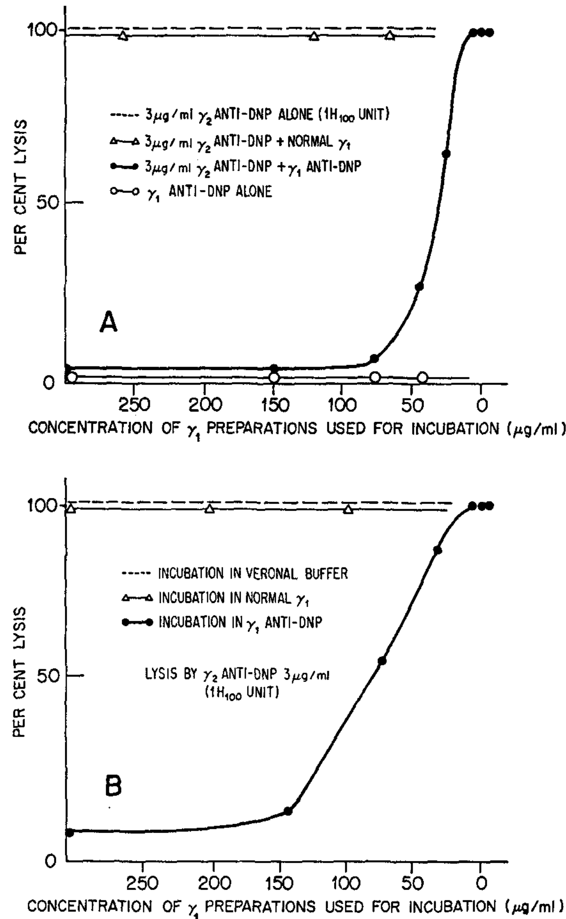

FIG. 1. Inhibition by purified γ_1 anti-DNP antibodies of passive hemolysis produced by the γ_2 antibodies isolated from the same sera. Fig. 1 *A*. Simultaneous incubations of DNP-BSA–coated SRBC with γ_1 and γ_2 anti-DNP antibodies. Fig. 1 *B*. Seueqntial incubation of DNP-BSA–coated SRBC with γ_1 and γ_2 anti-DNP antibodies.

B. Sequential incubation of hapten conjugate–coated erythrocytes with nonlytic γ_1 followed by lytic γ_2 antihapten antibodies: In these experiments, DNP-BSA–coated, tanned SRBC were incubated with varying amounts of γ_1 antibody, centrifuged, washed once with buffer, and subsequently exposed to the lytic action of γ_2 anti-DNP–BGG antibodies (one H_{100} unit).

Fig. 1 *B* illustrates the results obtained in these experiments; inhibition of lysis is comparable to that obtained by simultaneous incubation. Inhibition appears to be less complete than that illustrated in Fig. 1 *A*. The difference may be due to the loss of some antigen-coated SRBC during washing.

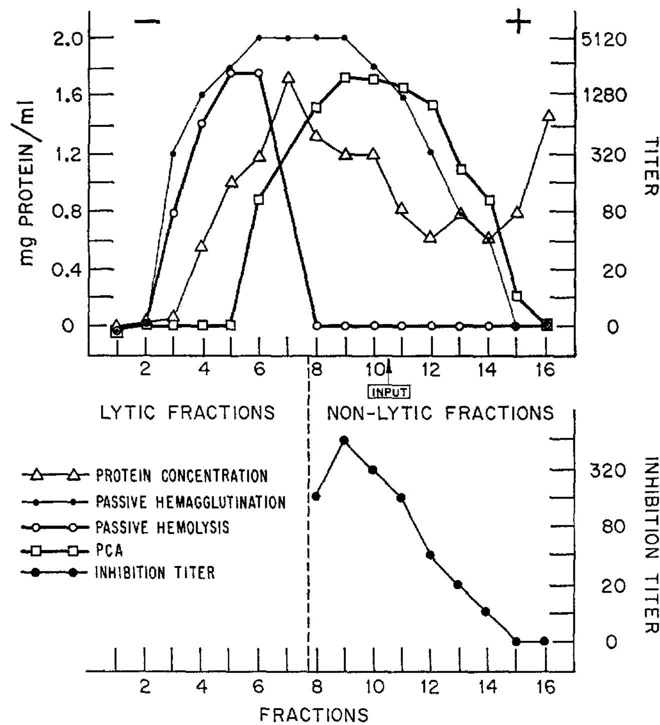

FIG. 2. Starch block electrophoretic separation of an anti-DNP-BGG guinea pig serum showing the inhibitory effect of the fast migrating fractions on passive hemolysis produced by one H_{100} unit of fraction 5, and correlating this activity with the PCA and passive hemagglutination titer of these fractions.

Similar results were again obtained with pool II γ_1 and γ_2 anti-DNP-BGG antibodies.

In all cases studied, the amount of γ_1 required to completely inhibit lysis provoked by one H_{100} unit of γ_2 was about 50 times the amount of γ_2 used.

II. Inhibition Experiments with Isolated γ_1 and γ_2 Globulin Fractions.—Serum obtained from guinea pig 1, hyperimmunized with DNP-BGG, was separated by starch block electrophoresis and results of tests performed on eluates are shown in Fig. 2. Antibodies capable of agglutinating DNP-BSA-coated, tanned erythrocytes were present in eluates obtained from the entire gamma globulin region. Slow migrating fractions 3 to 7 lysed these cells in the presence of complement and fixed complement in the presence of antigen (not shown). Fast

migrating fractions 6 to 15 contained guinea pig skin-sensitizing antibodies. Non-lytic fractions 8 to 16 were used to inhibit lysis provoked by lytic fraction 5.

Antigen-coated SRBC were incubated in serial dilutions of each non-lytic fraction 8 to 16, washed, and subsequently exposed to one H_{100} unit of lytic fraction 5. Results are plotted in the lower part of Fig. 2. The inhibition titer shown is defined as the highest dilution of fast fraction which is capable of reducing by 50 per cent or more, the lytic effect of one H_{100} unit of fraction 5. The inhibition curve parallels the curve of PCA activity of the fast fractions. Normal γ_1-globulin at a concentration of 1.6 mg/ml failed to have any inhibitory effect on lysis provoked by fraction 5.

Since failure to lyse sensitized cells could arise from unusual deviation or exhaustion of complement by components of the test system, it was necessary to assay the availability of complement for lysis in tubes showing complete inhibition. Therefore, after removal of cells from these tubes, 0.2 ml of optimally sensitized sheep red blood cells were added and incubated at 37°C for 15 minutes. Total lysis of the added cells was observed indicating the presence of adequate amounts of residual complement.

Inhibition of lysis might also arise through elution of antigen from the surface of tanned SRBC following incubation with specific antibody and subsequent experimental manipulations. In an attempt to test this possibility, one H_{100} unit of lytic fraction 5 was used to sensitize 0.1 ml of DNP-BSA–coated, tanned SRBC suspension during incubation at 37°C for 30 minutes. After centrifugation, washing, and resuspension in 0.5 ml of veronal buffer, the cells were reincubated at 37°C for 30 minutes and complement was then added. Complete lysis occurred within less than 7 minutes, indicating that no significant elution of antigen occurred under these experimental conditions.

III. Specificity of the Inhibitory Effect of γ_1 Immune Globulins.—The specificity of the inhibitory effect of$_2$ γ_1 antibodies on passive immune lysis provoked by their γ_2 counterpart was studied using two unrelated hapten conjugates: SAn-BGG and DNP-BGG. Antibodies directed against these haptens do not crossreact as demonstrated in Ouchterlony plates, passive immune lysis, and PCA.

Pool S anti-SAn–BGG (lytic) and pool F anti-SAn–BGG (non-lytic) were prepared as described. Fraction S anti-DNP–BGG (lytic) and fraction F anti-DNP–BGG (non-lytic) was obtained by starch block electrophoresis of guinea pig anti–DNP–BGG serum 6. The characteristics of these four preparations are summarized in Table II.

SAn-BSA–coated, tanned SRBC were incubated in serial dilutions of either fraction F anti-DNP–BGG, or in pool F anti-SAn–BGG. After centrifugation, these cells were resuspended in one H_{100} unit of pool S anti-SAn–BGG for subsequent lysis.

As illustrated in Fig. 3 *A*, the lysis of SAn-BSA–coated cells by pool S anti-

SAn–BGG is inhibited by the preincubation in pool F anti-SAn–BGG, but not by the preincubation in similar concentration of fraction F anti-DNP–BGG. Similarly (Fig. 3 *B*), the lysis of DNP-BSA–coated cells by fraction S anti-DNP–BGG, is specifically inhibited by preincubation in fraction F anti-DNP–

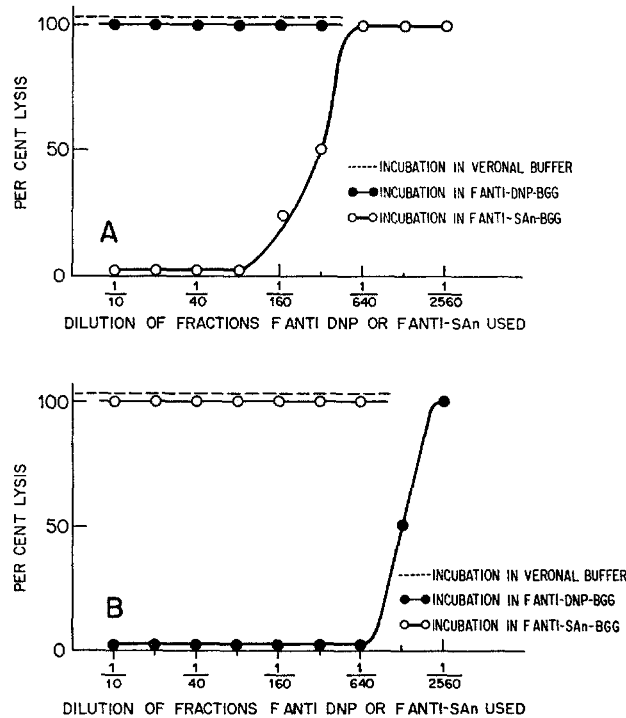

FIG. 3. Specific inhibition by guinea pig γ_1 anti-DNP or anti-SAn antibodies of passive hemolysis produced by one H_{100} unit of corresponding γ_2 fractions. Fig. 3 *A*. Lysis of SAn-BSA–coated cells sensitized with one H_{100} unit of pool S anti-SAn–BGG. Fig. 3 *B*. Lysis of DNP-BSA–coated cells sensitized with one H_{100} unit of fraction S anti-DNP–BGG.

TABLE II

Characteristics of Pool F and S Anti-SAn–BGG and of Fraction F and S Anti-DNP–BGG

	Protein concentration	Passive agglutination	Passive hemolysis H_{100}	PCA
	mg/ml			
Pool F anti-SAn–BGG	4.3	320	None (<10)	500
Pool S anti-SAn–BGG	3.3	240	60	None (<10)
Fraction F anti-DNP–BGG	5.4	20,000	None (<10)	5000
Fraction S anti-DNP–BGG	9.2	10,000	500	None (<10)

Titers are expressed as the highest dilution capable of producing definite agglutination, total lysis, and PCA reaction of 10 mm. Antigens used were SAn-BSA and DNP-BSA.

BGG, but not by incubation in pool F anti-SAn–BGG. Thus the same fast fraction which specifically inhibits lysis provoked by its own γ_2 counterpart, fails to inhibit lysis provoked by another γ_2 antibody directed against a different hapten.

DISCUSSION

Present experiments demonstrate an additional property of guinea pig 7S antibodies, namely, the ability of guinea pig $7S\gamma_1$ antihapten antibodies to inhibit passive immune lysis provoked by their $7S\gamma_2$ counterpart. Inhibition of lysis occurred under conditions of simultaneous incubation of hapten conjugate–coated erythrocytes with non-lytic γ_1 and lytic γ_2 antibodies, as well as under conditions of sequential incubation of cells with non-lytic antibodies followed by exposure to lytic antibodies.

The inhibitory effect appeared to parallel the skin-sensitizing ability of the γ_1 antibody preparations used. Inhibition of immune lysis was obtained only with γ_1 antibody of the same specificity as the lytic γ_2 antibody but was not obtained using normal guinea pig γ_1-globulin fractions or γ_1 antibody directed against a different hapten antigen.

Several experimental conditions require further comment. First, some globulin fractions used in these experiments had anticomplementary properties (12) or acquired them in the course of preparation; *i.e.*, lyophilization (13). However, the anticomplementary effect of eluates obtained from the γ_1 region of the starch block did not account for the inhibition of lysis observed. The experiments performed to test specificity of inhibition indicate that regardless of anticomplementary properties of the globulin solutions, only the specific γ_1 antibody inhibited lysis provoked by its γ_2 counterpart. Finally, it was shown that sufficient complement activity remained in the supernatant of tubes showing complete inhibition of lysis, to lyse sheep erythrocytes optimally sensitized with rabbit amboceptor.

It is also necessary to consider the possibility that apparent inhibition of lysis results from enhanced loss of antigen from hapten conjugate–coated, tanned erythrocytes. Under the usual experimental conditions, there is some elution of antigen from tanned erythrocytes (14). However, this loss of antigen does not significantly interfere with the test system used. Under the present experimental conditions there did not appear to be enhanced loss of hapten conjugate from cells incubated in large amounts of normal γ_1-globulin solutions nor in solutions of γ_1 antibody with a different hapten specificity. Furthermore, it is likely that solutions of γ_1 antibody of the same hapten specificity do not enhance loss of antigen from cells since it was experimentally shown that γ_2 antibody of the same specificity did not significantly elute antigen from tanned erythrocytes.

The present experiments suggest that the mechanism of inhibition of passive immune lysis is one of competition for antigen by guinea pig $7S\gamma_1$ and $7S\gamma_2$ antibodies. Since these two antibodies differ markedly in their biological prop-

erties, the outcome of competition determines, at least *in vitro*, the immunological consequences resulting from the union of antigen and antibody.

Competition for antigen has also been postulated to explain the specific inhibition by guinea pig $7S\gamma_2$ antibodies of passive cutaneous anaphylactic reactions provoked by guinea pig $7S\gamma_1$ antibodies (5). It is of interest that in both experimental situations, the ratio of the inhibiting to provoking antibody had the same order of magnitude.

The differences in immunological activities of these two types of guinea pig 7S antibodies, may be due to different characteristics of their piece III or H chains of the globulin molecule, since this part of the globulin molecule is responsible for the complement-fixing and skin-sensitizing property of antibodies in other species. In the rabbit, complement fixation has been shown to be a property of piece III obtained by papain digestion of rabbit antibody (15–17). Investigators employing the immunologically active fragments obtained by papain or pepsin digests of rabbit antibody have also observed specific inhibition of complement fixation and of immune lysis provoked by the native antibody (17, 18).

In our experiments, approximately 50 times as much γ_1 antibody was required to inhibit the lytic effect of one H_{100} unit of $\gamma_2$antihapten antibody. These experiments probably succeeded because (*a*) passive immune lysis involving a single hapten antihapten antibody system was studied, (*b*) the method of immunization and the animal species used produced large amounts of γ_1 antibody, (*c*) guinea pig $7S\gamma_1$ antihapten antibodies are probably no less avid for antigen than guinea pig $7S\gamma2$ antibodies, and (*d*) the experimental conditions selected limited the time available for exchange and rearrangement of antibody molecule on the surface of antigen-coated cells. Conversely, failure to demonstrate complete inhibition of lysis may be expected in systems involving complex antigens and those in which limited amounts of γ_1 antibody are produced.

The data presented illustrate that a non-lytic antibody such as guinea pig γ_1 can, under certain conditions of concentration and timing, inhibit hemolysis by the corresponding γ_2 complement-fixing antibody. The production of antibodies with biological properties similar to those of γ_1 guinea pig antibodies in significant amounts, by other mammalian species, has not been investigated and is presently being studied. The presence of antibodies with such properties in animals capable of showing immunological enhancement of tumors or grafts, may indeed help clarify some of the mechanisms of these puzzling phenomena.

SUMMARY

Passive immune lysis of antigen-coated erythrocytes provoked *in vitro* by guinea pig γ_2 antihapten antibodies in the presence of complement, is inhibited by guinea pig γ_1 antibodies directed against the same specificity.

This inhibition of the lytic effect of complement-fixing γ_2 antibodies is presumably due to the competition for antigen by non-lytic γ_1 antibodies.

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