SEROLOGIC DEMONSTRATION OF DUAL SPECIFICITY OF RABBIT BIVALENT HYBRID ANTIBODY*

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PLATE 5

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Treatment of rabbit 7S antibody with pepsin results in the removal of an inactive fragment, leaving a bivalent residue with an average molecular weight of 106,000 and a sedimentation coefficient of approximately 5S (1, 2). Subsequent reduction of one labile disulfide bond splits this residual molecule into two univalent, non-precipitating fragments which migrate as a single 3.5S peak (3) in the ultracentrifuge. These fragments closely resemble the univalent fractions of a papain digest (4) in a number of their properties.

The univalent 3.5S fragments obtained by peptic digestion and reduction of a specifically purified rabbit antibody can be recombined by oxidation, in yields up to 75 per cent, to give 5S antibody which again possesses precipitating activity (5). When a mixture of purified rabbit antibodies of two different specificities is reduced and reoxidized, the recombined product contains a considerable amount of bivalent 5S "hybrid antibody" (6, 7).[1] The recombination of fragments of two different specificities appears to be essentially random, as shown by quantitative analysis of the hapten-binding capacity of hybrid antiovalbumin-antibenzoate antibody before and after treatment with an adsorbent specific for the antiovalbumin (7).

Previous reports from these and other laboratories demonstrated that the 5S derivatives of several 7S rabbit antibodies retain most of the original agglutinating activity on a molar basis (8, 9); the 3.5S derivatives lack agglutinating properties but combine with the homologous antigen, as shown by inhibition of hemagglutination (9, 10) or by a modified antiglobulin reaction (8).

The present communication extends these hemagglutination studies to 5S antibodies obtained by reoxidation of the 3.5S univalent fragments. The re-

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[1] The phrase "hybrid antibody" will be used to designate 5S antibody molecules of dual specificity; "hybrid preparation" denotes the mixture of 5S antibodies formed in the procedures described below.

sults indicate that a reconstituted 5S antibody can induce passive hemagglutination, and that reconstituted 5S hybrid antibody preparations produce passive mixed agglutination of two visually distinguishable red cell types, each coated with a different antigen. With an artificial mixture of 5S antibodies (not hybridized), mixed agglutination does not occur; the two cell types are agglutinated in separate homogeneous clumps. In the presence of only one antigen, 5S "hybrid preparations" produce significant agglutination only at concentrations much greater than those required for agglutination of a mixture of coated cells. This weak agglutinating activity appears related to the monospecific bivalent 5S antibody, presumably formed in the reoxidation stage in making the hybrid preparation. However, attachment of hybrid antibody to homologous antigens does occur at lower concentrations, as shown by a modified antiglobulin (Coombs') reaction and by stepwise agglutination procedures.

Materials and Methods

Materials.—Bovine gamma globulin (BGG) and twice crystallized chicken ovalbumin (EA) were obtained from the Pentex Inc., Kankakee, Illinois; 2-mercaptoethylamine hydrochloride (MEA) was purchased from the California Corporation for Biochemical Research, Los Angeles.

Antisera.—Antisera to EA or to BGG were prepared by intravenous inoculation of 2 to 3 kg rabbits with 1 ml of a 1 per cent saline solution of the protein antigen three times weekly for 6 weeks, followed by subsequent bleedings and inoculations at weekly intervals. Antisera of high titer obtained from a number of bleedings of several rabbits were pooled. The antibodies were specifically purified by partial dissociation of a specific precipitate at pH 2.5 and fractionation at this pH with sodium sulfate (11). The 5S preparations were obtained from the purified antibodies by peptic digestion and precipitation with sodium sulfate (3). Amounts of antibody in precipitates were estimated as previously described (5). The percentages of protein in the antibody preparations precipitable by an optimal concentration of antigen were as follows: anti-EA, 88 per cent; "5S" anti-EA, 85 per cent; anti-BGG, 76 per cent; "5S" anti-BGG, 61 per cent. The $s_{20,w}$ values of the purified anti-EA and anti-BGG were 6.5 and 6.9S, respectively; the value for each of the pepsin-treated antibodies was 4.6S. A slight amount of faster moving material ($\sim$ 10S) was present in the 6.5 and 6.9S preparations.

Chicken antiserum to the 5S derivative of rabbit 7S gamma globulin (chicken anti-rabbit 5S) was prepared by a similar immunization schedule. Before use, the chicken antiserum was absorbed with human erythrocytes. Aliquots were also absorbed with BGG for use in antiglobulin reactions with red cells coated with BGG; this was done to ensure that the chicken antiserum used in such reactions was actually reacting with the rabbit antibody and not by cross-reaction with the BGG.

Measurements of Protein Concentrations.—Protein concentrations were estimated by measurement of the absorbancy of solutions at 280 mμ. The extinction coefficient used for γ-globulin is 1.50 optical density units per mg per ml; for 3.5 or 5S derivatives the corresponding value is 1.48.

Ultracentrifugal Analyses.—Sedimentation velocities were measured in a 4°C, 12 mm cell in the Spinco model E ultracentrifuge at 59,780 RPM. The temperature was maintained at 20° $\pm$ 0.2°C during each run. For correction of S values to $s_{20,w}$, the partial specific volume was taken as that of untreated antibody.

Hybrid Antibody.—Antibody of dual specificity was prepared from specifically purified

anti-EA and anti-BGG by a method similar to that described elsewhere (6). Thirty-five mg portions of each antibody preparation were mixed and treated with 1.4 mg of pepsin for 6 hours at 37°C and pH 4.5. The major 5S product was separated by precipitation with sodium sulfate (final concentration, 0.18 gm/ml), then reduced under nitrogen at pH 5.0 and 37°C for 90 minutes with 0.015 M mercaptoethylamine hydrochloride (MEA). The product had a sedimentation coefficient ($s_{20,w}$) of 3.5S. The reducing agent was removed by passage through a column of IR-120 cation-exchange resin at pH 4.5 in the cold room, and the 3.5S material was reoxidized by allowing the mixture to stand at neutral pH and room temperature. After a day, approximately 70 per cent had recombined to give a product with $s_{20,w} = 4.9$S. The latter was separated from the slower sedimenting material by precipitation with sodium sulfate (final concentration, 0.16 gm/ml). This product did not form precipitates when tested with a wide range of concentrations of either antigen. When 25 μg of ovalbumin and 40 μg of BGG were added as a mixture to 800 μg of the product, 330 μg of precipitate formed. Because of lack of sufficient material a range of concentrations of the mixture of antigens was not tested. The failure to precipitate with a single antigen, and the formation of precipitate with a mixture of antigens was reported previously (6), and is consistent with the presence of antibody of dual specificity in the product.

Specifically purified antiovalbumin was treated with pepsin, reduced and reoxidized by an essentially identical procedure. The $s_{20,w}$ value of the product was 4.7S. The fraction of the total protein precipitable by an optimal amount of ovalbumin was 55 per cent. The 5S anti-ovalbumin produced in this manner, by reoxidation of 3.5S fragments, could not be distinguished serologically, either qualitatively or quantitatively, from the 5S material obtained directly by treatment with pepsin.

Erythrocytes.—Human (type O, Rh-positive) and chicken whole bloods, obtained at weekly intervals, were mixed with equal volumes of Alsever's solution, and stored at 4°C. Prior to use in hemagglutination experiments, the erythrocytes were washed four times with 20- to 40-fold volumes of normal saline.

Passive Hemagglutination.—Antigens EA and BGG were coupled to human and chicken erythrocytes (rbc) respectively, by the chromic chloride method of Jandl (12). Prior to use, the BGG was absorbed with human and chicken erythrocytes to ensure the absence of hetero-antibody against these erythrocytes. In brief, 0.5 ml of a 50 per cent suspension of chicken rbc, 1 ml of a 2.5 μM solution of $CrCl_3$ and 2.5 ml of a 1 per cent BGG solution were mixed and allowed to stand at 20°C for 1 hour. Similar volumes of human cells, of 10 per cent EA, and of 15 μM $CrCl_3$ were mixed to provide EA-coated human red cells. The concentrations of $CrCl_3$ used were selected on the basis of Jandl's results (12). The concentrations of BGG and EA used were those which provided approximately maximal agglutination titers with specific antisera in preliminary tests. The "sensitized" erythrocytes were subsequently washed 6 times and suspended in saline. Two per cent suspensions of sensitized or non-sensitized (control) erythrocytes were used in agglutination reactions, which were carried out in 10 × 75 mm test tubes or on slides. In some experiments tannic acid (13) rather than $CrCl_3$ was used to bind the antigens to the indicator erythrocytes.

Serial dilutions, starting at 0.1 mg/ml, of purified anti-EA or anti-BGG, 5S anti-EA, 5S anti-BGG, or of "hybrid" 5S antibody preparations, were added to equal volumes of 2 per cent suspensions of coated or uncoated erythrocytes, and incubated for varying time intervals at room temperature. Dilutions were performed with separate pipettes to prevent carry over. The mixtures were studied microscopically (400-fold magnification) for agglutination, and the degree of clumping graded as 0 to ++++. The cell types (human, chicken, or both) present in the agglutinates were also ascertained, as were the types and approximate relative proportions of free (non-agglutinated) cells in mixed cell populations. Specific attention was directed to the possible presence in mixtures of three discrete types of clumps, one containing

solely human cells, another solely chicken cells, and a third containing a mixture of the two types of cells (mixed agglutinates). Comparative titers of different antibodies were determined by double blind readings of the mixtures of cells and antisera.

"Two-step" Agglutination Procedures.—In this procedure, the 5S "hybrid preparation" was added to human cells coated only with EA (EA cells). After incubation for 1 hour in 10 × 75 mm test tubes at 20° or 37°C, the mixtures were centrifuged for 45 seconds in a Clay-Adams serofuge and read for agglutination. In the second step the cells were thrice washed with 20- to 40-fold volumes of normal saline, and chicken antiserum prepared against the 5S derivative of rabbit gamma globulin (chicken anti-rabbit 5S) was added. The chicken antiserum, which contained approximately 60 μg antibody nitrogen per ml, was first diluted with 40 to 80 volumes of saline. Similarly diluted normal chicken serum served as a control. In some experiments the second step consisted of the addition of 0.1 ml of a 2 per cent suspension of chicken cells coated with BGG ("detector cells") to the washed EA cells ("primary cells"). In either case, the mixture was kept at room temperature for 1 hour, then examined microscopically for mixed agglutination.

Similar two-step experiments were performed by using BGG-coated cells as the primary cell in the first step, and, as the second step, adding either chicken anti-rabbit 5S or EA-coated detector cells.

Dispersal of Agglutinates with Sulfhydryl Reagent.—An equal volume of 0.1 M MEA in pH 7.2 buffered saline was added to the agglutinates obtained with various antibody preparations (5S anti-EA, 5S anti-BGG, the hybrid preparation, or artificial mixtures of 5S anti-EA and 5S anti-BGG). Agglutinates obtained with "7S" anti-EA or anti-BGG were similarly treated. The mixtures of antibody, agglutinated cells, and MEA were kept at 20°C for 2 hours. Buffered saline was substituted for MEA in simultaneous control experiments. If the agglutinates were dispersed by the MEA treatment, the cells were subsequently washed four times with isotonic saline, chicken antiserum to the 5S rabbit gamma globulin was added, and the mixtures were read for agglutination.

Dispersal of Agglutinates by Excess Antigen.—Reversal of agglutination by the addition of excess antigen was studied by the method of Lawler (14), as modified for mixed agglutinates by Milgrom *et al.* (15). Agglutinating mixtures were first allowed to stand for 1 hour at room temperature. The tubes were then centrifuged at 2000 RPM for 2 minutes and the supernatants were removed. The agglutinates were resuspended in 0.1 ml of 1 per cent EA, of 1 per cent BGG, or of a solution containing 1 per cent concentrations of both EA and BGG, and the resultant mixtures were observed microscopically after incubation for 30 minutes at room temperature. In other experiments, concentrations of antigen varying between 10.0 and 0.01 mg/ml were used. In those suspensions in which dispersal did occur, the cells were tested for residual attachment of univalent antibody with chicken antiglobulin reagent.

RESULTS

Table I demonstrates that coupling of antigens EA or BGG to human or chicken erythrocytes with $CrCl_3$ rendered the "coated" indicator cells agglutinable by the homologous, but not by the heterologous antibody. This was true whether a 7 or a 5S antibody preparation was used.

The results in Table II indicate that, within the limits of accuracy of the doubling dilution method employed for titration, the specifically purified 7S antibodies or their 5S derivatives possessed equivalent activity on a weight basis. Reduced and reoxidized 5S antiovalbumin also possessed agglutinating

activity which, by the doubling dilution method, was equivalent on a weight basis to that of the 7S preparation from which it was derived.

The use of two morphologically distinct erythrocyte populations (small round human cells and large, oval, nucleated chicken cells) permitted facile

TABLE I

Passive Hemagglutination Reactions of Specifically Purified Antibodies to Ovalbumin (EA) and Bovine Gamma Globulin (BGG) with Homologous and Heterologous Antigens

Antigen*	EA	BGG	EA	BGG	None	None
Cell‡	[H]	[Ch]	[Ch]	[H]	[H]	[Ch]
Antibody§						
Anti-EA						
7S	+++	0	+++	0	0	0
5S	+++	0	+++	0	0	0
Anti-BGG						
7S	0	+++	0	+++	0	0
5S	0	+++	0	+++	0	0
Buffer control	0	0	0	0	0	0

* The antigen was attached to the specified cell type by the $CrCl_3$ method (see text).
‡ [H] = human cells; [Ch] = chicken cells.
§ Antibody concentration: 0.1 mg/ml.

TABLE II

*Passive Agglutination Reactions of Specifically Purified 7 and 5S Anti-EA or Anti-BGG with Homologous Antigen**

	Log₂ dilution of antibody‡									
	0	1	2	3	4	5	6	7	8	Buffer
Anti-EA§										
7S	++±	++	+±	+±	+	±	0	0	0	0
5S (pepsin-treated 7S)	++±	++±	++±	++±	+	±	0	0	0	0
5S (reconstituted)‖	++±	++±	++±	++±	+	±	±	0	0	0
Anti-BGG¶										
7S	+++	++±	++±	+	±	0	0	0	0	0
5S (pepsin-treated 7S)	+++	++	++	+	±	0	0	0	0	0

* All antisera gave negative reactions with heterologous antigen at the initial dilution.
‡ Initial concentration of antibody, 0.1 mg/ml.
§ Human erythrocytes coated with EA were used for the agglutination tests.
‖ Reduced and reoxidized after pepsin treatment of 7S antibody.
¶ Chicken erythrocytes coated with BGG were used for the agglutination tests.

differentiation of the two cell populations, and ready identification of the cell types present in each agglutinate. As is evident in Table III, line 5, addition of moderate concentrations of anti-EA to mixed suspensions of human cells coated with EA, [H]-EA, and uncoated chicken cells, [Ch], produced homogeneous

TABLE III

Passive Mixed Agglutination Experiments with 5S Derivatives of Specifically Purified Antibodies*

Cells and antigens used	Antibody specificity (concentration, 0.01 mg/ml)			
	Anti-EA‡	Anti-BGG‡	Anti-EA and anti-BGG mixture	Hybrid
1. [Ch]	0	0	0	0
2. [H]	0	0	0	0
3. [Ch] + [H]	0	0	0	0
4. [H]-EA	+++ [H] only	0	+++ [H] only	0§
5. [H]-EA + [Ch]	+++ [H] only	0	+++ [H] only	0§
6. [Ch]-BGG	0	+++ [Ch] only	+++ [Ch] only	0‖
7. [Ch]-BGG + [H]	0	+++ [Ch] only	+++ [Ch] only	0‖
8. [Ch]-BGG + [H]-EA	+++ [H] only	+++ [Ch] only	+++ some [H] only; some [Ch] only	+++ mixed [H] + [Ch]
9. [Ch]-EA + [H]-EA	+++ mixed [H] + [Ch]	0	+++ mixed [H] + [Ch]	0¶
10. [Ch]-BGG + [H]-BGG	0	+++ mixed [Ch] + [H]	+++ mixed [H] + [Ch]	0

* [Ch] = chicken cells; [H] = human cells; [Ch]-BGG = BGG coupled to chicken cells; [H]-EA = egg albumin coupled to human cells; +++ = clumping; [H] + [Ch] = both human and chicken cells within each clump.

‡ Similar results were obtained at concentrations of 0.05 or 0.025 mg/ml.

§ Rare clumps of 2 or 3 cells of [H] at concentrations of >0.05 mg/ml.

‖ Rare clumps of 2 or 3 cells of [Ch] at concentrations of >0.05 mg/ml.

¶ Rare small clumps (2 to 4 cells) with only human or only chicken cells.

agglutinates composed solely of human cells; non-agglutinated chicken cells were readily identified in the suspension. Comparable results were obtained when chicken cells coated with BGG, [Ch]-BGG, were substituted for the uncoated chicken cells (Table III, line 8). Again only human cells were seen in the agglutinates produced by the addition of EA (Fig. 1 *a*).

Conversely, addition of anti-BGG rather than anti-EA to mixed cell popula-

tions containing [Ch]-BGG produced agglutinates composed entirely of large oval (chicken) cells. The human cells in the mixture, either uncoated (line 7) or coated with EA (line 8) were not agglutinated by the anti-BGG. The smaller human cells were randomly dispersed throughout the solution (Fig. 1 *b*). Comparable results (not listed) were obtained when the antigens coupled to the human and chicken cells were reversed; *i.e.*, with [Ch]-EA and [H]-BGG. Because of occasional difficulty in coupling BGG to human cells, the majority of subsequent titration experiments were performed with [Ch]-BGG and [H]-EA.

When artificial mixtures of 5S anti-EA and 5S anti-BGG (not hybridized) were added to a mixed suspension of [Ch]-BGG and [H]-EA, two distinct types of agglutinates were produced, one containing only human cells, the other only chicken cells (Table III, line 8; Fig. 1 *c*). In contrast, addition of the "hybrid

TABLE IV

*Inhibition by Soluble Protein Antigens of Passive Hemagglutination Caused by 5S Antibodies**

Antibody	Cells	Inhibitor (1 mg/ml)			
		Buffer	EA	BGG	EA + BGG
Anti-EA	[H]-EA	+++	0	+++	0
Anti-BGG	[Ch]-BGG	+++	+++	0	0
Hybid	[H]-EA + [Ch]-BGG	++	0	0	0
Buffer	[H]-EA + [Ch]-BGG	0	0	0	0

* Antibody concentration: 0.02 mg/ml.

preparation" to similar cell mixtures produced distinctive mixed agglutinates with multiple large clumps, all containing *both* human and chicken cells (Table III, line 8; Fig. 1 *d*). Large mixed agglutinates were observed throughout the suspension. Single non-agglutinated cells or small homologous clumps consisting of fewer than 5 cells of a single species were seen only occasionally.

All the experiments in Table III were repeated with cells coated with antigen by the use of tannic acid rather than chromic chloride. The results obtained were identical with those described above.

The dual specificity of the hybrid was demonstrated by inhibition experiments (Tables IV and V) in which cells coated by the chromic chloride and by the tannic acid methods were used. (It should be noted that the hybrid preparation presumably contained, in addition to the antibody of dual specificity, monospecific bivalent anti-EA and monospecific bivalent anti-BGG.) Either antigen (EA or BGG) inhibited the agglutination produced by addition of the 5S hybrid preparation to a mixture of the two types of coated cells (Table IV, line 3). Quantitative inhibition data are given in Table V.

In contrast, agglutination of [H]-EA by 5S anti-EA, or of [Ch]-BGG by 5S anti-BGG was inhibited by the homologous, but not the heterologous antigen

(Table IV, lines 1 and 2; Table V). The concentration of antigen necessary for complete inhibition was less than 0.02 mg/ml in each case (Table V).

High concentrations of the hybrid preparation agglutinated a cell suspension containing one cell type coated with antigen and uncoated cells of the other type (Table VI, rows 1 and 2). However, titers were 8 to 16 times greater when a mixture of cells coated with each of the antigens was used (Table VI, row 3). The agglutination of a single coated cell type is consistent with the presence in a hybrid preparation of monospecific bivalent antibody. The considerably greater titers observed with a mixture of coated cell types is in accord with the morpho-

TABLE V

*Inhibitory Effects of Antigens on Passive Hemagglutination Caused by 5S Specifically Purified Antibodies**

Concentration of EA or BGG	5S Anti-EA + [H] -EA‡		5S Anti-BGG + [Ch] − BGG‡		Hybrid + [H]−EA + [Ch]−BGG		
	Homologous antigen	Heterologous antigen	Homologous antigen	Heterologous antigen	EA	BGG	Both
mg/ml							
2.0	0	++	0	+	0	0	0
0.4	0	++	0	+	0	0	0
0.02	0	++	0	+	0	0	0
0.004	0	++	0	+	+±	0	+±
0.0008	0	++	+	+	++	++	++
Buffer	++		+		++		

* The antibody concentration in each experiment was 0.01 mg/ml.

‡ The symbols [H] and [Ch] refer to human and chicken erythrocytes, respectively.

logic evidence (mixed agglutination), for the presence of antibody of dual specificity.

The relatively low titer of the hybrid preparation against a single coated cell type was increased by the antiglobulin reaction to a level nearly as great as that observed in direct agglutination experiments utilizing both cell types (Table VI, rows 4 to 6). The antiglobulin reaction did not enhance the titer of the hybrid preparation against a mixture of cell types (rows 3 and 6), nor did it enhance the titer of 5S anti-EA against [H]-EA (rows 7 and 10). The enhancement, by the antiglobulin reaction, of the titer of the hybrid preparation against a single cell type is consistent with the attachment to red cells of hybrid molecules which are effectively univalent in the presence of a single antigen. The agglutinates formed in this case contained only the cell type coated with antigen. The univalent 3.5S fragments of anti-EA did not cause direct agglutination (Table VI, rows 13 to 15). However, positive antiglobulin reactions were

TABLE VI

Titers of Purified Antibody against Cells Coated with One or with Both Antigens

Antibody	Cell type and antigen	Row No.	Log₂ dilution of antibody*								
			0	1	2	3	4	5	6	7	Buffer
Hybrid Agglutination	[H]-EA + [Ch]	1	+++	+±	+	0	0	0	0	0	0
	[Ch]-BGG + [H]	2	+++	+	0	0	0	0	0	0	0
	[H]-EA + [Ch]-BGG	3	+++±	+++	+++	+±	+	+	±	0	0
Antiglobulin‡	[H]-EA + [Ch]	4	×§	×	×	+±	+	+	0	0	0
	[Ch]-BGG + [H]	5	×	×	+±	+±	+±	±	0	0	0
	[H]-EA + [Ch]-BGG	6	×	×	×	×	×	×	×	0	0
5S anti-EA‖ Agglutination	[H]-EA	7	++±	++±	++	++	+	±	0	0	0
	[Ch]-BGG	8	0	0	0	0	0	0	0	0	0
	[H]-EA + [Ch]-BGG	9	++±	+±	+	±	±	0	0	0	0
Antiglobulin	[H]-EA	10	×	×	×	×	×	×	0	0	0
	[Ch]-BGG	11	0	0	0	0	0	0	0	0	0
	[H]-EA + [Ch]-BGG	12	×	×	×	×	×	0	0	0	0
3.5S anti-EA Agglutination	[H]-EA	13	0	0	0	0	0	0	0	0	0
	[Ch]-BGG	14	0	0	0	0	0	0	0	0	0
	[H]-EA + [Ch]-BGG	15	0	0	0	0	0	0	0	0	0
Antiglobulin	[H]-EA	16	++±	++	++	+±	+	±	0	0	0
	[Ch]-BGG	17	0	0	0	0	0	0	0	0	0
	[H]-EA + [Ch]-BGG	18	+++	++	++	±	±	0	0	0	0

* Initial concentration 0.1 mg/ml.

‡ Equal volume (0.1 ml) of 1:10 dilution of chicken anti-rabbit 5S serum added to thrice washed cells.

§ ×, agglutination in saline prior to addition of antiglobulin reagent.

‖ This 5S anti-EA was obtained by reoxidation of 3.5S anti-EA (see text).

observed with the homologous antigen (rows 16 and 18). The activity was approximately equal on a weight basis to that of the hybrid preparation in an antiglobulin test with cells coated with EA (rows 4 and 6).

The attachment of hybrid antibody to a cell species coated with a single anti-

gen was also demonstrable by a stepwise agglutination procedure which produced typical mixed agglutinates (Table VII). In this procedure (see Materials and Methods) the cells coated with one antigen were exposed to hybrid antibody (0.02 mg/ml) and washed; coated detector cells of the second type were then added. In each case agglutination resulted.

When 3.5S monospecific antibody rather than hybrid antibody was used to

TABLE VII

*Stepwise Agglutination Method**

Antibody	Primary cell and "coat"	Detector cell and "coat"					
		[H]-EA	[Ch]-EA	[H]-BGG	[Ch]-BGG	[H]	[Ch]
Hybrid	[H]-EA	0	0	+++‡ [H]	+++§ [H] [Ch]	0	0
	[Ch]-EA	0	0	+++§ [H] [Ch]	+++‡ [Ch]	0	0
	[H]-BGG	+++‡ [H]	+++§ [H] [Ch]	0	0	0	0
	[Ch]-BGG	+++§ [H] [Ch]	+++‡ [Ch]	0	0	0	0
3.5S anti-EA	[H]-EA	0	0	0	0	0	0
	[Ch]-EA	0	0	0	0	0	0
	[H]-BGG	0	0	0	0	0	0
	[Ch]-BGG	0	0	0	0	0	0

* Antibody, 0.02 mg/ml, was incubated with an equal volume of coated primary cells for 1 hour at 20°C; cells were then washed three times and detector cells added.

‡ Homologous agglutinates; cells of only one species.

§ Mixed agglutinates; cells of both species.

coat the "primary cell," addition of the "detector cell" failed to produce agglutination (Table VII).

In another group of experiments (not tabulated) it was found that incubation of the hybrid preparation with soluble homologous antigen (1 mg/ml), prior to exposure to the primary cell, prevented attachment to the cells, as evidenced by failure of agglutination upon subsequent addition of detector cells; prior incubation with heterologous antigen had no such inhibitory effect.

The agglutination induced by the hybrid antibody in mixed cell suspensions of [H]-EA and [Ch]-BGG was reversed by the addition of 0.1 M mercaptoethylamine (MEA) (Table VIII, line 2). (This concentration of reducing agent rapidly splits the 5S molecule into two univalent fragments, reference 1.) However, the fragments thus produced remained attached to antigens on the dispersed cells. This was demonstrated by the subsequent use of the chicken

anti-rabbit 5S reagent. Its addition produced strong agglutination. Each clump contained both human and chicken cells. MEA also dispersed the agglutinates obtained when mixtures of 5S anti-EA and 5S anti-BGG (not hybridized) were added to mixed [Ch]-BGG and [H]-EA suspensions. Subsequent addition of antiglobulin resulted in mixed (heterogeneous) clumps (Table VIII, line 2).

TABLE VIII

Effects of Sequential Addition of Dispersing Agent and Antiglobulin Reagent on Agglutinates Induced by Various 5S Antibodies

Symbols in the table show all types in the agglutinates observed after each procedure.

Antibody*	Mixture of 5S anti-BGG + 5S anti-EA		5S anti-BGG		5S anti-EA		5S hybrid	
Antigen	[Ch]-BGG+[H]-EA		[Ch]-BGG+[H]		[H]-EA+[Ch]		[Ch]-BGG+[H]-EA	
Test method	A‡	B§	A‡	B§	A‡	B§	A‡	B§
Dispersing Agent								
1. None (buffer control)	[H] + [Ch]‖	¶	[Ch]	¶	[H]	¶	[Ch]-[H]**	¶
2. MEA‡‡	0	[Ch]-[H]**	0	[Ch]	0	[H]	0	[Ch]-[H]**
3. 1 per cent EA + BGG	0	0	0	0	0	0	0	0
4. 1 per cent EA	[Ch]	¶	[Ch]	¶	0	0	0	[Ch]
5. 1 per cent BGG	[H]	¶	0	0	[H]	¶	0	[H]

* 0.01 mg/ml.

‡ Antibodies, coated cells, and dispersing agent mixed together and examined after 1 hour for agglutination. Table shows types of agglutinates observed.

§ Antiglobulin test. (Chicken anti-rabbit 5S serum added to thrice washed cells after procedure A.)

‖ Separate homologous clumps of only chicken or only human cells.

¶ Agglutinates present prior to addition of antiglobulin reagent.

** Mixed agglutinates. Each clump contains both chicken [Ch] and human [H] cells.

‡‡ 0.1 M Mercaptoethylamine (see text for condition of use).

Agglutinates produced by mixing 5S anti-BGG and [Ch]-BGG in the presence of uncoated human cells were similarly dispersed by MEA. Subsequent addition of antiglobulin reagent resulted in the formation of clumps containing only chicken cells. When agglutinates made with [H]-EA and 5S anti-EA, in the presence of uncoated chicken cells, were dispersed with MEA, subsequent addition of chicken anti-rabbit 5S reagent resulted in clumps containing only human cells. The fact that MEA caused dispersal of each type of agglutinate is consistent with previous demonstrations that it cleaves the bivalent 5S molecules produced by pepsin, or by reoxidation of reduced 5S protein, into univalent fragments. The composition of the agglutinates formed in each instance after

the addition of antiglobulin reagent indicates that the univalent fragments liberated by MEA remained in combination with homologous antigen on the cells.

In other experiments (not tabulated) it was observed that addition of MEA did not result in dispersal of agglutinates made with 7S anti-EA and [H]-EA or with 7S anti-BGG and [Ch]-BGG.

Agglutinates induced by monospecific 5S anti-EA or 5S anti-BGG were also dispersed by addition of a solution of homologous antigen (Table VIII, lines 3 to 5). The results differed, however, from those obtained with MEA in that the dispersed cells did not agglutinate following addition of the antiglobulin serum. Thus the addition of excess soluble antigen, in contrast to MEA, evidently caused detachment of antibody.

Addition of either EA or BGG to the mixed agglutinates of [H]-EA and [Ch]-BGG induced by the hybrid was sufficient to cause dispersal (Table VIII). However, when only EA was added to the agglutinates, attachment of antibodies to cells coated with BGG persisted, as was demonstrable by the antiglobulin reaction. Similarly, addition of BGG to the same type of agglutinate resulted in dispersal, but antibody remained bound to cells coated with EA, as shown by the antiglobulin test. Presumably the antibodies included both the hybrid and monospecific bivalent antibody in the hybridized mixture, the latter in amounts insufficient to cause agglutination. Addition of both antigens to the agglutinates produced by the hybrid caused dispersal without demonstrable attachment; the antiglobulin reaction in this instance was negative (Table VIII, line 3).

DISCUSSION

The results of these investigations demonstrate the applicability of the mixed agglutination technique to the study of the properties of rabbit antibody of dual specificity (hybrid antibody). Previous reports (6, 7) have demonstrated the formation of hybrid antibodies following reduction and reoxidation of a mixture of two specifically purified, pepsin-treated antibodies of differing specificities. The present experiments provide a direct, visual confirmation of the mixed specificity of these molecules.

The mixed agglutination technique was first employed by Topley *et al.* with a mixture of 2 serologically unrelated and morphologically distinct bacterial species (16). Microscopic examination of the agglutinates induced by addition of a mixture of the 2 corresponding antiserums invariably revealed homologous agglutinates consisting solely of separate clumps of each bacterial species. Recent extensions of the mixed agglutination technique by Coombs and coworkers (17, 18) have demonstrated the unique applicability of such reactions for study of antigens on cell surfaces. The elegant experiments of Milgrom *et al.* (15) demonstrated that inert indicator erythrocytes of two morphologically distinct

species (human and alligator), coated *in vitro* with differing test antigens, can be readily utilized in mixed agglutination experiments for studies of specificity of rheumatoid serums and for the detection of molecules in such serums capable of reacting with two different antigens (human and rabbit gamma globulin). Jacot-Guillarmod and Isliker (19) have recently utilized mixed agglutination to demonstrate *in vitro* formation of antibody of mixed specificity by reduction and reoxidation of a mixture of anti-A and anti-B isoagglutinins of high molecular weight.

The mixed agglutination experiments reported here were carried out with 5S hybrid obtained by oxidation of a mixture of 3.5S fragments of anti-EA and anti-BGG. Such hybrid antibody was consistently found to yield mixed agglutinates of chicken and human erythrocytes when each cell type had been coated with one of the two homologous antigens, EA or BGG. In contrast, an artificial mixture of 5S anti-EA and 5S anti-BGG (not hybridized) produced separate clumps, each containing only a single type of erythrocyte.

The hybrid antibody also caused agglutination of a single species of erythrocyte, coated with a single antigen, but at a significantly lower titer than was observed with the mixture of coated erythrocytes. The agglutination of a single cell type is consistent with the presence of some bivalent antibody of single specificity in the hybridized mixture (7). For random association of equal amounts of fragments of specificities A and B, one would expect to obtain antibodies A-A, B-B, and A-B in the ratio 1:1:2. The A-A or B-B could well be responsible for the agglutination of a single, coated cell type. The species A-B should act as an effectively univalent antibody in an experiment with a single type of cell, and tend to inhibit agglutination. Apparently the amount present was insufficient to cause complete inhibition. A few experiments were carried out to test the inhibitory capacity of 3.5S (univalent) fragments of anti-EA; the minimum weight ratio of 3.5S anti-EA to 5S anti-EA required for complete inhibition of agglutination of [H]-EA was approximately 20 to 1. This high ratio is probably attributable to the presence of a large excess of antigenic sites on the coated cells, as compared with the amounts of antibody required for agglutination; it is consistent with the observation that a hybrid preparation is capable of agglutinating a single coated cell type.

The fact that agglutination of a mixture of coated cells by the hybrid antibody was prevented by an excess of either antigen (Table V) is consistent with a mechanism of agglutination in which the red cells having different antigenic coats are linked by molecules of mixed specificity.

Similarly, the mixed agglutinates formed by hybrid antibody could be dispersed by addition of either antigen (Table VIII). However, antibody combining sites of the other specificity remained in combination with erythrocytes having the corresponding antigenic coat, as shown by subsequent antiglobulin tests (Table VIII). Only the cell type having a coat different from the antigen

used for dispersal was agglutinated upon addition of the chicken anti-5S antiserum.

The results of stepwise agglutination tests were consistent with the attachment of the hybrid antibody to a single coated cell type (Table VII). When such antibody was used in an amount insufficient to cause agglutination, subsequent addition of cells coated with the second antigen resulted in agglutination. Individual clumps in this case contained both cell types. The titer of hybrid antibody for mixed agglutination by the stepwise procedure was found in separate experiments to be approximately the same as that for agglutination by addition to a mixture of coated cells.

The fact that each type of agglutinate could be dispersed by treatment with 0.1 M mercaptoethylamine is consistent with the chemical nature of the 5S antibody. In this molecule two univalent fragments are linked by a labile disulfide bond. Evidently the reducing agent cleaves the bond and thereby destroys the lattice in which the red cells are linked. However, the univalent fragments remain attached to the antigens coating the erythrocytes; on subsequent addition of antiglobulin reagent, agglutinates are again formed. When both coated cell types and a mixture of the two monospecific 5S antibodies were used for the first agglutination, separate homologous clumps formed. However, after dispersal with MEA and reagglutination with the chicken anti-rabbit 5S, mixed clumps were observed, as would be expected since chicken anti-5S reacts with each of the rabbit 5S antibodies.

Evidence suggesting the presence *in vivo* of multivalent antibodies with dual specificity against blood group A and B antigens in group O sera has been obtained by Dodd (20). Other possible interpretations of Dodd's data have been proposed and are discussed in detail by Kabat (21). The molecular size of these antibodies was not determined, but if they are indeed multivalent it would seem unlikely that they are 7S since the 7S antibodies of several other species have been found to be bivalent. Milgrom *et al.* (15) have described rheumatoid factors (macroglobulins) of mixed specificity directed against rabbit and human gamma globulins but these results are probably entirely explainable on the basis of antigenic determinants common to both rabbit and human gamma globulin. However, attempts to demonstrate the presence of 7S antibody of dual specificity in rabbits hyperimmunized with two antigens have been uniformly unsuccessful (22–27). Experiments of the types reported here should be useful as a model for determining whether very small amounts of hybrid 7S molecules are formed *in vivo*.

SUMMARY

Hybrid, bivalent antibody molecules bearing specific combining sites for both ovalbumin and bovine gamma globulin were produced by reoxidation of a mixture of the 3.5S fragments of the two specifically purified antibodies. The

dual specificity and the properties of the hybrid antibody were demonstrated by mixed agglutination and two stage agglutination experiments, and by test systems utilizing inhibition of agglutination or dispersal of agglutinates followed by antiglobulin reactions.

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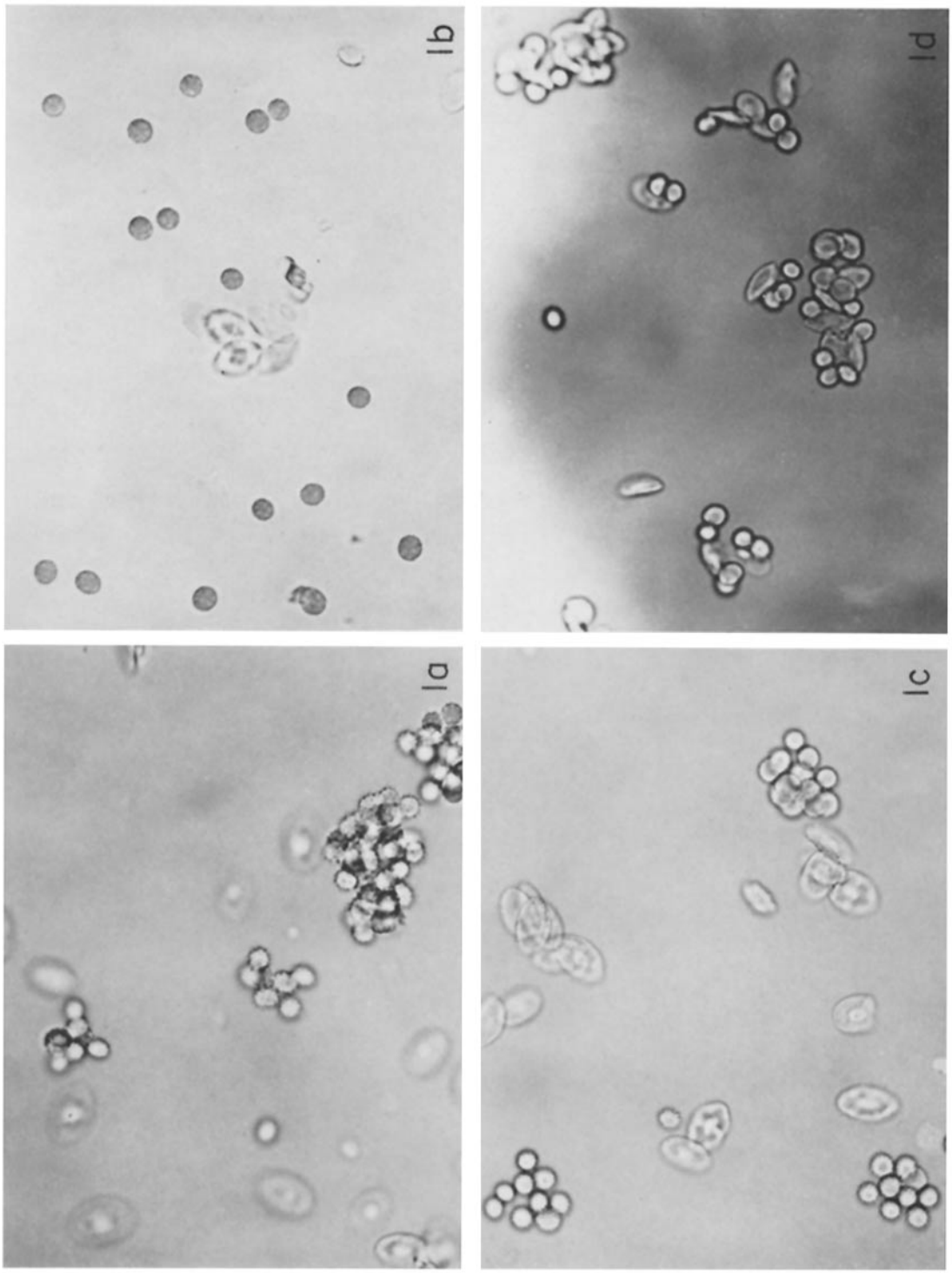
EXPLANATION OF PLATE 5

FIGS. 1 *a* to 1 *d*. Agglutinates made with [Ch]-BGG, [H]-EA, and the following 5S antibodies: 1 *a*, anti-EA; 1 *b*, anti-BGG; 1 *c*, mixture of anti-EA and anti-BGG; 1 *d*, hybrid preparation of anti-EA and anti-BGG. × 381.



(Fudenberg *et al.*: Serologic demonstration of dual specificity)