Intrawell Absorption of Antisera in a Template Thin-Film Gel Diffusion Method

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This report describes a method for in situ absorption of antisera within the template wells used in thin-film microgel diffusion. The intrawell absorption of antisera offers several advantages over previously reported techniques for detecting the presence of given antigens in biological preparations. The procedure is time saving, antigens are conserved, optimum antigen-antibody ratios are easily determined, and tests of several different absorbing antigens can be made on a single plate.

Antigen-antibody precipitation patterns obtained by double diffusion are frequently complex when the antigens are derived from biological materials. Precipitin bands may represent antigens from the components of the environment from which the product was derived. To obtain precipitin patterns for easier evaluation, the antiserum should be absorbed with the contaminant antigens. In the Björklund procedures (3, 4) using Ouchterlony plates (6), this absorption was accomplished by placing the absorbing or inhibiting antigen in the agar gel either by pouring the plate with agar solution mixed with the antigen or by diffusing the antigen into the gel by placing it in the wells before applying the reactants.

In this laboratory, the use of the template diffusion procedure of Wadsworth (7), as modified by Auernheimer and Atchley (2), suggested a new absorption technique which was designated as "intragel absorption." In this procedure, the template wells were considered as micro test tubes in which the serum is absorbed in situ. This report demonstrates the application of the intrawell absorption technique, using streptococcal and amoebic antigens.

MATERIALS AND METHODS

Apparatus. "Gel matrix forms" were obtained by cutting microscope slides into uniform 2.5- by 3.8-cm pieces. Single layers of vinyl plastic electrical tape, approximately 1.5-mm wide and 0.25-mm thick, were placed across the ends of each piece. Templates were 2.5- by 3.2-cm pieces cut from a 3.2-mm-thick acrylic plastic sheet. Holes (3.2 mm), 4 mm apart, were drilled in patterns suitable for gel diffusion tests. Capillary tubes, 76 mm long and 76 µl in capacity (Drummond Scientific Company, Broomall, Pa.) were used with Drummond Microcap bulb assemblies for convenient application of the solution to the wells. Drummond Ziprol applicators with Ziprol tubes were used for quantitative applications of 1 to 5 µl of solution. A repeating dispenser (Hamilton Company, Reno, Nev.) was used with a micro syringe for repeated applications of the same solution to a series of wells. Slide racks and diffusion chambers of convenient designs have been reported (2).

Reagents. The antigens used for absorption were sufficiently concentrated to permit use of relatively small volumes, such as 1 to 10 µl in a 25-µl well, thus allowing for 15 to 24 uliters of antiserum. Sufficient density of absorbing solution, over that of antiserum, was obtained in this study by adding a saturated solution of ammonium sulfate to make up 10 to 20% of the volume. The chemical properties of the antigen determine the choice of substances which can be used to increase the density. Group B type Ia streptococcal antiserum was produced in rabbits by intravenous inoculations of heat-killed and pepsin-treated cells, which were grown in Todd-Hewitt broth. Groups A and B polysaccharide antigens were obtained by the hot acid extraction of cells, using the Lancefield method (5). A human serum with a relatively high antibody titer to group A carbohydrate was used for gel diffusion tests. Entamoeba histolytica organisms and associated bacteria were grown on Cleveland-Collier medium plus rice starch and horse serum. Washed amoeba sediments were inoculated into rabbits subcutaneously. These sera contained amoebic antibodies and, in addition, antibodies to horse serum and bacteria, due to the difficulty of completely freeing the inoculum from such materials. The preparation of amoebic and associated bacterial antigens for gel diffusion has been described (1).

Gel diffusion procedure. For greater convenience, the method of forming the gel matrix has been modified from that previously reported (2). A gel
matrix form, tape side down, was placed on the center of a 2.5- by 7.6-cm micro slide that had been previously coated with agar by being dipped in hot 0.1% (wt/vol) agar solution and then dried in an upright position. The capillary space between the gel matrix form and the slide was filled with a hot solution of buffered agarose (1%, wt/vol, in phosphate-buffered saline [pH 7.5]).

After gelation, the gel matrix form was removed by gently sliding it off to one side, and a template was carefully slid onto the gel layer adhering to the slide. Density-adjusted absorbing antigen was applied in precisely measured amounts to the antiserum wells by means of the Ziptrol device. The desired amount of solution was drawn into the capillary tube and expelled while the tip of the tube was centered within the well, close to the bottom. Care was taken that the injected sample spread evenly over the bottom. The well was then carefully filled with antiserum by means of a capillary tube fitted with a bulb assembly. After a diffusion period of 1 to 3 days, the slides were eluted with three changes of normal saline during a 6-h period, treated with 1% (wt/vol) tannic acid solution to enhance precipitin band intensity, and photographed on high-contrast paper using dark-field illumination.

RESULTS

Three applications of the intrawell absorption technique are presented. Group B streptococci possess both common and type-specific polysaccharides. Precipitin bands for both kinds of polysaccharide are shown in Fig. 1. Figure 2 shows only a type-specific carbohydrate band after intrawell absorption of the type Ia antiserum with the common carbohydrate.

FIG. 1. Center well: rabbit antiserum to type Ia cells. Wells 1, 2, and 3: carbohydrates of group B types Ia, II, and III, respectively, diluted 1:100.

FIG. 2. Center well: lower layer, type II carbohydrate, undiluted, plus 25% saturated (NH₄)₂SO₄, 5 μlitters; upper layer, rabbit antiserum to type Ia cells, 20 μlitters. Wells 1, 2, and 3: same as in Fig. 1.

FIG. 3. Center well: group A carbohydrate, 4.8 μg/ml. Well 1: human serum possessing Ab to group A carbohydrate, unabsorbed. Wells 2, 3, and 4: lower layers, 10% N-acetylglucosamine, N-acetylgalactosamine, or L-rhamnose, respectively, plus 25% saturated (NH₄)₂SO₄, 5 μlitters; upper layers, same human serum as in well 1, 20 μlitters each well.

Group A streptococcal polysaccharide contains N-acetylglucosamine as the antigenic determinant (Fig. 3). Nearly complete inhibition of the polysaccharide antibody was obtained with N-acetylglucosamine by intrawell absorption, but not with N-acetylgalactosamine or L-rhamnose.
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A rabbit antiserum to a crude amoebic inoculum produced several antibodies not only to *E. histolytica* but to horse serum and to associated bacteria as well. Figure 4 shows the gel diffusion pattern obtained using these reagents. An attempt to prevent appearance of nonamoebic precipitin bands by placing horse serum in the gel resulted in a partial removal of such antibodies but also produced a heavy band about the serum well (Fig. 5). The use of the intrawell absorption technique (Fig. 6) resulted in the removal of precipitins to contaminant antigens without disturbance of the pattern.

**DISCUSSION**

The intrawell absorption of sera with antigen as described above has a number of advantages over the Björklund (3, 4) procedures. These advantages are as follows. (i) It is simpler to place the inhibiting antigen in a well than to prepare an agar and antigen mixture or to diffuse large quantities of the antigen into the gel. (ii) Considerable time is saved, especially over that of the second procedure of Björklund which requires 3 to 5 days to prediffuse the antigen into the agar (3, 4). (iii) Antigen preparations are conserved by this intrawell technique. (iv) Several ratios of antigen to antiserum may be applied to one slide to determine the optimum proportions for complete inhibition. These data can then be extrapolated to determine the amount of antigen required to absorb a large quantity of antiserum. (v) Tests of several different absorbing antigens can be made on one plate. (vi) A method is provided for determining specific antibodies in large numbers of sera; nonspecific antibodies are inhibited or removed with a minimum expenditure in time and materials.

The intrawell absorption technique thus offers an ideal method for absorbing sera and detecting specific antigens or antibodies at a minimal cost in time and reagents.
LITERATURE CITED

1. Atchley, F. O., A. H. Auernheimer, and M. A. Wasley. 1963. Precipitate patterns in agar gel with sera from human amebiasis and Entamoeba histolytica antigen. J. Parasitol. 49:313-315.

2. Auernheimer, A. H., and F. O. Atchley. 1962. Modifications of micro agar precipitin test. Amer. J. Clin. Pathol. 38:548-553.

3. Björklund, B. 1952. Specific inhibition of precipitation as an aid in antigen analysis with gel diffusion method. Proc. Soc. Exp. Biol. Med. 79:319-324.

4. Björklund, B. 1952. Serological analysis of components in hemopoietic tissue. Proc. Soc. Exp. Biol. Med. 79:324-326.

5. Lancefield, R. C. 1928. The antigenic complex of Strep- tococcus haemolyticus. I. Demonstration of a type specific substance in extracts of S. haemolyticus. J. Exp. Med. 47:91-103.

6. Ouchterlony, Ö. 1948. In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta Pathol. Microbiol. Scand. 25:186-191.

7. Wadsworth, C. 1957. A slide microtechnique for the analysis of immune precipitates in gel. Int. Arch. Allerg. 10:555-560.