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Double Diffusion in Agarose: Precipitin Lines which are not the Result of Antigen–Antibody Reactions

E. GARDNER AND L. T. ROSENBERG

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California, U.S.A.

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Summary. Precipitin lines were observed in double diffusion tests between mouse gut tissue homogenates and serum from the same animal. Other tissues failed to produce this result. The line-forming components of the tissue are lipoidal while serum reactants are free of lipid. Purified γ-globulin, IgG, does not react with tissue constituents. An IgM component can be identified as one reactant. Lipopolysaccharides extracted from *E. coli* 014, Forssman antigen and Kidd–Friedewald antigen could not be demonstrated as reactants. It appears that certain tissue components which may be lipoproteins, and lipid free serum components, one of which is IgM, react in a way which resembles immunological interactions but which are believed not to be so.

INTRODUCTION

We report the observation that tissues of the gut from mice react with the serum of the same mice to give precipitin lines in double diffusion tests. For reasons described below we believe these reactions are not immunological in nature. Despite the fact that the specific chemical basis for these reactions is as yet unknown, they may occur in circumstances which can confuse the interpretation of certain types of immunological experiments, particularly those concerned with the study of autoimmune phenomena.

MATERIALS AND METHODS

Tissues of the gut and other organs were obtained from two inbred strains of mice designated B10.D2 new line and B10.D2 old line maintained by this laboratory (Tachibana and Rosenberg, 1964).

Etherized animals were perfused with about 40 ml of 0·15 N NaCl which suffices to bleach the liver and kidneys. Organs were removed and washed with cold Tris buffered saline (TBS) and then homogenized in the cold in a tissue grinder equipped with a motor driven Teflon pestle. A minimal amount of the TBS was added to facilitate the homogenization. These preparations were stored in the freezer at about −8°.

Tris buffered saline solution was 0·14 M in NaCl and 0·01 M in Tris brought to a pH of 7·5 by the addition of HCl. The final solution was made 5×10^{-3} M in Mg^{++} and $1{\cdot}5\times10^{-3}$ M in Ca^{++} and 0·1 per cent in gelatin.

Gel diffusion studies were carried out in a 1 per cent washed agarose–barbiturate buffer medium (agarose is a product of Maine Colloid Inc.). The barbiturate buffer, pH 8·6, ionic strength = 0·075, was composed of diethyl barbituric acid and sodium diethyl barbiturate. Immunoelectrophoretic (IEP) studies were carried out on microscope slides using the same medium at 10–15 V/cm for 90 minutes in the cold. The supporting electrolyte was also barbiturate buffer. The lines were allowed to develop at room temperature and the results recorded after 24 hours.

The γ-globulin fraction was isolated from mouse serum on a DEAE-cellulose column (Campbell, Garvey, Cremer and Sussdorf, 1963) with 0·01 M phosphate buffer containing 0·01 M-NaCl, as the eluting medium. The eluted material was concentrated and precipitated with half-saturated ammonium sulphate. The material was removed from the ammonium sulphate, redissolved in TBS, and IEP studies with a polyvalent rabbit anti-mouse serum revealed the single characteristic γ-globulin line.

The macroglobulin fraction of mouse serum was isolated on a Sephadex G-200 column using the same elution medium described above. The material contained in the first peak was collected, concentrated and passed through the column a second time. IEP studies of a concentrate of this eluant produces two or three lines with a rabbit anti-mouse serum. One of these was the IgM line.

Both IgM and IgG solutions were made 0·1 M in 2-mercaptoethanol (ME) to produce the normally expected structural changes.

The small amount of Forssman antibody present was absorbed from normal mouse serum by repeated (three times) treatment with an equal volume of sheep red blood cells. The supernatant serum from the last centrifugation, somewhat diluted, was used in the tests described below.

Centrifugation to separate lipoproteins was carried out by layering serum or tissue homogenates on a 5·2 M NaBr solution and spinning at 30,000 rev/min for 18 hours at 4° in an International SB head (Dalmasso and Müller-Eberhard, 1966). Fractions were collected by piercing the bottom of the centrifuge tube. The lower layer was dialysed against 6 litres of TBS in the cold for 60 hours. The dialysate was then concentrated by pervaporation in equilibrium with TBS solution. This was labelled the protein (P) fraction. The lighter components were labelled the lipoprotein (L) fractions. These L fractions were subsequently treated with desoxycholic acid in TBS buffer to solubilize them prior to their use in gel diffusion tests.

Lipopolysaccharide was extracted from *E. coli* 014 by the method of Westphal, Lüderitz and Bister (1952). A 1 mg/ml solution of this preparation in normal saline was used in the tests described here. An antiserum against *E. coli* 014 was raised in mice and this serum contained precipitating antibodies against the lipopolysaccharide preparation.

RESULTS

We have observed the formation of precipitation lines in agar gel diffusion plates. These lines arise as a result of a reaction between the gut tissues of a 'normal' mouse and its own serum. Similar responses were found in reactions between these same tissues and the serum of the guinea-pig, sheep, rabbit, goat and man.

The basic observation (see Fig. 1) was made when a series of whole tissue homogenates placed in adjacent wells of a double diffusion plate were permitted to react with whole serum from the same animal. Tissues of the small intestine, large intestine, and stomach

of the mouse produced two or three lines. Homogenates of liver, kidney, spleen, lung and brain tissue produced none.

In an effort to determine the origin of these reactions the tissue homogenates and whole serum were subjected to centrifugation in a high density medium. The fractions which appeared were isolated and separately tested by the gel diffusion and immunoelectrophoretic methods.

The lighter (L) layers produced precipitation lines with either whole serum or with centrifuged lipid free serum in gel diffusion tests. The heavier (P) fractions did not. These experiments indicate that the lipoprotein components associated with IgM are not involved.

The application of the electrophoretic gel precipitation method provided additional information.

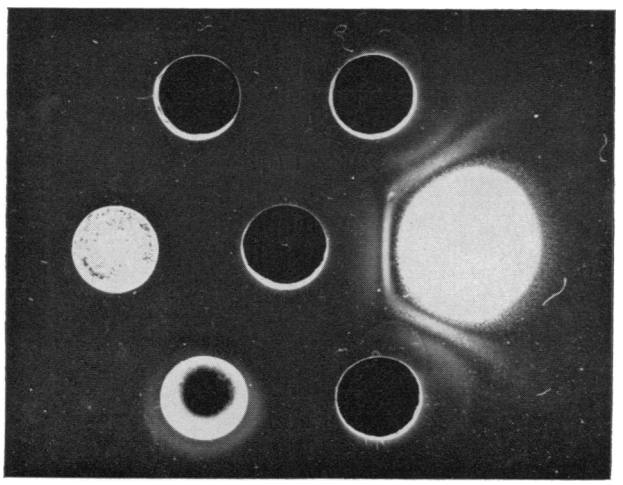

FIG. 1. Agar gel diffusion reaction between gut tissue homogenate of a B10.D2 old line mouse (right), the serum of the same mouse (centre), the serum of another B10.D2 old line mouse (above right) and the serum of another B10.D2 new line mouse (below right). The other wells contain materials which do not contribute any information.

(1) Upon electrophoresis, whole serum produced lines in reaction with the tissue homogenates and the lipoprotein (L) fractions. These lines appeared in the regions designated as α-globulin, and β- to γ-globulin.

(2) When the whole tissue homogenates were subjected to electrophoresis and permitted to react with whole serum, multiple lines were again produced which indicate that the reactants migrate slightly toward the anode at the pH used. The L fractions of homogenate produced similar lines of precipitation after electrophoresis.

Since one of the lines of precipitation formed when serum is fractionated electrophoretically occurred in a region where γ-globulin is found, we studied purified γ-globulin as well as a fraction rich in macroglobulin. The results of these experiments are shown in Fig. 2. The wells labelled (a–f) contain: (a) IgG, (b) mercaptoethanol treated IgG (ME-IgG), (c) mercaptoethanol treated macroglobulins (ME-GM), (d) macroglobulins (GM), (e) normal mouse serum, and (f) tissue homogenate. The centre well contains a polyvalent rabbit anti-mouse serum. As can be seen, purified γ-globulin failed to produce

a line in a double diffusion reaction with whole tissue homogenate. The tissue homogenate and mouse serum reacted to produce the expected precipitin lines. The antiserum produced, as expected, lines with serum, IgG, mercaptoethanol treated IgG, and macroglobulins. The lines seen between the centre well and the tissue homogenate (f), were unchanged when normal rabbit serum was substituted for the antiserum. Of particular interest are the two sets of lines of identity. The lines of identity between the well containing the tissue homogenate and the wells containing the serum and antiserum represent one set. The serum fraction containing IgM isolated by column chromatography has three components which are recognized by the antiserum. One of these components forms a line of identity with a line formed between the antiserum and the IgG fraction, whole serum, and tissue homogenate. The necessary condition for this to occur is, of course, a common reactant. We suggest that this common factor is light chain in the serum constituents and a substance related to light chain in the tissue constituents. No precipitin line is formed near the well containing 2-mercaptoethanol treated macroglobulin. This same result was

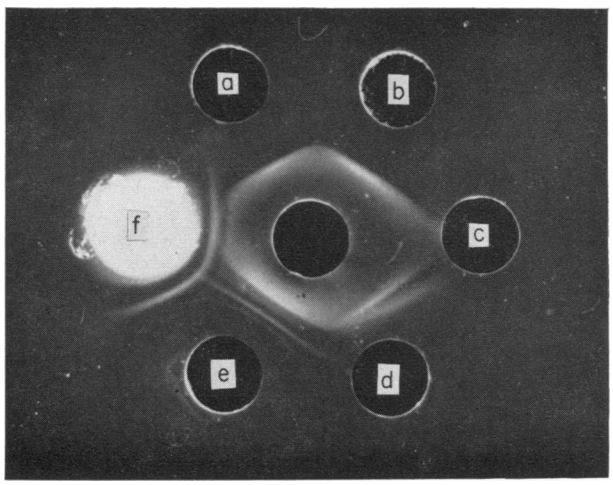

FIG. 2. Rabbit anti-mouse serum (centre) with: (a) IgG, (b) ME-IgG, (c) ME-GM, (d) GM-, (e) mouse serum, and (f) tissue homogenate. Note that of the three lines between antiserum in the centre and tissue homogenate (f), two are also found between it and normal serum (e). ME designates material treated with 2-mercaptoethanol, GM designates macroglobulin serum fraction.

obtained when the treated macroglobulins underwent electrophoresis and were allowed to react with an antiserum. Presumably the antigenic determinants recognized by our antiserum were eliminated by the mercaptoethanol treatment. This is a surprising result.

We conclude that one of the lines formed between serum and tissue extract includes as a reactant, IgM. We believe that the IgM is not reacting as an antibody, that is, by its antibody combining site, but by some other portion of the molecule. We have not as yet tested this belief.

We considered the possibility that the tissues of the gut contain lipopolysaccharide (LPS) antigens capable of cross-reacting with serum antibodies produced in response to enteric bacteria found in the gut. Antisera directed against *E. coli* 014 are known to cross-react with many coliforms as well as with other Enterobacteriaceae (Kunin and Beard,

1963). Gut tissue homogenate was tested against normal serum, crude *E. coli* 014 lipopolysaccharide extract, and mouse anti-014 serum. Multiple lines of precipitation, as before, were produced between the normal serum and the intestinal tissue. The 014 LPS antigen extract produced a weak line with the anti-014 serum. The 014 LPS antigen produced no evidence of interaction with the precipitin lines formed between tissue and serum. Thus there was no deflection of the line of precipitation formed between serum and tissue extract and this line continued up to the well containing 014 antigen.

A well-known heterogenetic antigen, the Forssman antigen, is found associated with tissue particles (Furth and Kabat, 1940). The tissue distribution of Kidd–Friedewald antigen (Kidd and Friedewald, 1942) parallels the occurrence of Forssman antigen in species where they occur (Dumonde, 1966). Since the antigen is also found on the surface of sheep red cells (Forssman, 1911), it is to be expected that anti-Forssman antibodies would be removed from serum by absorption with sheep red cells. However, mouse serum treated in this way still produced lines of identity with unabsorbed serum as well as with serum from other animals in precipitin tests with tissue homogenate. Forssman antibodies are thus not considered to be involved in these reactions; and in the absence of any evidence indicating antibody specificity, we draw a similar conclusion with respect to Kidd–Friedewald antibodies.

DISCUSSION

The reactions reported here seem to us to be important in two respects. They may be the result of interactions of serum and tissue components whose physiological significance is as yet not understood. Since not all tissues tested give these lines the interactions may be peculiar to gut function. Secondly, studies of autoimmune phenomena often rely upon the formation of precipitin lines grossly indistinguishable from those we have described here. We would urge the application of stringent criteria before a reaction is characterized as the result of antigen–antibody interactions.

There appear to be few immunological precedents to explain our observations. There are several examples of lines formed in double diffusion tests which are not based on antigen–antibody interaction. Peetom, Rose, Ruddy, Michell and Grabar (1960) reported on a 'non-specific' precipitin line formed in a reaction between red cell haemolysate and serum from the same subject. They concluded that antibodies were not involved but that haptoglobin was a possible reactant. Subsequent examination of this reaction led to the finding that the serum reactive component is albumin (Wilson and Warren, 1962) and that the red cell lysate reactant is methaemoglobin (Kunzel and Bundschuh, 1968). Leonard and Thorne (1961) observed the formation of precipitin lines in a reaction between certain serum proteins and γ-glutamyl polypeptides. The lines were sensitive to salt concentration and required a pH of 7 or less. Other evidence implicated serum lysozyme as one of the precipitants. Concanavalin A and a large number of polysaccharides (Goldstein and Lucy, 1965) and serum glycoproteins (Leon, 1967) form precipitin lines in agar gel diffusion tests. The structural basis of specificity in this instance is known. In recently published experiments (Sjöquist, Forsgren, Gustafson and Stalenheim, 1967) a protein obtained from the cell wall of *Staphylococcus aureus* (Protein A) was found to precipitate human and guinea-pig γ-globulins *in vitro*. The precipitin lines observed in agar diffusion tests resemble antigen–antibody reactions but in this case the combining site has been located in the Fc region. Since the antigen combining site is in the Fab region of the

antibody molecule, this reaction is, in the immunological sense, another example of the class of phenomena reported here. We are unaware of other similar reports. We consider that the lines of precipitation which were observed in these experiments originate in undefined chemical interactions.

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