REVERSE IMMUNE CYTOADHERENCE:

A new technique for detection of receptor γ-globulin on lymphocytes

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ABSTRACT

Sho-tone Lee

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A new technique for detection of receptor γ-globulin on lymphocytes

A reverse immune cyto-adherence technique utilizing hybrid antibody is described, which is able to demonstrate γ-globulin-like receptors on the surface of the normal mouse spleen cells. The hybrid antibody carries an anti-γ globulin site on one side and an anti-ferritin site on the other. The hybrid antibody fixed on the cell surface by its anti-γ globulin site, in the meantime attracts a ferritin coated sheep red blood cell by its anti-ferritin site, a rosette is thus formed with a single spleen cell surrounded by a layer of ferritin coated sheep red blood cells.

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LITERATURE REVIEW AND INTRODUCTION

The recognition of antigen, the production of antibody subsequent to antigenic stimulation and the regulation of γglobulin synthesis in animal tissue cells have been subjects of conjecture for many years. Antigenic stimulation followed by cell proliferation and differentiation and antibody production have been studied recently (1, 2, 3, 4), however, the question of how antigen interact with the cell to induce antibody production is still unsolved. It is not surprising that many years before anything was known about the chemical nature of antigens and antibodies, Ehrlich formulated the ingenious side chain theory of antibody formation at the end of last century. He assumed that the animal organisms produce various types of cells with 'receptors', which were complementarily adjusted to the injected or invading bacterial or cellular antigens. According to his theory, each antigen combined with closely fitting receptors on the surface of these cells and thus stimulated the regeneration of the homologous receptors which later passed into the blood as antibodies. The term 'receptor' has survived up to the present day. Burnet's clonal selection theory (5) of antibody formation is based on the idea of existence of receptors which act like antibodies for the incoming antigens. More recently, Jerne (6) and Mitchison (7) postulate that such receptors arepreformed antibodies since only antibody recognizes antigen. With the present day knowledge of

protein synthesis and the genetic inheritance of the cells, the term 'receptor' used by Burnet (5) Jerne (6) and Mitchison (7) may not be the same as that of Ehrlich's, however, these authors still emphasize that at least part of these receptors bears the resemblance to the antibody molecules whether they act as antibodies or just serve as stations to transport the informations (8, 9, 10) to the cell after antigenic stimulation for antibody synthesis.

No direct evidence is available for the presence of such Ig-like receptors on the surface of lymphocytes or other immunologically competent cells. However, considerable indirect evidence suggests their presence. The most important are the findings of Sell and Gell that anti-allotypic antisera can induce blastogenic transformation indicating that 'something of the nature of an Ig molecule' acts as specific 'receptor' for the anti-allotypic antiserum (11, 12). Human lymphocytes show the same blastogenic transformation when cultured with horse (13) or monkey (14) antisera to human γglobulins or their light chains. Although γglobulin synthesis occurs in the cells transformed by antigens, the relationship of the proliferative activity induced by anti-γ-globulin and γglobulin production is not clear. In rabbit, lymphocytes stimulated with anti-allotypic antisera show blastogenic transformation but no γglobulin synthesis is detected (15).

A technique is developed to demonstrate the existence of receptors of γglobulin-nature on the surface of normal lymphoid cells, utilizing a hybrid antibody which has an anti-γG site on one side and an anti-ferritin site on the other. The hybrid antibody

fixes on the lymphoid cell surface by its anti-γG site and in the meantime attracts a ferritin coated sheep red blood cell (SRBC) by its anti-ferritin site, thus a rosette is formed with a lymphoid cell surrounded by a layer of ferritin coated SRBC. The technique detects the γglobulin-like receptors on the cell surface as antigens, a completely reversed system of direct immune-cytoadherence (ICA) as described by Zaalberg (16) and Biozzi et al (17).

MATERIALS AND METHODS

I. Sephadex G-100 and G-200 gel filtration

The Sephadex G-100 and G-200 columns were packed according to the instructions given by Pharmacia Ltd. (Montreal, Canada). The complete swelling of the Sephadex gels was carried out at room temperature for three days in the desired buffer. The gels were added gradually to an excess of buffer with mixing to facilitate dispersion of the beads in the swelling medium. Decanting and stirring were carried out intermittently during swelling. Before packing, the air bubbles trapped in the gel-buffer mixture (1:1 in volume) were removed by a vacuum pump. A Sephadex laboratory column was mounted vertically on a stand and 15-20 cm of buffer was poured into it. Any air bubbles trapped in the polyethylene disc were removed by forcing the buffer back and forth through the disc by a syringe attached to the outlet tubing. The gel-buffer mixture was dispersed evenly and poured down the column gradually until it reached the top of the column. The outlet was kept at the same level with the top of the gel slurry. After about half an hour, when approximately 10 cm of bed was settled, the outlet was lowered, so that the pressure was equal to 1 cm. As the packed bed rose, the outlet was lowered to maintain an optimum pressure equal to one tenth of the packed bed length. To prevent boundaries, the excess buffer was removed and another portion of gel slurry was added before the previous portion had settled completely. Care was

taken not to disturb the packed bed during addition of the gel slurry. After the column was packed, the upper surface of the bed was protected by inserting a sample applicator. The column was then allowed to equilibrate for 24 hours at 10-15 cm pressure at a rate of about 20 ml/hour. Before starting an experiment, about 5 mg blue dextran dissolved in 3 ml buffer were filtered through the column in order to check the homogeneity of the packing and determine the void volume.

In the experiments, the samples were allowed to filtrate the column at a rate of 5-10 ml/hour, under a pressure head of 2-3 cm.

II. DEAE-cellulose ion-exchange chromatography

The DEAE-cellulose ion exchange column was packed according to the method of King (18). The dry DEAE-cellulose ion-exchanger (Carl Schleicher and Schuell Co., Keene, N.H. .89 meq/gm dry weight) was washed by suspending it in .5N NaOH+ .5N NaCl with stirring. After settling for 30 minutes, the cloudy supernatant was decanted. The cellulose was then resuspended in IN NaCl. The process of stirring and decanting was continued for once or twice and the cellulose was filtered by suction on Büchner funnel through filter paper. The moist cake was suspended in IN HCl, immediately filtered by suction, and washed with distilled water until the pH was neutral. The ion-exchanger was then equilibrated to the desired pH and molarity by suspending and washing with the starting buffer. The cellulose-buffer mixture was prepared in thick slurry

form which was first dispersed in a waring blender and then the trapped air bubbles were removed by a vacuum pump. The uniform suspension was poured into the column. The cellulose was allowed to settle to 4 or 5 cm length at the bottom of the column with the outlet closed. Excess buffer was removed with the outlet open and more slurry was added until the column was packed. A 3-5 cm column of the buffer above the bed was left behind to avoid any disturbance of the packed bed with next addition of cellulose suspension. The whole process of packing was carried out under the atmospheric pressure. The upper surface of the bed was protected by inserting a piece of filter paper or a sample applicator. The DEAE-cellulose ion-exchange column was run under a pressure generally greater than atmospheric pressure at a rate of 30 to 40 ml per hour.

III. Immunoelectrophoresis

The method was that of Grabar and Burtin (19). The glass slides (25 x 76 mm) were first coated with 2 ml .5% melted agar (Difco Noble) in distilled water and dried at 80° C for 4 hours. In the immunoelectrophoretic experiments, 3 ml 2% melted agar in .025M barbital buffer pH 8.5 were layered on the coated slides. The agar gel was allowed to solidify for a few minutes, and the required wells and troughs were cut. The materials to be tested were placed in the wells and the slides were then placed in electrophoretic apparatus and ran at 14 ma. approximately 70 to 75 volts for three and a half hours. The slides were then removed from the apparatus, the troughs were filled with proper antisera and placed in a moist chamber at

room temperature overnight to allow the development of the precipitation lines. The slides were washed in .9% saline for 24 hours, desalted in distilled water for 8 hours, and then dried overnight by placing filter paper on the slides. The dried slides were stained with amido black (1 gm/1000 ml sodium acetate buffer) for 10 minutes, decolorized in acetic acid (acetic acid: methanol: water = 150: 750: 750 in volume) for another 10 minutes and then air dried.

IV. Ouchterlony gel diffusion

Coated slide as described in immunoelectrophoresis was used. It was layered with 3 ml 1.5% melted agar in .15M saline. The gel was allowed to solidify for 5 to 10 minutes in a moist environment. Wells were cut in circle using a hole puncher. Required patterns of antigen-antibody precipitation reaction were arranged by filling the wells with antigen or antibody solution. The slide was placed in a moist chamber at room temperature overnight for the precipitation lines to develop. It was then washed and stained as described for immunoelectrophoretic slides.

V. Coating of sheep red blood cells (SRBC) (20)

The formalinized sheep red blood cells were used in all the experiments. The method of formalinization was that of Wede (21). Sheep red blood cells in Alsever's solution were washed 3 or 4 times with .15M saline. One volume of 8% washed SRBC was incubated with an equal volume of 3% formaldehyde pH 7 (adjusted by .IN NaOH)

for 18 to 24 hours at 37°C. The cells were washed 4 times with distilled water, and finally suspended in distilled water as a 10% suspension and stored at 4°C. From this 10% suspension, a 2% suspension was prepared for tanning and coating. Two ml of the 2% suspension were processed in the following manner using .15M phosphate buffered saline pH 6.4 in all the steps of this procedure: the cells were washed three times, suspended in 2 ml of a .0025% tannic acid in buffer and incubated at 37°C for half an hour. Then they were washed once, resuspended in 2 ml of buffer containing .2 mg protein/ml and incubated at 37°C for one hour. The coated cells were washed three times and finally suspended in 1.5 ml of buffer containing .25% human serum albumin. This resulted in a suspension of approximately 2.5% cells.

VI. Mice

Inbred BALB/C male mice from Jackson Laboratories, Bar Harbor, Maine, were used in all the experiments. The mice were fed with tap water and dry food ad libitum.

VII. Antigens

1. Mouse γG myeloma protein

This was isolated from ascitic fluid of a transplantable mineral oil (M.O.) induced tumour. Ten ml ascitic fluid equilibrated in .005M phosphate buffer pH 8 were chromatographed on a DEAE-cellulose ion-exchange column (22 x 500 mm) packed in the same buffer.

The proteins were eluted from the column stepwise by increasing the molarity of the buffer to .OIM and .033M pH 8, while the remaining protein was washed out with .5N NaCl. The eluates were collected in 10 ml fractions by an automatic fraction collector and the protein content in each tube was determined by measuring the optical density (O.D) at 280 mu in a Zeiss spectrophotometer (fig. 1). The tubes from each step were pooled and concentrated by pervaporation in .15M borate buffered saline pH 8 to a concentration of 10 mg/ml. Each fraction was examined by immunoelectrophoresis using a rabbit antiserum to mouse serum (fig. 2 a and b). The immunoelectrophoretic results indicated the presence of γG myeloma protein in .005M, .OIM and .033M fractions. In order to exclude trace contamination of transferrin, each fraction was passed through a Sephadex G-200 column equilibrated with .15M borate buffered saline pH 8. Generally, two peaks were obtained (fig. 3). The first peak was pure γG myeloma protein as revealed to be a single line by immunoelectrophoresis (fig. 4) and Ouchterlony gel diffusion using the rabbit antiserum to mouse serum (fig. 5). The isolated protein was concentrated by pressure filtration to about 10 mg/ml and stored at -20°C.

2. Ferritin

Horse spleen ferritin, twice crystallized, Cadmium free (Nutritional Biochemical Corp., Cleveland, Ohio).

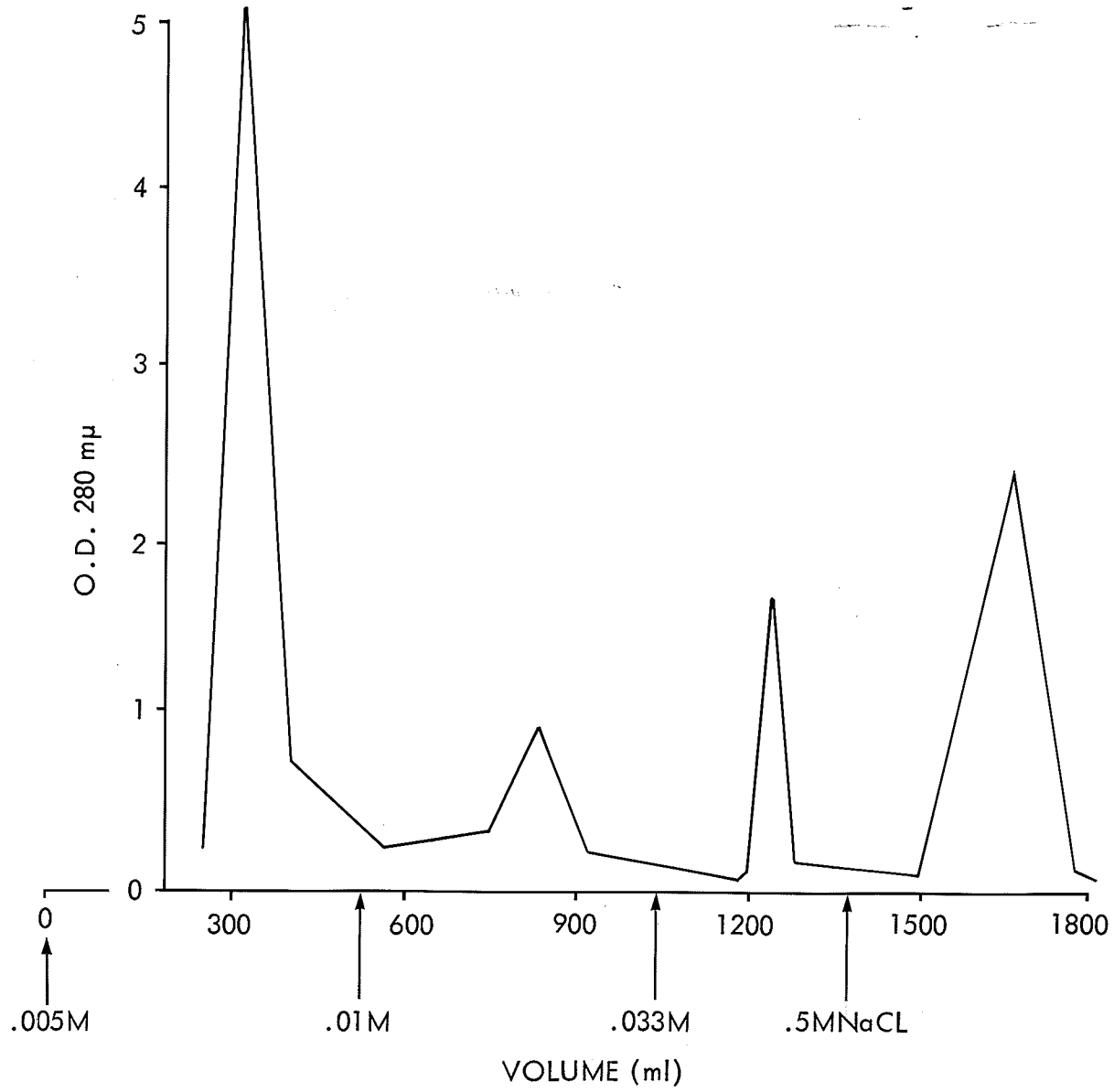

Fig. 1. DEAE-cellulose column chromatography of ascitic fluid of γG tumor bearing mouse. A stepwise elution was used by changing the molarity of phosphate buffer as indicated.

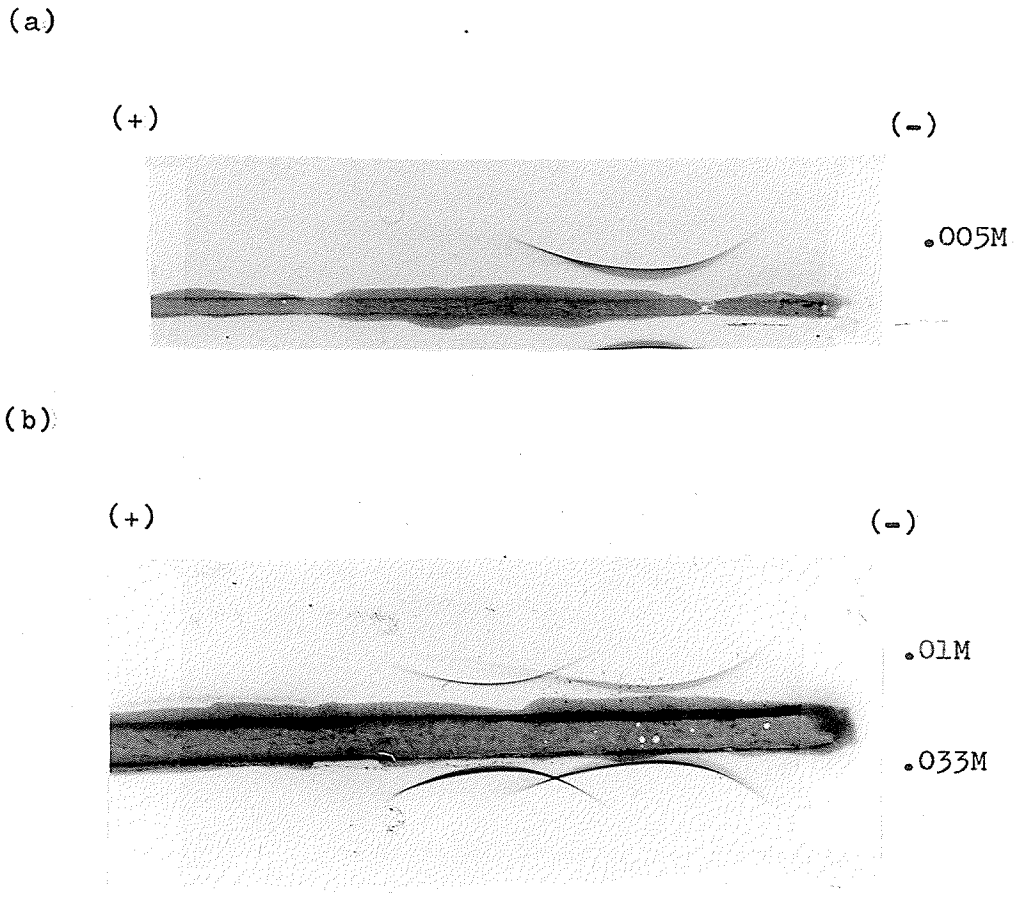

Fig. 2. a and b. Immunoelectrophoretic patterns of different fractions isolated from DEAE-cellulose column. The wells contained different fractions as indicated. The troughs contained rabbit antiserum against mouse serum.

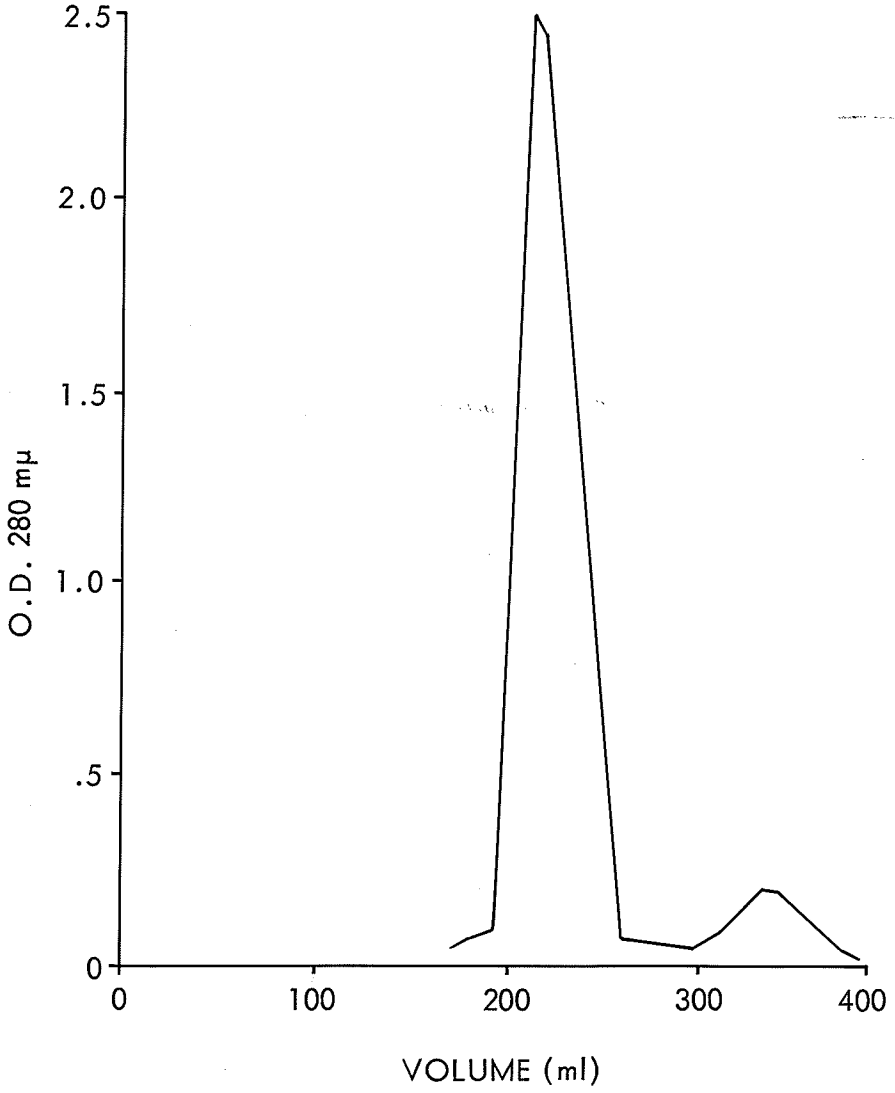

Fig. 3. Gel filtration on Sephadex G-200 column of .005M fraction from DEAE-cellulose column chromatography.

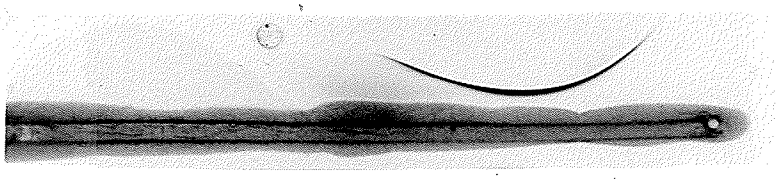

Fig. 4. Immunoelectrophoretic pattern of mouse γG myeloma protein isolated from the first peak of Sephadex G-200 gel filtration, developed with a rabbit antiserum against mouse serum.

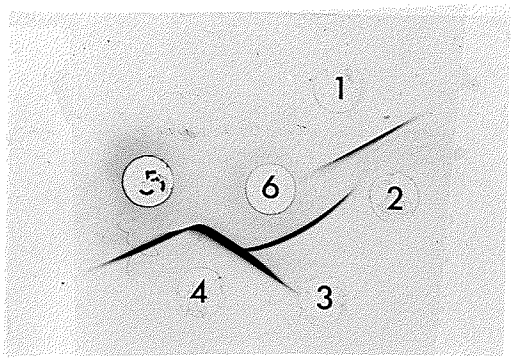

Fig. 5. Ouchterlony double gel diffusion to demonstrate the antibody activities of specifically purified rabbit anti-ferritin and anti-mouse γG antibodies and the purity of the γG myeloma protein isolated from Sephadex G-200 gel filtration, developed by a rabbit antiserum against mouse serum. (1) ferritin, (2) specifically purified rabbit anti-ferritin antibody, (3) mouse Bence Jones protein, (4) mouse γG myeloma protein isolated from Sephadex G-200 gel filtration, (5) rabbit antiserum to mouse serum and (6) specifically purified rabbit anti-mouse-γG antibody.

VIII. Rabbit immunization and collection of rabbit antisera

A group of two or three rabbits were injected either with mouse γG-myeloma protein isolated from Sephadex G-200 column or with horse spleen ferritin intra-muscularly for five times with 1 mg/ml protein per injection per week. The antigens were emulsified in complete Freund's adjuvant (Difco). One week after the fifth injection, the rabbits were test bled through the vein of the ear or by heart puncture. The antibody activity of each antiserum was checked by immunoelectrophoresis. Once the rabbits showed the proper antibody activities, they were bled constantly by heart puncture once a week for four or five times, then a booster shot of 1 mg protein was given to elevate the antibody level. In each bleeding, 50 ml of rabbit blood was collected, after standing at room temperature for two hours, the serum was separated from blood clot by centrifugation at 3,000 rpm for 15 minutes. The serum from each individual rabbit was pooled and stored at -20°C.

IX. Purification of specific antibody

1. Purification of anti-ferritin antibody

(i) Determination of optimal precipitation range of anti-ferritin antiserum

The method of precipitation was that of Campbell et al (22). To .5 ml of 1:2 dilution of anti-ferritin antiserum, increasing

amounts of ferritin were added as shown in Table I. The tubes were incubated for half an hour at 37°C and then left at 4°C for two days. At the end of this period, the precipitates were removed by centrifugation at 3,000 rpm for 30 minutes at 4°C and washed three times with cold .15M NaCl. Protein determination of the precipitates were made by dissolving them in .5M NaOH using the Lowry method (23) as shown in Table II. The optimal range is determined as being 3 mg of ferritin for .5 ml 1:2 dilution of antiserum. Therefore a total of 12 mg is required for optimal precipitation for 1 ml rabbit anti-ferritin antiserum.

(ii) Isolation of specific anti-ferritin antibody

Using the data obtained from precipitation curve above, 120 mg ferritin were added to 10 ml anti-ferritin antiserum in a 12 ml test tube. The tube was first incubated at 37°C for half an hour and left at 4°C overnight. The heavy precipitates were isolated by centrifugation at 5,000 rpm at 4°C for half an hour and washed three times with ice cold .15M NaCl. 10 ml of IN HCl was added to dissolve all the precipitates which were then separated from ferritin by ultracentrifugation at 35,000 rpm (135,000 g) for 60 minutes in a model L- 2 Beckman ultracentrifuge using WL-39 rotor. The ferritin came down to the bottom of the tube, the supernatant containing antibody globulins was pipetted out carefully and immediately neutralized by addition of 7 ml .2M phosphate buffer pH 7.2. Heavy precipitates came down during neutralization, which were insoluble in phosphate buffer.

TABLE I

PRECIPITATION PROCEDURE OF FERRITIN WITH RABBIT ANTISERUM TO FERRITIN

Tube No.	Control	1	2	3	4	5	6	7	8	9	10
Ferritin-mg.	-	.25	.5	.75	1.0	1.5	2.0	3.0	4.0	5.0	6.0
Volume - ml.	-	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Borate buffered saline-ml .15M pH. 8	.5	-	-	-	-	-	-	-	-	-	-
Rabbit anti-ferritin antiserum - ml (1/2 dil.)	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5	.5
Apparent Precipitation	-	+	+	++	++	++	+++	++++	+++	++	++

TABLE II

DETERMINATION OF PROTEIN IN PRECIPITATES USING THE LOWRY METHOD

Tube No.	Blank	1	2	3	4	5	6	7	8	9	10
Water-ml.	.8	.6	.6	.6	.6	.6	.6	.6	.6	.6	.6
Precipitate-ml. in $\frac{M}{2}$ NaOH	0	.2	.2	.2	.2	.2	.2	.2	.2	.2	.2
Copper reagent - ml.	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
	Stand at room temperature for 10 minutes										
Folin-Ciocalteau phenol reagent-ml.	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4	.4
	Mix thoroughly after each addition, and then stand for 30 minutes										
$O.D_{750\ mu}$	0	.093	.119	.129	.162	.175	.195	.236	.225	.218	.206

They were removed by centrifugation at 10,000 rpm for 10 minutes. The clear colorless supernatant was collected and concentrated by pressure filtration to about 15 mg/ml and stored at 4°C. The anti-ferritin activity of the preparation was confirmed by Ouchterlony gel diffusion technique (fig. 5) using a solution of 1 mg/ml.

The preparation contained only γ-globulin as revealed by a goat anti-rabbit serum antiserum (Hoechst Pharmaceutical Co., Kansas City, Mo.) on immunoelectrophoresis as shown in fig. 6. About 25 mg antibody globulins were isolated from 10 ml rabbit serum.

2. Purification of anti-mouse γG antibody

This was achieved by using γG myeloma protein aggregated with bis-diazotized-benzidine (BDB). The method of aggregation was that of Ishizaka et al (24) and Bernier and Cebra (25). The BDB solution was prepared according to the method of Herbert (20).

(i) Aggregation of mouse γG myeloma protein

To 50 mg γG myeloma protein in 50 ml .IM phosphate buffer pH 6.8, 50 ml freshly thawed 1:15 dilution of BDB solution was added, the mixture was allowed to stand at room temperature for 5 hours. The heavy insoluble precipitates were isolated by centrifugation at 3,000 rpm for 15 minutes at 4°C and washed three times with the same buffer to remove free BDB.

(ii) Isolation of specific anti-γG antibody

50 mg washed BDB-mouse-γG aggregates were added to 10 ml

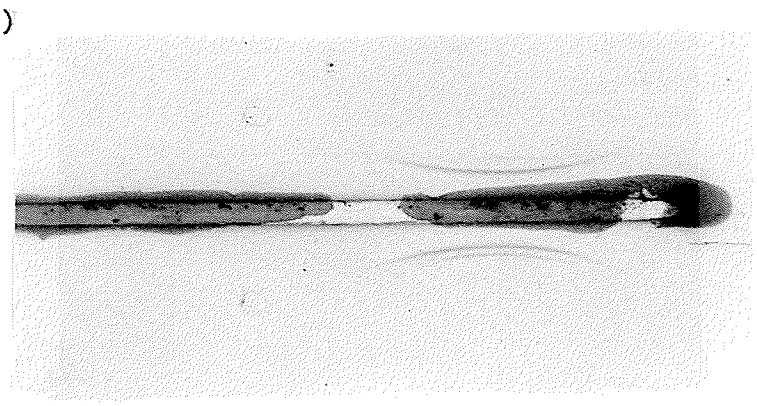

Fig. 6. The specifically purified rabbit anti-ferritin (upper well) and anti-mouse-γG myeloma protein antibodies (lower well) were developed with a goat antiserum to rabbit serum (trough). Each antibody formed one line in the γ-globulin region.

rabbit anti-γG antiserum. The absorption was carried out at 37°C for half an hour and then continued at 4°C overnight. The aggregates were isolated and washed three times. The antibody globulins were dissociated from the aggregates by addition of 4 ml .IM glycine-HCl buffer pH 2.5 and left for one hour at 4°C. The antibody globulins were then separated by centrifugation at 15,000 rpm at 4°C. The supernatant was immediately neutralized by addition of 2 ml .2M phosphate buffer pH 7.2. The antibody activity and the γG nature of the preparation were checked by Ouchterlony and immunoelectrophoretic techniques (fig. 5 and 6). The purified antibody reacted both with mouse γG and mouse Bence Jones protein (fig. 5).

The antigen aggregates were washed twice with glycine-HCl buffer and once with .IM phosphate buffer pH 6.8. The washed antigen aggregates were used repeatedly for 10 times without losing absorption capacity.

From 50 ml rabbit anti-γG antiserum, approximately 100 mg antibody globulins were collected by repeated absorptions.

X. Determination of per cent specifically precipitable protein in purified antibody preparation

The precipitation procedure for γG-anti-γG (Table III) and ferritin-anti-ferritin (Table IV) were carried out as described before (22). To a known amount of antibody in .5 ml volume, increasing amounts of antigen were added. The tubes were incubated at 37°C for half an hour and left at 4°C for two days. At the end

TABLE III

DETERMINATION OF PER CENT OF SPECIFICALLY PRECIPITABLE PROTEIN IN THE ANTI-MOUSE γG ANTIBODY PREPARATION

Tube No.	Control	1	2	3	4	5	6	7
Specific rabbit anti-mouse γG antibody - O.D 280 mu.	.55	.55	.55	.55	.55	.55	.55	.55
Volume - ml.	.5	.5	.5	.5	.5	.5	.5	.5
Mouse γG - O.D 280 mu.	-	.0156	.0312	.0625	.125	.25	.5	1.0
.15M Borate-buffered saline pH. 8 - ml.	.5	-	-	-	-	-	-	-
O.D 280 mu. reading of supernatants	.55	.396	.305	.167	.087	.208	.64	1.35

TABLE IV

DETERMINATION OF PER CENT OF SPECIFICALLY PRECIPITABLE PROTEIN IN THE ANTI-FERRITIN ANTIBODY PREPARATION

Tube No.	Control	1	2	3	4	5	6	7	8
Sp. purified rabbit anti-ferritin - O.D.	.6	.6	.6	.6	.6	.6	.6	.6	.6
Volume - ml.	.5	.5	.5	.5	.5	.5	.5	.5	.5
Horse spleen ferritin - mg.	-	.0156	.0312	.0625	.125	.25	.5	1.0	2.0
.15M Borate saline pH. 8 - ml.	.5	-	-	-	-	-	-	-	-
O.D. 280 mu reading of supernatants	.598	.44	.369	.29	.182	.367	1.09	2.94	3.1

10% TCA precipitation

To each supernatant (1 ml in volume), 1 ml 20% TCA added to precipitate all the protein. The precipitates were removed by centrifugation after standing at room temperature for half an hour. The supernatants were read at 280 mu.

O.D. reading of supernatants	.01	.019	.022	.022	.023	.043	.116	.279	.310
Multiply by 2 (dilution factor)	.02	.038	.044	.044	.046	.086	.232	.558	.62

of incubation, the supernatants from each tube were saved and the precipitates were stored at 4°C. Determination of specifically precipitable antibody globulins was carried out by measuring the O.D of supernatants in each tube at 280 mu (Table III and IV). The tube which gave the lowest O.D reading in the series was taken as the tube of highest specifically precipitable antibody globulins.

The calculation of per cent precipitable antibody globulins was based on the assumption that in the tube with lowest O.D reading, all the active antibody globulins were precipitated together with all the homologous antigens, so that the O.D reading of the supernatant represented the amount of non-specifically precipitable protein which was present in the original antibody preparation. Therefore, for anti-γG antibodies:

Supernate with lowest reading at 280 mu = .087 O.D

Specifically precipitable antibody protein = .55-.087 = .463 O.D

Per cent specifically precipitable anti-γG antibody $= \frac{.463}{.55} \times 100 = 84\%$

For ferritin anti-ferritin system, however, due to the non-specific color contributed by ferritin, a 10 per cent trichloroacetic (TCA) precipitation was carried out to remove all the protein molecules (Table IV) in the supernatants, the O.D of each tube was measured again to determine the amount of O.D contributed by the non-specific coloring substances. Therefore, for anti-ferritin antibodies:

Supernate with lowest reading at 280 mu = .182 O.D.

Reading of supernate after TCA precipitation = .046 O.D.

Non-precipitable protein in supernate = .182-.046 = .136 O.D.

Specifically precipitable anti-ferritin antibody = .60-.136 = .464 O.D.

Per cent of specifically precipitable anti-ferritin antibody = $\frac{.464}{.60} \times 100 = 77\%$

XI. Preparation of F (ab')$_2$ (5S) fragments of specifically purified anti-ferritin and anti-γG antibody globulins.

The specifically purified anti-ferritin and anti-γG antibody globulins were digested separately by enzyme pepsin (Mann Research Laboratories, New York) according to the methods of Nisonoff et al (26) and Utsumi and Karush (27). The antibody globulin sample at a concentration of 1-2% was incubated with pepsin at a protein-enzyme ratio between 100:1 to 200:1 in .1M sodium acetate buffer pH 4 at 37°C. The incubation time was about 4 to 5 hours. The digestion was stopped by neutralization with 1N NaOH to about pH 8, and the sample was chromatographed on a Sephadex G-100 column (23 x 970 mm) equilibrated with .15M borate buffered saline pH 8 to remove the non-digested 7S globulins (fig. 7).

XII. Hybridization of F(ab') 2 fragments of anti-ferritin and anti-γG antibodies.

Hybrid antibody molecules of two specificities were prepared according to the method of Nisonoff and Rivers (28). 45 mg F(ab')2

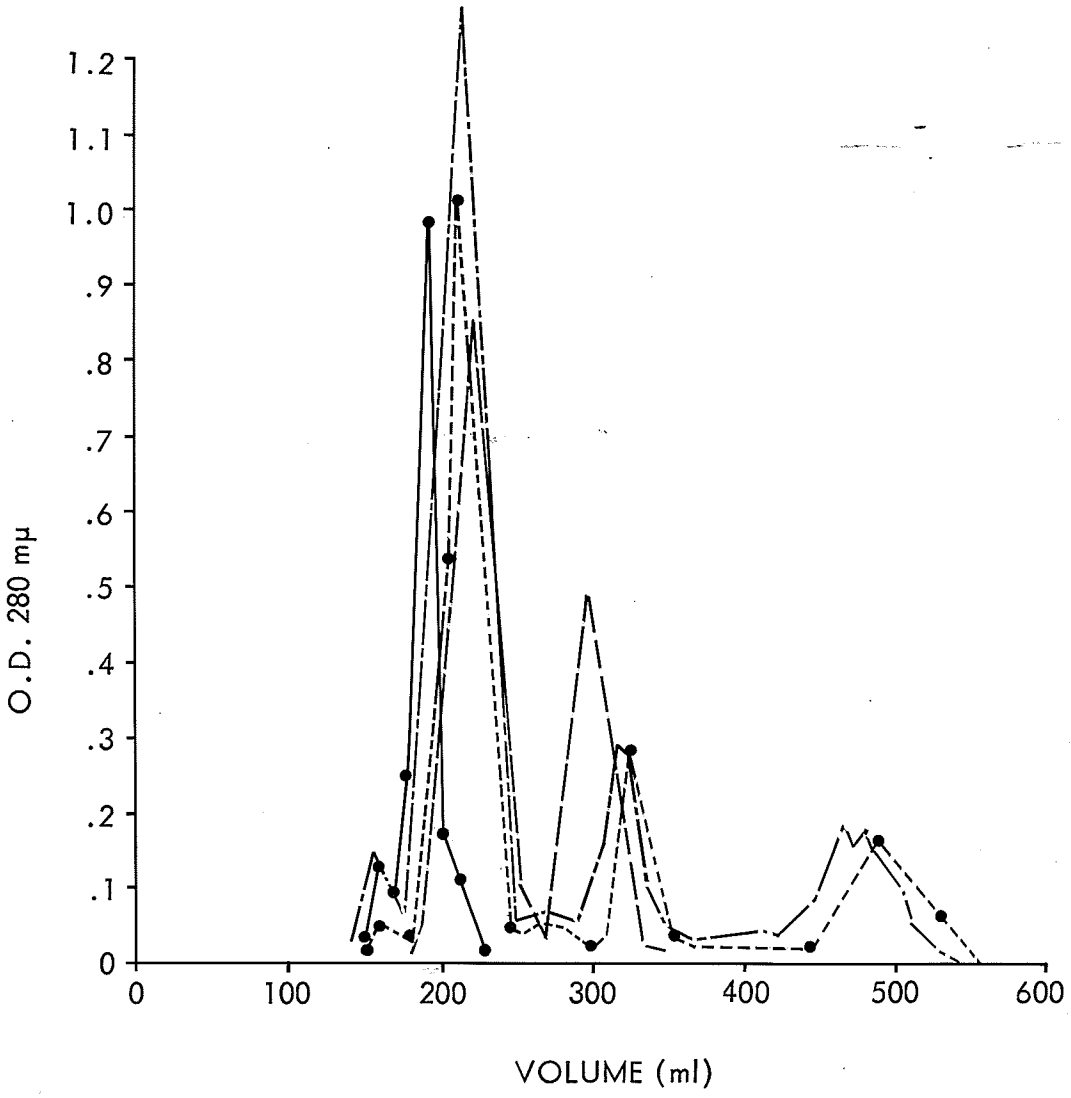

Fig. 7. Gel filtration on Sephadex G-100 column of (1) human 7S γG globulin as a marker (●——●), (2) pepsin digestion of specifically purified rabbit anti-ferritin antibody (— — —), (3) pepsin digestion of specifically purified rabbit anti-mouse γG antibody (- - - - - -), and (4) reoxidized preparation of rabbit anti-ferritin (5S) and anti-mouse γG (5S) antibodies (————).

fragments of anti-ferritin antibody were mixed with 35 mg F(ab')2 fragments of anti-γG antibody in 4 ml volume and dialysed against 1,000 ml .IM sodium acetate buffer pH 5 overnight at 4°C. 2-Aminoethanethiol Hydrochloride (2-Mercapto-ethylamine/HCl) (Matheson Coleman and Bell, Norwood, Ohio) was added to a final concentration of .015M and the mixture incubated under nitrogen at 37°C for 60 minutes. The reducing agent was then removed by passage through a column (10 x 200 mm) of cation exchange resin AG50 WX4 (mesh 100-200, BioRad Laboratories, Richmond, Calif.) packed in .IM sodium acetate buffer pH 5. The eluate from the column read at O.D 280 mu in a spectrophotometer was immediately neutralized with IN NaOH and reoxidized gently by stirring at room temperature in an atmosphere of oxygen for two hours. F(ab') fragments that had failed to dimerize were removed by chromatography on Sephadex G-100 column (fig. 7). The F(ab')2 fraction was concentrated by pressure filtration to 5 mg/ml and stored at 4°C.

XIII. Hybrid antibody purification

The 5S reoxidized material obtained from Sephadex G-100 column consisted of many possible combinations. Table V showed the different possible combinations after hybridization and their abbreviations. In order to isolate aγG-aF recombinants the following method was used: About 10 mg of 5S reoxidized material in 2 ml volume were absorbed with 20 mg BDB-aggregated ferritin. The recombinants that would be recovered after acid elution and neutralization were

TABLE V

TABLE OF ABBREVIATIONS

FRAGMENTS	RABBIT γG	ABBREVIATION
F(ab') (Univalent)	Anti-ferritin	aF
	Anti-mouse γG myeloma protein	aγG
	Normal rabbit γG	Nγ.
$F(ab')_2$ (Bivalent)	Anti-ferritin	$(aF)_2$
	Anti-mouse γG myeloma protein	$(a\gamma G)_2$
	Normal rabbit γG	$(N\gamma)_2$
$F(ab')_2$ hybrid Recombinants after reoxidation	1. Anti-mouse γG) Anti-ferritin)	aγG-aF
	2. Anti-mouse γG) Normal rabbit γG)	aγG-Nγ.
	3. Anti-mouse γG) Anti-mouse γG)	aγG-aγG
	4. Anti-ferritin) Anti-ferritin)	aF-aF
	5. Anti-ferritin) Normal rabbit γG)	aF-Nγ.
	6. Normal rabbit γG) Normal rabbit γG)	Nγ-Nγ.

a γG-aF, aF-Nγ and aF-aF. In the second step, about 5 mg of the material recovered from the first absorption was absorbed again with 10 mg BDB-aggregated mouse γG-myeloma protein. After acid elution, neutralization, the preparation was stored at 4^{o}C. The preparation contained aF-aγG hybrid molecules only. About 2 mg of hybrid antibody was isolated out of 10 mg originally used for absorption, a recovery of 20%.

The specificity of the preparation was first checked by capillary hemagglutination in which the preparation showed no hemagglutination with ferritin coated sheep red blood cells and only very weak agglutination (1 to 2 tubes dilution) with mouse γG coated sheep red blood cells. However, using a mixture of both types of sheep red blood cells (1:1 in volume) a positive reaction was obtained to a $\log_2$ dilution of 20. The most convincing results of the true hybrid nature of the preparation was revealed by Ouchterlony gel diffusion method (fig. 8) where the hybrid antibody (1 mg/ml) did not give a precipitation line against ferritin (1 mg/ml) or mouse γG (1 mg/ml) when used alone, but a strong line was obtained against a mixture of the two proteins (ferritin: γG=2.5: 1 by weight) in a concentration of 1 mg/ml.

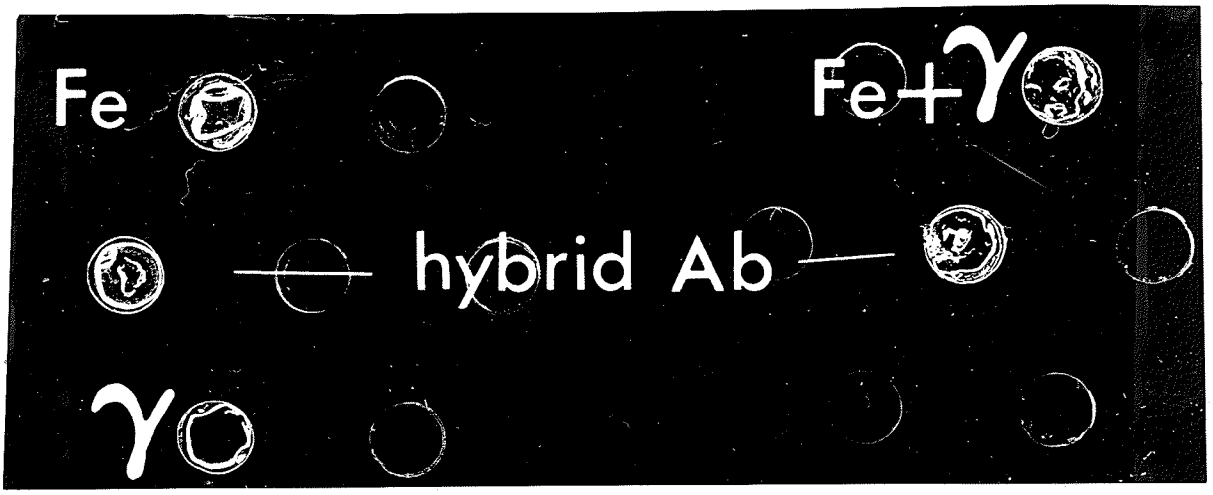

Fig. 8. Ouchterlony double gel diffusion of purified aγG-aF hybrid antibody (Hybrid Ab) against ferritin (Fe) ferritin + mouse γG myeloma protein (Fe +γ) and mouse γG myeloma protein (γ).

EXPERIMENTAL

I. Technique

Suspensions of normal spleen cells of inbred BALB/C male mice (age 12-16 weeks) were examined in all the experiments. The suspension was prepared by teasing the spleen with forceps in cold Hank's balanced salt solution (Microbiological Associates Inc., Bethesda, Maryland) after the mouse was sacrificed with ether. Once the cells were in suspension, small tissue clumps, which were unbreakable by forceps, were aspirated through a 21 gauge needle gently for 3 or 4 times to free the trapped cells. The cell suspension was then sieved through a stainless steel cloth (200 mesh/inch) to remove the tissue debris. The cells were then washed three times in the cold at a speed of 1000 rpm. The washed cells were counted in duplicate in 2% acetic acid in a hemacytometer.

To 10^6 washed spleen cells in Hank's solution, .1 mg aγG-aF hybrid antibody was added. The volume was made up to 1 ml with the same solution. The mixture was incubated for half an hour at $37^{\circ}C$. 30 cmm of 2.5% ferritin coated sheep red blood cells (approximately 100 SRBC/spleen cell) were added to the mixture which was well mixed, incubated for half an hour at $37^{\circ}C$ and then left at $4^{\circ}C$ overnight. In each experiment, a control without hybrid antibody was set up under the same experimental condition.

Bellco slides (Bellco Glass Inc., Vineland, N.J.) with chambers, 20 mm square and a depth of 10 u were used for counting the cells. The

incubated sample was mixed well by gentle flipping and introduced into the chamber by a Pasteur pepitte. The whole chamber was covered completely by a 22 mm square glass coverslip. The total number of lymphoid cells and the number of rosette forming cells were counted under phase contrast microscope (Zeiss) using a 40 objective lens. From each sample tube, a duplicate count of 1000 cells was made, in the meantime the cells forming rosette were recorded (Table VI). Typical rosettes were shown in fig. 9 a, b, c, d. A spleen cell surrounded at least by four red cells was counted as a rosette.

II. Controls of the method

(1) Spleen cell concentration

One million spleen cells/ml were chosen in the final test system because at this concentration, small clumps or aggregate of cells were much reduced or eliminated.

(2) Ferritin coated SRBC concentration

Concentration of 50 SRBC, 100 SRBC, 200 SRBC and 400 SRBC/ spleen cells were tested, while the levels of spleen cells (10^6 cells) and hybrid antibody (.1 mg/ml) were kept constant. Besides the reduction of rosette forming cells at 50 SRBC/spleen cell concentration, at all the other concentrations, the number of rosette forming cells remained more or less the same. 100 SRBC/spleen cell concentration was chosen because the counting was easier.

TABLE VI

NUMBER OF ROSETTE FORMING CELLS IN SPLEENS OF NORMAL BALB/C MALE MICE (AGE: 12-16 WEEKS)

EXPERIMENT NO.	ROSETTE FORMING CELLS/1000 SPLEEN CELLS
1	292
2	394
3	372
4	384
5	299
6	321

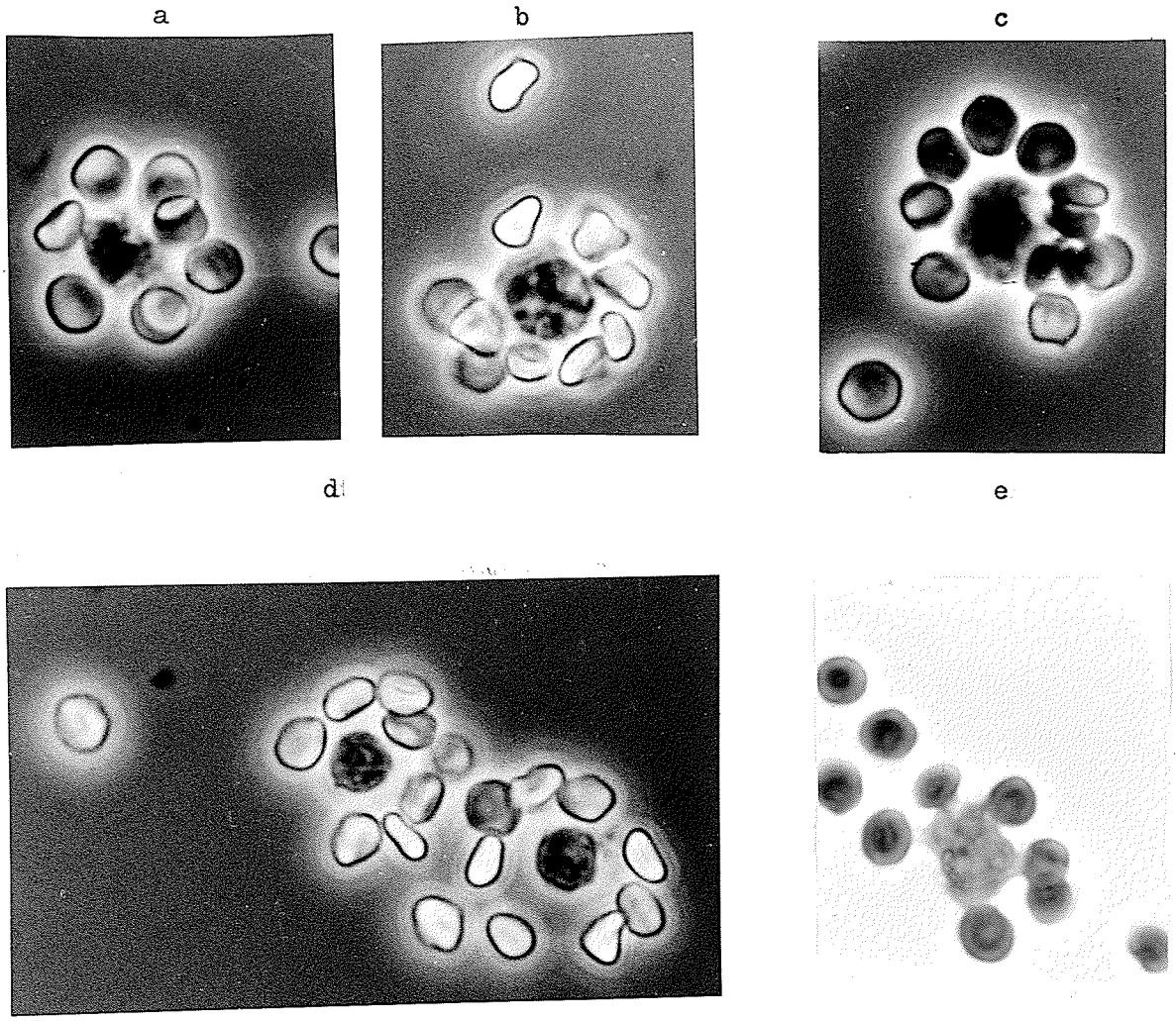

Fig. 9. a, b, c and d, rosette forming cells from normal BALB/C male mice spleen as seen under phase contrast microscope (a, b and c x 1200, d x 1000). e, Giemsa stain of rosette forming cell showing physical contact of spleen cell with the surrounding ferritin coated sheep red blood cells (x 600).

(3) Hybrid antibody concentration

Experiments with .05 mg/ml, .2 mg/ml, .5 mg/ml and .9 mg/ml hybrid antibody concentrations were tested, while the levels of ferritin coated SRBC (100 SRBC/spleen cell) and spleen cells (10^6 cells) were kept constant. Slight declination of rosette counts was found when higher concentrations (.5 mg/ml and .9 mg/ml) of hybrid antibody were used. With .05 mg/ml and .2 mg/ml hybrid antibody, the rosette counts were about the same and the maximal possible number of rosette forming cells was detected.

(4) Inhibition of rosette formation by papain F(ab') fragments of anti-mouse γG antibody

Rabbit anti-mouse γG antibody globulin was digested by papain (Worthington Biochemical Corp., Freehold, New Jersey) at a ratio of substrate to enzyme of 100:1 by weight in the presence of .01M L-cysteine, .002M EDTA, .1M phosphate buffer pH 7, for three hours at 37°C. The papain F(ab') fragments obtained from Sephadex G-100 (23 x 970 mm) gel filtration was used for inhibition test. The rabbit anti-mouse γG antibody used for papain digestion was the same preparation as that used for hybrid antibody production.

Complete inhibition of rosette formation by spleen cells was achieved when 5 mg papain F(ab') fragments were added to 10^6 spleen cells prior to the addition of hybrid antibody as described in the usual test system.

(5) Time of incubation

Rosette counts made after one or two hours incubation were always lower than that of overnight incubation at 4°C. The formation of rosette by spleen cells seemed dependent on the settling of ferritin coated SRBC to be in close contact with the spleen cells.

(6) Temperature of incubation

Two identical samples were arranged for study. The first sample was incubated at 37°C for one hour and then left at 4°C overnight as described in the usual system. The other was arranged so that the addition of hybrid antibody and ferritin coated SRBC to the spleen cell preparation was all done at 4°C and then left overnight at the same temperature without exposing the sample to any higher temperature throughout the whole process. The rosette counts in both samples were found to be the same.

DISCUSSION

Judging from the specificity of the hybrid antibody and the total inhibition of rosette forming capacity of the spleen cells by papain F(ab') fragments of anti-mouse γG antiserum, the receptors for the anti-γG antiserum as detected by the reverse rosette technique are molecules or structures of the nature of γ-globulins which are detected as antigens on the surface of the spleen cells. The anti-γG site of the hybrid antibody fixes on the surface of the cells through these Ig-like structures and in the meantime attracts a ferritin coated SRBC by its anti-ferritin site. A rosette is thus formed with a spleen cell surrounded by a single layer of SRBC. The evidence that these Ig-like structures are on the surface of the spleen cell is not only shown by the positions of the ferritin coated SRBC forming a layer on the surface of the cell, but also by the study of Giemsa stain. When the rosette preparations were stained by Giemsa, the rosette forming cell was shown to be in physical contact with the SRBC as shown in fig. 9 e.

The numbers of rosette forming cells per 1000 spleen cells (Table VI) in suspension refer only to the counts in our experiments and do not necessarily reflect numbers of cells carrying γG receptors. Since the anti-γG antiserum made to mouse γG myeloma protein contains anti-γchain antibodies as well as anti-light chain antibodies (fig. 5); the number of cells with which the hybrid antibody can react is higher than the number of cells carrying only γG receptors

and lower than the total number of cells carrying any of the other γ-globulin receptors. If one cell carries more than one class of Ig receptors the figures might approach the total number of cells potentially able to react with this technique.

This technique can be used in the study of a number of fundamental questions relating to the cellular events during the immune response, such as the ultrastructure of the rosette forming cell, the fate of the receptor during the differentiative stages from lymphocyte to plasma cell, the unipotentiality vs the pluripotentiality of the immunologically competent cells in terms of potential of γ-globulin synthesis, etc.

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