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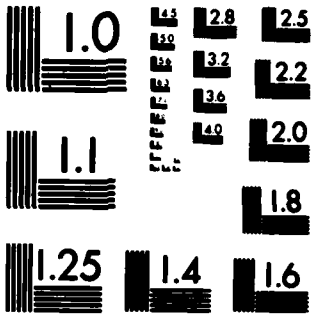

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A MODIFIED DIFFUSION IN GEL — ENZYME LINKED IMMUNOSORBENT ASSAY, (DIG-ELISA) FOR QUANTITATION OF SPECIFIC ANTIBODY

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A Modified Diffusion in Gel - Enzyme Linked Immunosorbent Assay, (DIG-ELISA) for Quantitation of Specific Antibody

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Summary

When using the Enzyme-Linked Immunosorbent Assay (ELISA) for detection and measurement of antigens and antibodies the initial step of adsorbing the antigen or antibody onto the carrier surface is critical. Attempts to produce an evenly dispersed tightly bound coating over the entire carrier surface are not always successful. Once bound to the support surface a significant amount of the antigen or antibody can be lost as a result of the several wash steps required throughout the test.

This report presents the results of one attempt to overcome these problems by immobilizing an antigen in agar to act as a carrier support for capture of specific antibody.

Adenovirus antigen was diluted in 1% Noble agar which had been cooled to 45°C. Five ml per dish of this mixture was added to 15 x 60 mm polystyrene tissue culture dishes. The agar was then allowed to evaporate to form a very thin film attached to the bottom of the tissue culture dish. Five ml of 2% Noble agar was added to the antigen containing film. After hardening, evenly spaced 3 mm wells were cut into this agar overlay and the wells filled with the test antibody. The antibody was allowed to remain in contact with the agar film for 90 minutes at room temperature to allow for the antigen-antibody complex to form. After incubation the agar overlay containing the antibody wells was removed from the dish leaving the agar film undisturbed. Goat anti-rabbit IgG labeled with the enzyme alkaline phosphatase in PBS-Tween was added to the agar film for an additional 90 minute incubation period. The goat anti-rabbit alkaline phosphatase conjugate was removed from the dish and 5 ml of substrate (p-nitrophenyl phosphate 1g/ltr), dissolved in 1% agarose was overlayed onto the agar film. After 45 minutes the colored zones of reactivity were visualized with the aid of oblique light.

In these experiments immobilization of antigen in an agar film appeared to provide a more even dispersal and to reduce loss of the antigen. The simplicity of this procedure lends itself to handling large numbers of clinical specimens for viral/bacterial identification and would be useful in disease outbreaks to rapidly identify the causative agent.

INTRODUCTION

Enzyme-labeled immunosorbent assay (ELISA), described by Engvall and Perlman (1) and by Van Weemen and Schuurs (2), has been used to identify and quantify antigens and/or antibodies. Advantages of the ELISA system are stability of reagents, reduction of potential health hazards and the need for less expensive equipment. A major limitation of the conventional solid phase ELISA is loss (leaching) of the adsorbed antigen or antibody during the numerous wash procedures (3). Covalent linking of the antigen or antibody to the solid-phase surfaces reduces the leaching (4,5), but variations in sensitized supports and the relatively high color development in the negative controls often make the ELISA results difficult to interpret.

Elwing and Nygren quantified class-specific antibodies in a modified ELISA procedure called diffusion in gel-enzyme linked immunosorbent assay (DIG-ELISA) (6). Plastic plates were coated with antigen then antibody was diffused through an agar overlay. The antigen-antibody reaction zone was detected by alkaline phosphatase (requiring a photographic technique) or by horseradish peroxidase (visible to the naked eye).

This paper reports a modification of the DIG-ELISA technique. Agar containing the antigen is placed in plastic plates and allowed to dry to a film. Anticipated benefits of immobilizing the antigen in such a solid support system were a more even antigen distribution over the plate, a decrease in antigen leaching, and detection of antigen-antibody reaction with the naked eye.

MATERIALS AND METHODS

Antigens and antibodies. Adenovirus types 4 and 7 were used as antigens in this test system. Both virus types were propagated in HEp#2 cultures maintained in serum free media. When the infected tissue cultures showed four plus cytopathology, the cells were disrupted by three freeze-thaw cycles centrifuged and the cell free supernatants were used as antigens in these experiments.

Anti-adenovirus types 4 and 7 sera were produced in New Zealand white rabbits. After heat inactivation at 56°C for 30 minutes the whole serum was used for titrations against its specific antigen.

Agar and Agarose. Noble agar (Difco Laboratories) was used for production of both the agar film and the agar overlay in which 3 mm wells were cut for the serum dilutions. The substrate containing overlay was agarose, type III, (Sigma Chemical Co.).

Polystyrene Petri dishes. Costar (#3006) 15 x 60 mm tissue culture dishes and Falcon (#1007) 15 x 60 mm non-tissue culture dishes were used.

DIG-ELISA Technique. Dilutions of the adenovirus antigens, types 4 and 7 were made in a 1% concentration of Noble agar which had been dissolved in deionized water and cooled to 45°C. Five ml aliquots of the antigen containing agar were placed in each 15 x 60 mm petri dish. After the agar had solidified at room temperature the dishes were transferred to a 37°C incubator equipped with a

fan for air circulation. After complete evaporation of moisture from the agar (approximately 30 hours) a very thin agar film was firmly attached to the surface of the dishes. The dishes were then stored at 4°C until used, (usually within 72 hours). Duplicate dishes were coated with adenovirus 4 and 7 antigens diluted in carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C.

Five ml of 2% Noble agar dissolved in deionized water were put in each dish and allowed to solidify at room temperature. Evenly spaced 3 mm wells were cut into the agar overlay. Care was taken not to cut through the antigen containing film with the agar punch. The wells were then filled to slightly overflow with 12 microliters of the antibody dilutions.

The sera were allowed to diffuse through the agar for 90 minutes at room temperature. The 2% agar overlay was then gently teased from the dishes with a thin spatula. Careful removal of the agar layer left the antigen containing agar film undisturbed. All dishes were then washed for 5 minutes at room temperature with 0.15 M NaCl. The wash fluids were removed by decanting. Three ml of a 4% suspension of bovine serum albumin (BSA) was added to each dish and allowed to stand at room temperature for 90 minutes. The BSA was removed by decanting and the dishes were washed for one 5 minute interval with 0.15 M NaCl. After draining, 5 ml of alkaline phosphatase conjugated goat anti-rabbit IgG in PBS-Tween was added. After 90 minutes at room temperature, unbound conjugate was removed by washing with .15 M NaCl. The substrate, p-nitrophenyl phosphate (1g/1tr), was contained in 1% agarose which had been dissolved in 0.05 m carbonate buffer and cooled to 45°C. Five ml of the substrate containing agarose was added to each dish. Forty-five minutes after addition of the substrate overlay the dishes were examined by holding the inverted dish over a view box with oblique light and the colored areas of the enzyme-substrate reaction were outlined with a fine point marking pen for measurement.

RESULTS

Intensity of color development from the enzyme and its substrate and serum antibody levels obtained from this modified procedure were compared to those obtained using the procedure described by Elwing and Nygren (6). As can be seen in Table 1 repeated titration of the identical antiserum gave consistently higher end points in dishes with the agar film than in those in which antigen was coated directly on to the plastic. Areas of color development in the agar film were more intense and could easily be seen with the naked eye. In both the tissue culture and non-tissue culture dishes in which the antigen was coated directly on to the plastic the areas of color development were faint and poorly defined. Attempts to produce a smooth firmly attached agar film in the non-tissue culture dishes usually resulted in formation of air bubbles in the film with subsequent detachment and loss of the entire film during the wash stages of the procedure.

TABLE I

Results of repeated titrations, (DIG-ELISA), of Anti-Adenovirus-7 rabbit serum in tissue culture dishes with and without an antigen containing agar film. Positive sign (+) indicates areas of color reaction visible with the naked eye.

Run No.		1:10	1:20	1:40	1:80	1:160	1:320	Normal Rabbit Serum 1:10
1	With agar film	+	+	+	+	+	±*	0
	Without agar film	+	+	0	0	0	0	±*
2	With agar film	+	+	+	0	0	0	0
	Without agar film	+	+	+	0	0	0	±*
3	With agar film	+	+	+	+	+	0	0
	Without agar film	+	+	0	0	0	0	0

* Faint color present

DISCUSSION

In our hands addition of an antigen containing film in the polystyrene tissue culture dish had several advantages over the bare antigen coated plastic for the capture of antibody. Immobilization of the antigen in agar appeared to provide a more even dispersal and to lessen the loss of antigen.

Use of the tissue culture dish proved far superior to the non-tissue culture dish for production of the antigen containing agar film. In most instances even after treatment of the non-tissue culture dishes with 95% ethanol or detergents, the agar film contained numerous air bubbles and was not firmly attached to the plastic surface. As a result the agar film was usually lost upon removal of the agar overlay containing the serum wells or during the washing processes.

Attempts to reduce the concentration of agar from 1% to 0.5% for preparation of the antigen containing film were not satisfactory. During the evaporation process the 0.5% agar overlay tended to retract toward the center of the dish causing detachment from the walls of the dish and leaving an uneven film with areas of the dish surface bare of agar.

In our laboratory we are currently investigating the feasibility of producing an antibody containing agar film in tissue culture dishes which would serve as specific antigen capture. Antigen could then be identified by the ELISA technique. This procedure would be useful in disease out-

breaks to rapidly identify the causative agent. Its simplicity lends itself to handling large numbers of clinical specimens for bacterial/viral identification.

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Diffusion in Gel-Enzyme Linked Immunosorbent Assay
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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

A method was developed to quantify specific antibody by immobilizing an antigen in an agar film.
In our model system, adenovirus types 4 and7 in 1% agar was allowed to dry to a thin film in 15 x 60 mm plastic tissue culture dishes. This antigen containing "film" was used to "capture" specific antibody. Specific antiserum was allowed to diffuse from wells in a gel layered over the antigen containing agar film. After the antibody had diffused, the agar layer was removed and alkaline phosphate conjugated anti rabbit antiserum was layered over the plate. After

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incubation, this was removed and the plate was layered with a substrate containing agarose. Within 45 minutes, zones of reactivity were visualized with the aid of oblique light. The method proved to be sensitive and end points could be accurately identified with the naked eye.