REVERSE IMMUNE CYTOADHERENCE:

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

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ABSTRACT

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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).

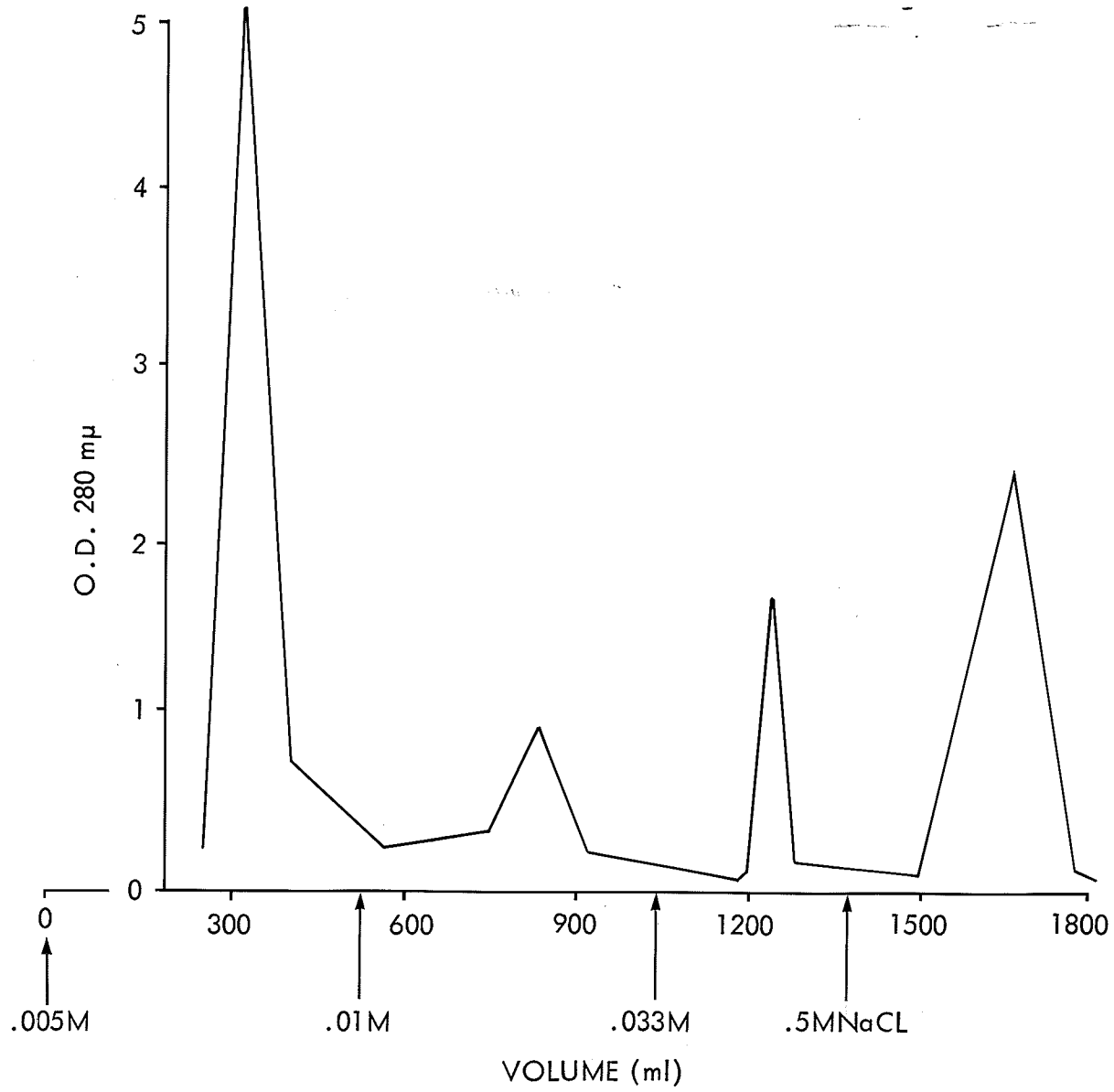

Fig. 1. DEAE-cellulose column chromatography of ascitic fluid of γG tumor bearing mouse. A stepwise elution was used by changing the molarity of phosphate buffer as indicated.

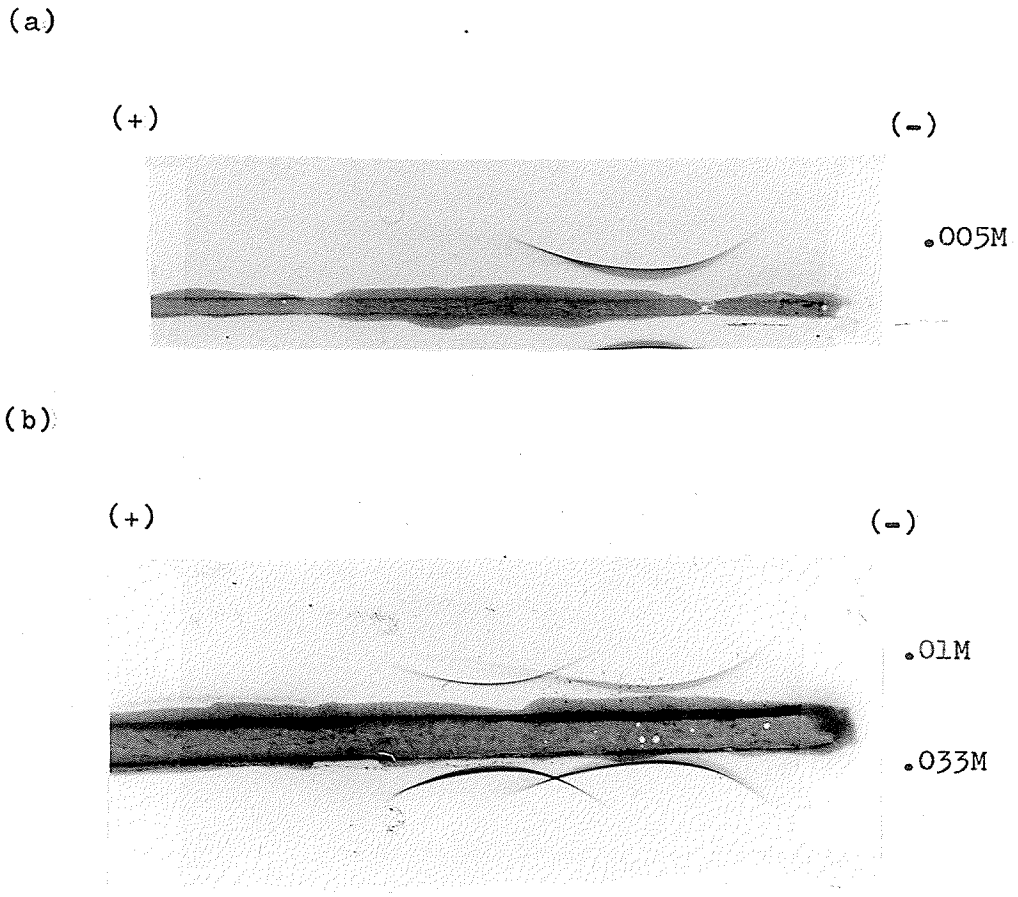

Fig. 2. a and b. Immunoelectrophoretic patterns of different fractions isolated from DEAE-cellulose column. The wells contained different fractions as indicated. The troughs contained rabbit antiserum against mouse serum.

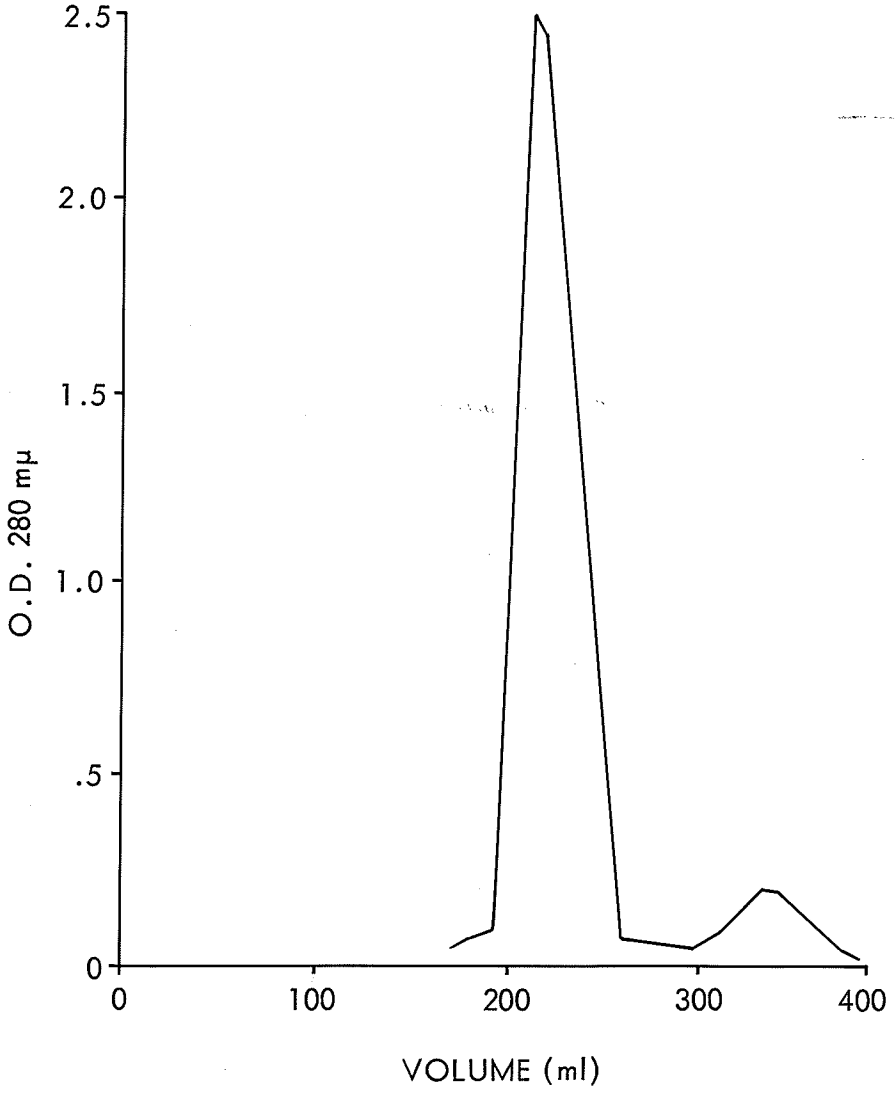

Fig. 3. Gel filtration on Sephadex G-200 column of .005M fraction from DEAE-cellulose column chromatography.