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Research Article

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SEROLOGIC STUDIES OF PROTEOLYTIC FRAGMENTS OF RABBIT AGGLUTINATING ANTIBODIES *

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The reaction of the bivalent rabbit antibody molecule with papain (1, 2) or pepsin (3) and a reducing agent results in the release of two univalent fragments. The evidence for univalence is provided by the capacity of the fragments of hydrolyzed antiprotein antibody to inhibit specifically the homologous precipitin reaction (2), and by direct measurements of the maximal number of moles of hapten that can be bound by a mole of active "fragments" after hydrolysis of antihapten antibody with papain (4, 5, 6). The present communication describes another property of the univalent fragments, namely, their capacity to combine with, but not agglutinate, red blood cells of the appropriate antigenic type.

Whether unfragmented 7S "incomplete" antibodies are univalent is not known. This appears unlikely, at least in the case of "incomplete" anti-Rh_0, since it is difficult to understand how treatment of red cells with trypsin or other proteolytic enzymes could render them agglutinable by univalent antibody.

The data presented here indicate that univalent fragments of antibody can react specifically with antigens of erythrocytes and that the reaction does not cause agglutination, even when the cells are first treated with one of several proteolytic enzymes. That the fragments combine with the erythrocytes is shown by the occurrence of agglutination on subsequent addition of an untreated antibody specific for the univalent fragments.

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MATERIALS AND METHODS

Human erythrocytes

Ten ml of whole blood obtained from the same group O Rh_0 donor at 2-week intervals was mixed with an equal volume of Alsever's solution and stored at 4° C. Cells were discarded after 10 days. Human Oh^{A2} erythrocytes were obtained from a subject with blood of the "Bombay" (7) type and stored in a similar fashion.

Antisera

Rabbit antisera to human erythrocyte antigens (anti-HEA) were prepared by injection of rabbits weighing 4 kg intramuscularly once a week for 8 weeks with 5 ml of a mixture of Freund's complete adjuvant and a 50 per cent saline suspension of thrice-washed human red cells. Two rabbits were immunized with group O erythrocytes and two others with group Oh^{A2} "Bombay" erythrocytes. The antisera prepared against O cells and those prepared against Oh^{A2} cells were pooled separately. The γ-globulin fractions of the anti-HEA antisera were obtained by starch-block electrophoresis (8). The 7S components of the γ-globulins were obtained by diethylaminoethyl cellulose column chromatography (9) of the materials derived from the starch-block procedure. The resultant preparation showed only one peak, approximately 7S, on analytical ultracentrifugation. For some experiments further purification of the anti-HEA materials was achieved by absorption on and elution from packed, washed erythrocyte stroma (10). Residual hemoglobulin was removed from the antibody by precipitation with sodium sulfate.

For antihuman globulin rabbits were immunized with human γ-globulin (Pentex Co., fraction II) by repeated intravenous inoculations with 1 to 2 ml of a 1 per cent solution of the protein in 0.16 M saline given over a period of at least 2 months. As a control, rabbit anti-chicken ovalbumin was prepared by a similar method of immunization. Antisera of high titer obtained in a number of bleedings of several animals were pooled.

Antisera to derivatives of rabbit 7S γ-globulin were prepared as follows. Chicken antiserum to the 5S fragment of rabbit 7S γ-globulin, obtained by digestion with pepsin, was prepared by intravenous inoculations. Before use the antiserum was diluted to contain 100 μg of antibody nitrogen per ml, precipitable by the homologous antigen. Goat antisera to rabbit 7S γ-globulin and to its

papain 3.5S fragments (1) were obtained through the courtesy of Dr. Melvin Cohn, Stanford University. The antibody nitrogen concentrations with the homologous antigens were 388, 68, and 220 μg per ml for the antisera prepared against piece I, piece III, and the intact 7S material, respectively. The antisera directed against piece I and against the 7S γ-globulin were diluted to 100 μg of antibody nitrogen per ml before use.

Gamma globulin fractions

These were prepared from each of the antisera by the sodium sulfate precipitation technique of Kekwick (11), then dialyzed against 0.1 M sodium acetate. The fractions were heated at 56° C for 20 minutes before use, and sodium azide, final concentration 0.1 per cent, was added as a preservative. With the exception of the anti-HEA, the various antibody γ-globulins were then absorbed with half their volumes of washed, pooled, human erythrocytes until the reagents no longer agglutinated 2 per cent saline suspensions of such pooled red cells. The chicken and goat antirabbit γ-globulin reagents cross-reacted with human 7S γ-globulin; hence, these were also absorbed with pooled human fraction II before use with an amount of human fraction II equivalent to 20 per cent by weight of the amount of rabbit γ-globulin precipitated in slight antibody excess. Complete absorption of antibody activity against human 7S γ-globulin was demonstrated by loss of agglutinating activity against human O Rh_o-positive cells coated with an incomplete anti-Rh_o antiserum. Protein concentrations were estimated by absorbancy at E_{280} mμ by use of an extinction coefficient of 1.5 for a solution containing 1 mg of 7S protein per ml. For purified 5S or 3.5S protein, the absorption coefficients based on Kjeldahl analysis, assuming a nitrogen content of 16 per cent, were found to be 1.48.

Preparation of antibody fragments

To insure that results were reproducible, several 5S and 3.5S preparations were made from each rabbit antibody γ-globulin by treatment with pepsin or papain, respectively; 3.5S fragments were also prepared with pepsin and mercaptoethylamine (MEA). Digestion of rabbit 7S antibody globulin with pepsin, 2 per cent by weight, in 0.1 M acetate buffer at pH 4.5 was performed as described elsewhere (3). After digestion, the reaction mixture was adjusted to pH 8.0, the protein was precipitated with 18 per cent sodium sulfate and dissolved and dialyzed against saline-borate buffer at pH 8.0. The protein so obtained showed a single peak in the ultracentrifuge with $s^\circ_{20,w}$ of 4.8 ± 0.2S. The yields were 45 to 55 per cent of the weight of γ-globulin used. To obtain reduced 3.5S pepsin-treated fragments, 17 to 40 mg of the globulin was treated with pepsin, 2 per cent by weight, in the presence of 0.015 M MEA at pH 4.5 for 8 hours at 37° C. The reaction was stopped with 0.05 M iodoacetamide, and the mixture was adjusted to pH 8.0 and dialyzed against saline-borate buffer at pH 8.0 overnight in the cold. Each of the resulting materials had only one major peak on analytical ultracentrifugation, with $s^\circ_{20,w}$ values of 3.4 ± 0.1S. Some smaller peptides were also present, which are normally removed from 5S preparations by precipitation with sodium sulfate. No faster-moving components were observed (Figure 1).

Hydrolysis of the 7S antibody γ-globulin or the eluted anti-HEA with papain was accomplished as described by Porter (2); 80 mg of the globulin was treated with papain, 1 per cent by weight, in the presence of 0.01 M cysteine, 0.002 M sodium ethylenediaminetetraacetate and saline-phosphate buffer at pH 7.0, $\mu = 0.16$. The reaction was carried out at 37° C for 2 hours. After dialysis the papain was inactivated with 0.005 M iodosobenzoate and the mixture was dialyzed again against cold saline-borate buffer at pH 8.0. In each case there was essentially complete breakdown to 3.5 ± 0.02S as determined by ultracentrifugation.

Other Materials

Chicken ovalbumin, pepsin, and papain were obtained as twice-crystallized preparations from the Worthington Biochemical Corp. The first two were stored as dry

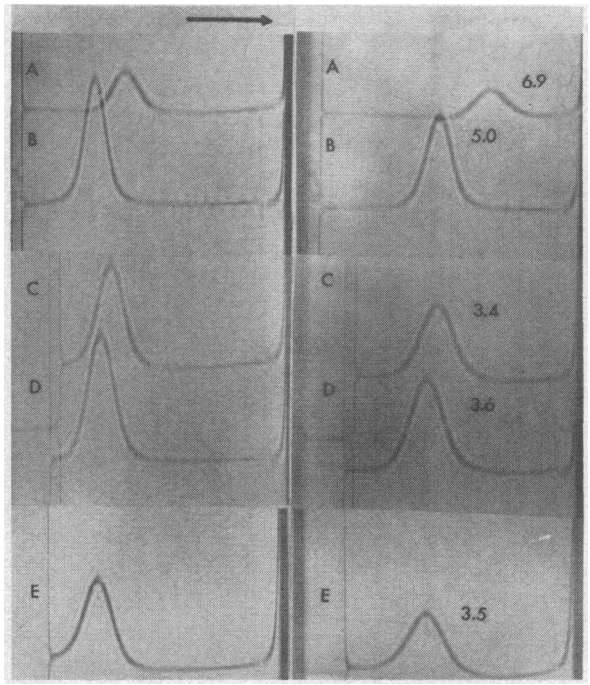

FIG. 1. PHOTOGRAPHS OF SCHLIEREN PATTERNS TAKEN IN A SPINCO MODEL E ULTRACENTRIFUGE. Samples in sodium chloride-borate buffer, $\mu = 0.16$, pH 8.0, except B, which is in 0.1 M sodium acetate at pH 7.0. Sedimentation is from left to right; 48 minutes at 59,780 rpm in column on left, 80 minutes in column on right. A, rabbit antihuman γ-globulin (gamma globulin fraction). B, treated with pepsin and precipitated with sodium sulfate. C, reduced with 0.01 M mercaptoethylamine. D, rabbit antihuman γ-globulin treated with papain and 0.01 M cysteine. E, same globulin treated with pepsin and 0.015 M mercaptoethylamine. The numerals are $S^\circ_{20,w}$ values.

powders at − 20° C; the papain was stored as an aqueous suspension in the refrigerator. L-Cysteine was obtained from the Nutritional Biochemicals Company and 2-mercaptoethylamine hydrochloride from California Corporation for Biochemical Research.

Serologic methods

Anti-HEA reagents. 1. Specificity. The anti-HEA reagents, both those prepared against O cells and those prepared against Oh^{A2} cells, produced direct agglutination of saline suspensions of human erythrocytes; no enhancement of titer [1] was obtained with the use of chicken or goat antirabbit γ-globulin antisera. The anti-HEA reagents showed no apparent preferential specificity against the erythrocytes of 100 random group O donors, nor against a selected panel of 8 group O donors whose erythrocytes contained the antigens D, C, E, c, e, C^w, K, k, M, N, S, s, P, Le^a, Le^b, Vel, Jk^a, Fy^a, or Lu^a, in varying combinations. Titers obtained against cells of varying ABO type, however, suggested anti-H specificity in that $O > A_2 > A_1 > B$. Anti-H specificity could not be demonstrated conclusively by precipitation reactions with H substance,[2] but purified H substance produced partial inhibition of agglutination. These antibodies will subsequently be referred to as anti-HEA.

2. Agglutination. Serial dilutions of the rabbit 7S, 5S, and papain and pepsin-MEA 3.5S preparations of anti-HEA antibodies were tested against 2 per cent suspensions of human O Rh_o-positive erythrocytes by incubation of equal 0.1-ml volumes of antibody dilution and of cell suspensions at 37°, 20°, and 4° C for 1 hour in 10 × 75-mm serologic test tubes. At the end of this period, the tubes were centrifuged for 45 seconds at 1,000 rpm in a Clay-Adams serologic centrifuge and read for agglutination with the aid of a hand lens. Results were recorded as O to +++++. Tubes giving negative readings were tested with goat and chicken antirabbit γ-globulin antisera.

3. Sensitization. One-ml samples of serial dilutions of 3.5S preparations of rabbit anti-HEA γ-globulin were incubated at 4°, 20°, and 37° C for times ranging from 1 to 18 hours, with equal volumes of 2 per cent buffered saline suspensions of human O cells. After being washed three times with 20-fold volumes of 0.85 per cent sodium chloride, the cells were reconstituted to their original volume in buffered saline. Samples of 0.1 ml were removed and placed in 10 × 75-mm test tubes, and serial dilutions of chicken and goat antisera prepared against the 7S, 5S, and 3.5S components of rabbit γ-globulin were added. Readings were obtained as above. In this way "block titration" of the various reagents was accomplished. Tubes were read for agglutination at 1 minute and again at 1 hour.

4. Other test methods. The rabbit anti-HEA was also tested against a 2 per cent concentration of red cells suspended in a high-protein medium, 30 per cent albumin, and against red cells modified by the proteolytic enzymes trypsin, papain, and ficin, according to standard serologic methods (12, 13). Both O Rh_o and O rr cells were used. A high-titer "incomplete" anti-Rh_o antiserum, obtained from an Rh-negative woman sensitized during pregnancy, served as a control.

Antihuman S γ-globulin

Two per cent suspensions of human O Rh_o red cells were sensitized at 37° C for 1 hour with equal volumes of varying dilutions of an incomplete anti-Rh_o antibody, with a titer of 2,000 by indirect antiglobulin (Coombs) test. Varying amounts of anti-Rh_o activity "units" were present in the sensitizing solutions, a "unit" being arbi-

[1] Doubling dilutions, here and in all other titrations, were made with separate pipettes to prevent carry-over.

[2] This anti-H did not precipitate. According to Dr. W. M. Watkins (personal communication), most anti-H sera, regardless of titer, are nonprecipitating.

TABLE I

Agglutination end-points of 5S and 3.5S derivatives of 7S anti-HEA *

Experiment	Preparation			
	7S	5S	3.5S (papain)	3.5S (pepsin-MEA †)
1. Eluate of 7S anti-HEA no. 1				
Saline agglutination	8		2	
Coombs antiglobulin reaction	‡		9	
2. Whole 7S γ of anti-HEA no. 1				
Saline agglutination	7	9		2
Coombs antiglobulin reaction	‡	‡		9
2. Eluate of 7S anti-HEA no. 2				
Saline agglutination	6		0	
Coombs antiglobulin reaction	‡		6	

* Values indicate the number of two-fold dilutions of a 1 mg per ml solution. Anti-HEA = rabbit antisera to human erythrocyte antigens.

† MEA = mercaptoethylamine.

‡ No enhancement of saline titer by antiglobulin.

TABLE II

Agglutination of human type O erythrocytes sensitized by univalent fragments of rabbit antibody

Sensitizing dose, in mg/ml, of papain 3.5S fragments of 7S rabbit γ-globulin	Antibody, in μg/ml												
	Chicken anti-5S			Goat anti-I			Goat anti-III			Goat anti-7S rabbit			
	5.0	1.0	0.2	5.0	1.0	0.2	3.0	1.0	0.2	5.0	1.0	0.2	Saline
1. Anti-HEA *													
1.0	++	++	+	+++	+++	++	++	++	+	++	++	±	0
0.025	+++	+	±	+	+++	±	+	+	±	+	+	0	0
0.005	±	0	0	0	+	±	+	+	0	±	0	0	0
2. Rabbit anti-ovalbumin													
1.0†	0	0	0	0	0	0	0	0	0	0	0	0	0
3. Saline control	0	0	0	0	0	0	0	0	0	0	0	0	0

* As in Table I.
† Similar negative reactions obtained with concentrations ranging from 0.005 to 0.10 mg per ml.

TABLE III

Inhibition by γ-globulin of agglutinating activity of chicken antiserum to rabbit 5S fragments *

Fragments	γ-Globulin added, in mg/ml						
	2.0	1.0	0.25	0.06	0.015	0.0003	Saline
Human II	+++	+++	+++	+++	+++	+++	+++
Rabbit II	0	0	+	++	+++	+++	+++

* Test system: human O Rh_0 cells sensitized by rabbit 3.5S anti-HEA (abbreviation as in Table I). Chicken anti-5S rabbit fragments, 5 μg antibody nitrogen per ml, used for agglutination.

TABLE IV

Reaction of 7S, 5S, and 3.5S preparations of anti-HEA with enzyme-modified cells, and with cells suspended in 30 per cent bovine albumin *

Preparation		Protein concentration = 1 mg per ml/2^n											
		no. = 0	1	2	3	4	5	6	7	8	9	10	Saline
I. Anti-HEA													
Papain-treated cells	7S	++++	++++	++++	++++	++++	+++	++	+	±	0		0
	5S	++++	++++	++++	++++	++++	+++	+++	++	+	±		0
	3.5S	+++	++	++	0	0	0	0	0	0	0		0
Trypsin-treated cells	7S	+++++	+++++	+++++	++++	+++	+++	+++	+	trace	0	0	0
	5S	+++++	+++++	+++++	++++	+++	+++	+++	++	++	+	0	0
	3.5S	++	++	++	±	0	0	0	0	0	0	0	0
Ficin-treated cells	7S	+++++	+++++	+++++	++++	++++	+++	++	±	0	0		0
	5S	+++++	+++++	+++++	++++	+++	++	++	++	±	0		0
	3.5S	++	++	++	±	0	0	0	0	0	0		0
Untreated cells (saline control)	7S	++++	++++	++++	++++	+++	++	+	±	0	0		0
	5S	+++++	++++	++++	++++	+++	+++	++	+	±	0		0
	3.5S	+	±	0	0	0	0	0	0	0	0		0
30% Albumin	7S	+++++	++++	++++	++++	++++	++	0	0	0			0
	5S	+++++	+++++	+++++	++++	++++	++	+	0	0			0
	3.5S	+	±	0	0	0	0	0	0	0			0
II. Control anti-Rh serum													
Coombs		+++	+++	+++	+++	++	++	++	++	±	0	0	0
Trypsin		++++	++++	++++	+++	+++	++	±	0	0	0	0	0
Papain		++++	++++	++++	+++++	+++	+++	++	±	trace	0	0	0
Ficin		++++	++++	++++	++++	+++	+++	++	+	±	0	0	0
Saline		0	0	0	0	0	0	0	0	0	0	0	0
Albumin		+++	++++	+++	++	+	±	0	0	0	0	0	0

* The 5S and 3.5S fragments were prepared with pepsin, or with pepsin and 2-mercaptoethylamine, respectively. Abbreviation as in Table I.

trarily defined as 2,000 multiplied by the dilution used; e.g., a 1 : 500 dilution contains 4 units of activity. After incubation, the sensitized cells were washed four times with 20-fold volumes of buffered saline and distributed in 0.1-ml amounts into 10 × 75-mm serologic test tubes. Block titrations were performed by addition of 0.1 ml of serial dilutions of 7S, 5S, and 3.5S preparations of the γ-globulin of the rabbit antiserum against human γ-globulin. Similarly treated rabbit antiovalbumin was added to a control series of red cells. Agglutination was read as described above, both at 1 minute and again after incubation at 37° C for 1 hour. If no agglutination occurred, the cells were again washed four times. After the addition of varying dilutions of chicken or goat antirabbit γ-globulin, the mixtures were observed for agglutination.

In some experiments, reversal of agglutination was studied by addition of equal volumes of 0.1 M MEA in pH 7.2 phosphate buffer to the agglutinated mixture of Rh-sensitized cells and the rabbit antihuman globulin preparations.

RESULTS

Several pepsin 5S and papain and pepsin-MEA 3.5S preparations were made of each of the 7S antibody γ-globulins tested. These preparations invariably exhibited only one peak on ultracentrifugal analysis; no appreciable concentrations of faster-moving material were detectable. Some small peptides, however, were present in the fragments made with pepsin and MEA. Further immunoelectrophoretic experiments failed to demonstrate unsplit 7S material. These methods, however, are relatively insensitive, and by these criteria perhaps as much as 2 to 3 per cent, and probably at least 1 to 2 per cent, of the total γ-globulin remained undigested (see below).

Anti-HEA. As is evident from Table I, pepsin 5S derivatives of the 7S rabbit anti-HEA retained full activity, as measured by agglutination titers against human O cells. Similar results were observed with human A or B cells, although titers generally were somewhat lower. The 3.5S material derived from the 7S anti-HEA, however, was almost completely devoid of agglutinating activity; nonetheless, combination of the 3.5S material with antigenic receptor sites on the red cells did occur, as shown by agglutination upon subsequent addition of goat or chicken antisera prepared against rabbit γ-globulin or its proteolytic fragments (Table II). Addition of such goat or chicken antirabbit globulin sera to red cells previously exposed to 3.5S preparations of rabbit antiovalbumin antibody failed to produce agglutination (Table II). Further, agglutination of cells coated with 3.5S anti-HEA by the chicken antiserum was prevented by prior incubation of the antisera with rabbit γ-globulin; human γ-globulin failed to exert

TABLE V

*Comparative inhibitory effects of papain and of pepsin-mercaptoethylamine 3.5S derivatives of 7S γ-globulin of rabbit antihuman γ-globulin**

Inhibitory material †	Optical density of specific precipitate ‡
None (0.33 ml saline)	0.104
Pepsin-MEA fragments	0.025
Papain fragments	0.023

* Inhibition of precipitation of human γ-globulin by 7S rabbit antihuman γ-globulin.

† In each test, 2 mg of antibody globulin (rabbit antihuman γ-globulin) was used together with an optimal amount of antigen (23 μg human γ-globulin). For precipitation-inhibition tests, 4 mg of fragments derived from the antibody globulin was used. In each case, the untreated antibody was added last.

‡ At 280 mμ, dissolved in 1.0 ml 0.02 N NaOH.

TABLE VI

Agglutination of sensitized Rh_0 cells by 7S or 5S preparations of rabbit antihuman γ-globulin

Units of anti-Rh_0 antibody used for sensitization	Concentration of rabbit antihuman γ-globulin (γ-globulin fraction) in mg/ml												
	5.0		1.0		0.25		0.05		0.01		0.002		5†
	7S	5S	7S	5S	7S	5S	7S	5S	7S	5S	7S	5S	Papain 3.5S
160	+++	+++	+++	+++	+++	+++	+	++	±	0	0	0	0
40	++++	++++	++++	++++	++++	++++	+++	++++	+	++	0	0	0
10	+++	+++	+++	+++	+++	++	++	++	±	±	0	0	0
4	+++	+++	++	++	++	±	+	±	0	0	0	0	0
1	+	±	±	0	0	0	0	0	0	0	0	0	0
Saline	0	0	0	0	0	0	0	0					

* Unit, by arbitrary definition, =2,000 × dilution used.

† Identical results with papain 3.5S material in concentration ranging from 0.002 to 1.0 mg per ml.

TABLE VII

Summary of three-dimensional block titrations

Antirabbit γ-globulin		3.5S Rabbit antibody added														
		0.2 mg antihuman γ-globulin				0.02 mg antihuman γ-globulin				0.2 mg antiovalbumin				Saline control		
Species and antigen	Antibody nitrogen	160*	40*	4*	Saline	160*	40*	4*	Saline	160*	40*	4*	Saline	160*	40*	4*
	μg/ml															
Chicken anti-5S	20.0	++++	++++	+++	0	+++	+++	++	0	0	0	0	0	0	0	0
	1.0	++	+	±	0	++	0	0	0	0	0	0	0	0	0	0
	0.20	++	±	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.05	++	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Goat anti-I	20.0	++++	++++	++++	±	+++	+++	+++	0	0	0	0	0	0	0	0
	1.0	+++	+++	+++	0	+++	+++	+++	0	0	0	0	0	0	0	0
	0.20	+++	++	0	0	++	++	++	0	0	0	0	0	0	0	0
	0.05	++	+	0	0	++	+	±	0	0	0	0	0	0	0	0
	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Goat anti-7S	20.0	+++	+++	++	+	+++	+++	+++	0	0	0	0	0	0	0	0
	4.0	+++	+++	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.4	+	++	0	0	0	0	0	0	0	0	0	0	0	0	0
	.08	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Goat anti-III	4.0	+++	+++	++	0	+++	+++	+++	0	0	0	0	0	0	0	0
	.20	+++	++	0	0	0	0	0	0	0	0	0	0	0	0	0
	.08	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Saline		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Units of anti-Rh_0 antibody used for sensitization, as in Table VI.

TABLE VIII

Sensitizing capacities of papain and pepsin-mercaptoethylamine 3.5S fragments of rabbit antihuman globulin

Preparation (mg/ml antibody nitrogen)		Papain fragments (mg/ml protein nitrogen)					Pepsin-MEA fragments (mg/ml protein nitrogen)					3.5S anti-ovalbumin[7] (mg/ml protein nitrogen)	
		0.008	0.002	0.001	0.0005	Saline	0.008	0.002	0.001	0.0005	Saline	0.1	0.001
Goat anti-7S	1.0	+++	+	±	0	0	+++	+	±	0	0	0	0
	0.20	++	0	0	0	0	++	0	0	0	0	0	0
Goat anti-I	0.20	+++	++	+	0	0	+++	++	±	0	0	0	0
	0.05	++	+	±	0	0	+++	+	±	0	0	0	0
Chicken anti-5S	0.20	++	±	0	0	0	++	+	0	0	0	0	0
Saline		0	0	0	0	0	0	0	0	0	0	0	0

such a neutralizing effect (Table III). Attempts were made to inhibit the agglutinating activity of intact 7S anti-HEA by prior incubation of erythrocytes with 3.5S anti-HEA at temperatures of 4°, 20°, and 37° C. The molar ratios of 3.5S to 7S material varied from 2:1 to 200:1, and incubation periods ranged from 1 to 24 hours. In all instances the univalent fragments failed to inhibit agglutination of the red cells by untreated rabbit antibody.

The 7S, 5S, and 3.5S preparations of rabbit anti-HEA produced against type O human cells gave results similar to the comparable materials derived from antisera produced against Oh^{A_2} "Bombay" cells.

Table IV indicates that the 3.5S preparation of the anti-HEA no. 1 produced only very weak agglutination of human O Rh cells suspended in 30 per cent albumin or of such cells previously treated with 0.1 per cent papain, ficin, or trypsin. Similar results were obtained with the papain 3.5S derivatives of anti-HEA no. 2. In contrast, an "incomplete" 7S anti-Rh antibody that gave no direct agglutination of O Rh_0 cells in saline gave full agglutination if the red cells were enzyme-treated or if the suspending medium contained 30 per cent albumin.

It is obvious from Table IV that 5 to 6 tube differences in titers were observed with the 3.5S preparation, compared with the 5S and 7S preparations of anti-HEA by these methods of detecting "incomplete" antibodies. With the particular preparations used for this experiment, this difference in titer, from 32- to 64-fold, corresponded almost exactly to the difference in direct agglutination titers as recorded in Table I. It is therefore highly probable that the antibody activity detected by these methods in the 3.5S preparation that was used represents undegraded 7S anti-HEA.

Antihuman γ-globulin. By precipitin criteria, approximately 4 per cent of the rabbit 7S γ-globulin and 3 per cent of the rabbit 5S γ-globulin were antihuman γ-globulin. With both the 7S and 5S materials, precipitation went through an optimum with 23 μg of antigen. These figures are corrected for estimated antigen in the precipitate. Tests for inhibition of precipitation were carried out with 4 mg of 3.5S material, at optimal antigen concentration (23 μg) against 2 mg of untreated, unabsorbed rabbit 7S γ-globulin containing 4 per cent antihuman γ-globulin. Inhibition was nearly complete with the 3.5S derivatives produced both by papain and by pepsin-MEA, and the results with the two systems were almost identical (Table V). Similar 3.5S fragments of antiovalbumin failed to block antihuman γ-globulin activity.

Table VI demonstrates that the pepsin 5S derivative of 7S rabbit antihuman γ-globulin antibody retains full activity as a serologic reagent, as is shown by the agglutination in indirect Coombs tests of human Rh_0 red cells sensitized with an "incomplete" anti-Rh antibody. Similar results were obtained with use of the 5S derivatives in direct antiglobulin Coombs tests of the washed red cells of two patients with Coombs-positive, acquired antibody hemolytic anemia of the so-called "gamma" antibody (14) variety. [Negative results were obtained in direct Coombs tests of the red cells of a patient with acquired antibody hemolytic anemia of the "non-gamma" (14)

TABLE IX

Comparative activities of 7S, 5S, and 3.5S preparations of rabbit antibody to human γ-globulin with human O Rh_0 cells *

Preparation	Lowest concentration of protein producing agglutination (mg/ml protein nitrogen)		
	Direct agglutination	Subsequent reaction with	
		Goat anti-I	Chicken anti-5S
7S	0.00150	†	†
5S	0.00075	†	†
Papain 3.5S	None	0.00075	0.00150
Pepsin-mercaptoethylamine 3.5S	None	0.00075	0.00150

* Cells sensitized by 160 units of "incomplete" anti-Rh_0; units as in Table VI.
† No enhancement.

variety with both the 7S and 5S rabbit antihuman γ-globulin preparations.]

The 3.5S derivatives of the 7S γ-globulin of rabbit antibody to human γ-globulin, produced either by papain hydrolysis or by MEA treatment of the pepsin 5S digest, failed to agglutinate the sensitized Rh_0 cells; however, these 3.5S fragments combined with their homologous antigen, human 7S γ-globulin, as shown by subsequent agglutination when goat or chicken antirabbit γ-globulin antisera were added. The data shown in Table VII are representative of the results obtained with the papain 3.5S material in a three-dimensional block titration, i.e., with varying concentrations of anti-Rh antibody, of 3.5S material, and of antirabbit γ-globulin antibody. Note that 3.5S antiovalbumin failed to combine with the sensitized cells. Only two concentrations of the 3.5S material are listed in this table, but Table VIII lists in greater detail the results obtained with other concentrations of the papain 3.5S fragments. Note that sensitizing activity was retained at concentrations as low as 0.03 μg per ml of antibody nitrogen.[3] Table VIII also demonstrates that the pepsin-MEA 3.5S fragments were serologically indistinguishable from the papain 3.5S fragments.

No augmentation of titer was demonstrable by use of the chicken or goat antisera to rabbit γ-globulin or to its 5S or 3.5S derivatives with subagglutinating doses of the 7S or 5S rabbit antihuman globulin (Table IX). The 3.5S material possessed the full activity of the parent 7S antibody insofar as can be ascertained within the limits of error of the doubling-dilution serologic technique. Antibody activity was detectable at concentrations of 0.06 μg per ml of antibody nitrogen with the intact material, as measured by use of the chicken antiserum (Table IX). The calculations above were made by using the values 4 per cent and 3 per cent, respectively, for the antibody content of the 7S and 3.5S preparations. Activities of the papain and pepsin-MEA 3.5S preparations were comparable. Further, direct addition of MEA to agglutinates produced by 5S antihuman γ-globulin and Rh_0 cells sensitized

[3] Or, 0.001 mg per ml of protein nitrogen, of which 3 per cent is antibody.

TABLE X

*Reversal by mercaptoethylamine of agglutination of Rh-sensitized red cells by 5S preparation of rabbit antihuman γ-globulin**

Preparation	γ-Globulin added, mg/ml 0.2	0.02	0.002	0.0005
7S + Buffer	+++	+++	++	0
0.1 M MEA	+++	+++	+++	0
5S + Buffer	+++	+++	++	0
0.1 M MEA	0	0	0	0

* Cells sensitized by 40 units of blocking anti-Rh_0 antibody.

TABLE XI

Reversal of agglutination by reduction with mercaptoethylamine, yielding dispersed, sensitized cells

Preparation	Anti-Rh_0 blocking antibody: 40 Units*, mg/ml 5S rabbit antihuman γ-globulin added: 0.2	0.02	0.002	Saline	4 Units: 0.2	0.02	0.002	Saline
1. Reversal of agglutination †								
a. Buffer	+++	+++	+	0	+	±	trace	0
b. MEA, 0.01 M	0	0	0	0	0	0	0	0
c. MEA, 0.10 M	0	0	0	0	0	0	0	0
2. Antiglobulin reaction on samples above‡								
a. Buffer	§	§	§	§	§	§	§	0
b. MEA, 0.01 M	+++	++	++	0	+++	++	+	0
c. MEA, 0.10 M	++++	+++	++	0	+++	++	+	0

* Unit = 2,000 × the dilution used.

† Equal volumes of 5S material of 2 per cent cell suspension of sensitized cells and of MEA or buffer added to each tube. Readings after 2 hours at 20° C.

‡ Cells for Part 1 washed and antiglobulin reagent added. Results listed obtained with goat anti-I (1 μg per ml antibody nitrogen). Similar results obtained with goat antirabbit 7S and chicken antirabbit 5S in comparable concentrations and with goat anti-III in somewhat higher concentration.

§ No enhancement.

by blocking anti-Rh resulted in reversal of agglutination (Table X), presumably by breaking the disulfide bond linking the univalent fragments of the 5S antihuman γ-globulin. Addition of appropriate chicken or goat antirabbit antibodies to the dispersed cells revealed the presence of univalent fragments attached to the Rh_0-positive sensitized cells (Table XI). This serologic activity of cells "sensitized" in this reverse fashion was not significantly different from the serologic activity of cells sensitized by 3.5S fragments prepared by pepsin-MEA or by papain digestion. Attempts were made to inhibit the agglutinating activity of the intact 7S antihuman γ-globulin by prior incubation of sensitized Rh-positive cells with 3.5S preparations of antihuman γ-globulin. Varying ratios of 7S : 3.5S materials and varying conditions of incubation were used, as in similar experiments with the anti-HEA (see above). In all instances the univalent fragments failed to inhibit the agglutination of the sensitized cells by intact antihuman γ-globulin.

DISCUSSION

Porter has shown that hydrolysis of rabbit 7S γ-globulin antibody with papain yields three fragments, two of which possess combining sites; the third fragment, III, is inactive but crystallizable (2). It has been demonstrated that Porter's chromatographic fractions I and II do not correspond to two fragments of a given molecule (15, 16). Nevertheless, his other evidence strongly suggests that the molecule contains one inactive and two active subunits. The capacity of the two active fragments to block precipitation in the homologous system indicates that they are univalent, and as indicated in the introduction, this has been confirmed by measurements of the maximal hapten-binding capacity of the active fragments.

Nisonoff and co-workers (3, 17) have demonstrated that successive treatments of 7S rabbit antibody globulin with pepsin at pH 4 to 5 and one of several disulfide-splitting reagents at pH 8, where pepsin is inactive, similarly yield univalent fragments. These split products exhibit a sedimentation pattern and coefficient, 3.5S, identical with those of a papain digest, and are separable into two active and one inactive fractions on carboxymethyl cellulose under conditions identical with those used by Porter for a papain digest (18). Fraction III is partially degraded on peptic digestion and may be almost completely hydrolyzed on prolonged treatment. The proteolysis with pepsin alone results in reduction of the sedimentation coefficient from 6.5S to 5S through loss of fragment III, but the capacity to form specific precipitates is retained, indicating that the product is still bivalent (3). Univalent 3.5S fragments can be separated from the bivalent rabbit antibody molecule by subsequent reduction of a single labile disulfide bond with cysteine, 2-mercaptoethylamine, or other sulfhydryl reagents (19).

The data presented here demonstrate that 3.5S univalent fragments of agglutinating antibody retain the capacity to combine with molecules of homologous antigen attached to the surface of erythrocytes, even though the capacity to produce agglutination is lost.

The combination results in sensitization of the red cells. Similar results were obtained whether the antigen was one that occurred naturally on the red cell or took the form of an "incomplete" anti-Rh antibody attached to its surface. In the latter instance, the rabbit antibody used, antihuman γ-globulin, was capable of combining specifically with the "incomplete" anti-Rh antibody. That combination of the univalent fragments with antigens on the red cells had occurred was shown by the addition of another, complete antibody, prepared in chickens, with specificity for the univalent fragments. When this was done, agglutination occurred. Similar results were obtained with goat antisera to rabbit γ-globulin fragments I and III or to rabbit 7S γ-globulin. That goat anti-III was effective suggests that the fraction III used to immunize the goat was contaminated by univalent fragments or by undegraded γ-globulin. An alternative explanation is that certain antigenic determinants characteristic of rabbit gamma globulin may be shared by fragments I and III so that an antiserum produced in another species against rabbit fragment I cross-reacts to a greater or lesser degree with fragment III, and vice versa. In any event, fragment anti-III possessed distinct antibody activity against fragment I; the amount of antibody nitrogen precipitated from a goat anti-rabbit fragment-III antiserum by a purified preparation of fragment I (isolated by column chromatography and giving but one band on immuno-

electrophoresis) was approximately 6 per cent of the antibody nitrogen precipitated by the purified homologous antigen (20). This ratio is not very dissimilar from the relative end-points obtained with fragments anti-I and anti-III in antiglobulin experiments in which moderate doses of the 3.5S rabbit material—e.g., 0.02 mg per ml—were utilized for sensitization (Table VII).

When human anti-Rh was used to sensitize the erythrocytes, the sequence of molecules presumably taking part was: Rh antigen, human anti-Rh (incomplete), univalent rabbit antihuman γ-globulin, and chicken antirabbit. The chicken antibody was prepared against a peptic digest of rabbit antibody, which consists essentially of a linkage of the two univalent fragments.

The identical serologic behavior of the 3.5S fragments derived by papain hydrolysis or by pepsin-MEA hydrolysis provides further evidence for the identity of the univalent fragments derived by these two methods. The results obtained with the pepsin-treated 5S rabbit antibodies are consistent with the hypothesis that these molecular fragments are bivalent and are split into univalent subunits on reduction of a disulfide bond. Cells were found to be directly agglutinable by the appropriate 5S antibody, but agglutinates were dissolved upon addition of a reducing agent; the dispersed cells could then be shown to be "sensitized" with univalent antibody. The same treatment with reducing agent had no observable effect on agglutinates prepared with untreated 7S rabbit antibodies. The binding of univalent antibody to antigenic sites was demonstrable after addition of MEA to the 5S antibody agglutination system and the resultant reversal of agglutination. The serologic behavior of this test system did not differ significantly from that of 3.5S fragments produced by papain or by combined pepsin and MEA treatment.

The retention of some serologic activity of bivalent 5S material derived from 7S rabbit antibody to sheep erythrocytes has been demonstrated by Amiraian and Leikhim (21) and by Gyenes and Sehon (22). It is interesting, however, that 5S—both antihuman γ-globulin and anti-HEA—materials possessed in full all the serologic properties of the parent antibodies, as would be expected if each 5S preparation were composed solely of the two univalent fragments. Further, the reaction times of these 5S materials were identical to those of the parent 7S antibodies at 37°, 20°, and 4° C, although the 5S antisheep erythrocyte preparation reportedly produced agglutination only after 16 hours at 4° C (21).

It has recently been found (15) that the univalent fragments from a given molecule are present either in chromatographic fraction I, or in fraction II, but not in both. Hence, "sensitizing" studies with the separated fragments were not attempted, since starch-block separation of the rabbit antihuman γ-globulin gave materials with comparable specificity and activity in areas obtained from the fast, middle, and slow γ-globulin region (23). Further, Goodman (20) and Porter (2) have demonstrated that fractions I and II derived from papain-digested rabbit γ-globulin are antigenically indistinguishable when tested by goat [4] or rat antisera prepared against either of the fractions.

In the present study, univalent fragments did not inhibit agglutination by untreated rabbit antibody. In contrast, univalent fragments of rabbit antibody are powerful inhibitors of the precipitin reaction. A probable explanation of the difference is the large number of antigenic sites present on erythrocytes. Only a small percentage of these sites are ordinarily occupied during agglutination, and a very large concentration of univalent fragments would be required to block all of them. The presence of a surplus of antigenic sites may also account for Porter's observation (2) that univalent fragments failed to inhibit the precipitation of pneumococcal polysaccharide with homologous untreated antibody of univalent fragments. Some inhibition of hemagglutination, however, has been observed by Amiraian and Leikhim with 3.5S digests of rabbit antisheep erythrocyte antibodies (21).

In the present experiments, 3.5S preparations occasionally produced direct agglutination when added in extremely high concentration to the red cell suspensions. Such serologic activity never amounted to more than 1 to 3 per cent of the parent 7S material, as measured by doubling-dilution agglutination methods. Although the 3.5S materials exhibited single ultracentrifugal

[4] The same goat antisera, obtained from Dr. Melvin Cohn, were used in our study as in Goodman's experiments (20).

peaks and single immunoelectrophoretic bands, it is highly probable that the residual agglutinating activity is attributable to contamination by a small amount of undegraded rabbit antibody. Force is lent this hypothesis by the results obtained through serendipity in one experiment involving only partial reduction of 5S fragments to 3.5S.[5] Here approximately 30 per cent of the material, as calculated from the ultracentrifugal plates, remained in the 5S form; serologic experiments demonstrated approximately 25 per cent of agglutinating activity in the mixed 5S and 3.5S preparation as compared with the 5S. The presence of small amounts of undegraded material despite ultracentrifugal criteria of purity requires caution in evaluation of the validity of experiments purportedly demonstrating the "enhancing" effect of Porter fractions I or II in complement fixation or other test systems (21).

It is conceivable that the undegraded 7S material, rather than "univalent" 3.5S fragments, was responsible for the "incomplete" antibody activity. This possibility, however, appears remote for several reasons. First, it is unlikely that undegraded material, which accounts for no more than 1 to 3 per cent of the original 7S material, would produce titers of "incomplete" antibody activity equivalent to those of the "complete" antibody activity of the original 7S. If this were the case, a 30- to 100-fold increase in the titer of the original 7S material when tested by the antiglobulin method would also be anticipated. No such increase, however, was demonstrable. Further, the titers of "incomplete" antibodies derived by treatment of the 7S material with papain or with pepsin-MEA were almost exactly equal on a molar basis to the titers of "complete" antibody in the undegraded parent material. If the "incomplete" activity were due to undegraded 7S, it would be necessary to assume that 7S material exists in two forms: *a*) a major population with "complete" antibody activity completely susceptible to enzymatic degradation, and *b*) an additional minor population with "incomplete" antibody activity, completely resistant to enzymatic digestion, but possessing far greater activity per unit on a molar basis than the major population. This possibility, although unlikely, cannot be entirely excluded.

Of special interest is the failure of 3.5S univalent fragments of rabbit antibody to produce direct agglutination of red cells suspended in high-protein media, 30 per cent albumin, or with red cells penetrated by proteolytic enzymes. The blocking antibodies demonstrable by indirect antiglobulin test in various human antibody systems—e.g., anti-Rh—almost invariably produce positive reactions in such test systems. These blocking antibodies have been termed "univalent" in standard texts on blood groups (13) and hematology (24) since Wiener's introduction of this term in 1945 (25). The results obtained in this study suggest that the truly univalent 3.5S fragments of 7S rabbit antibody display serologic characteristics differing from those of the "incomplete" anti-Rh antibodies. Indeed, it is difficult to see why pretreatment with proteolytic enzymes should cause cells to be agglutinated by a univalent antibody preparation. It seems more reasonable to assume that incomplete antibodies may be present on slightly indented areas, or may combine with insufficient energy to overcome mutual repulsive forces of the negatively charged red cell surfaces at short distances. A second antibody may increase the distance of separation.

Support of this hypothesis is provided by preliminary experiments showing that the 3.5S derivatives of "incomplete" human anti-Rh antibodies produced by papain and cysteine fail to agglutinate Rh-positive cells treated with ficin, papain, or trypsin, in contrast to the behavior of the untreated "incomplete" antibody; similarly the antibody fragments produced no significant agglutination when the cells were suspended in 30 per cent albumin. A Coombs-type reaction was obtained when rabbit antihuman globulin was used with the papain-treated Rh antibodies.

SUMMARY

Univalent 3.5S fragments of rabbit 7S antihuman red cell agglutinating antibodies prepared with papain or pepsin and mercaptoethylamine reacted specifically with their homologous antigens, but failed to produce agglutination of the erythrocytes. Prior treatment of the cells with proteolytic enzymes or suspension in 30 per cent albumin still failed to produce agglutination.

[5] In this experiment, 5S fragments were made by pepsin without the concomitant use of a reducing agent. Partial reduction to 3.5S was then accomplished by subsequent treatment with mercaptoethanol for 2 hours.

Combination of the fragments with the homologous antigen was demonstrable by agglutination upon subsequent addition of chicken or goat antibody specific for the univalent fragments of rabbit γ-globulin. Similar results were obtained with univalent fragments of rabbit antibody to human γ-globulin by use of human cells sensitized with human γ-globulin. The results are consistent with current theories as to the mechanism of action of bivalent and univalent antibodies, and provide another example of the properties of univalent subunits of antibody.

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