Localization of Virus Antigens by Enzyme-labelled Antibodies

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SUMMARY

Three enzymes—horseradish peroxidase, alkaline phosphatase, and glucose oxidase—were used as markers in antibody conjugates employed for the detection in the light microscope of either or both T antigens and structural antigens of three viruses: SV40, adenovirus 12, and rat K virus, in infected cell cultures. The specificity of reaction and localization of the antigens was the same with each enzyme and identical with those revealed by fluorochrome-conjugates. By using enzymes with reaction products of different colours, two antigens were revealed simultaneously in a single preparation.

INTRODUCTION

Antibodies labelled with fluorescent compounds have been widely used for the detection of antigens (Coons, Leduc & Conolly, 1955). Recently, the use of enzymes as markers was introduced (Avrameas & Uriel, 1966; Nakane & Pierce, 1966). Enzymes are selected that produce coloured products in cytochemical reactions, which are visible by optical microscopy. In the present paper we have employed several enzyme-labelled antibodies to detect virus antigens and have compared the results with those obtained with fluorescein labelling.

METHODS

Virus antigens. This technique was applied to three viruses under study in our laboratory: SV40 (Granboulan et al. 1963), adenovirus 12 (Cassingena & Tournier, 1965) and rat K virus (Vasquez & Brailovsky, 1965). The first two are oncogenic for newborn hamsters (Eddy et al. 1962; Trentin et al. 1962), and in infected cells they induce new nuclear antigens (T antigens) distinct from structural antigens. These T antigens are detectable by immunofluorescence (Pope & Rowe, 1964a, b; Rapp et al. 1964).

Cell cultures. Several different types of cell were used in this study. These included continuous cell lines maintained by serial subculture, MA104 cells (rhesus) (Microbiological Associates Inc., Bethesda, Md., U.S.A.), KB cells (human) (Eagle, 1955), locally developed sublines of rat or hamster cells transformed by SV40 and also secondary cultures of kidney cells from the rat, hamster and man. The cells were grown in Leighton tubes in media described elsewhere (Tournier et al. 1967).

Infection time. The cells were infected 24 hr after subculture by viruses inoculated at infection multiplicities between 1 and 10. The times after infection at which the cells were fixed varied with the virus and the antigen under study. Thus, for SV40, T antigen was observed after 24 hr and structural antigen 48 hr after infection. For adenovirus 12 these times were, respectively, 18 and 36 hr after infection.
Fixation. Before fixation, the coverslips were washed three times with phosphate buffered saline (PBS). Of the fixatives used routinely with cell smears or imprints, acetone was best in conserving cell structure adequately and retaining the antigen + antibody reaction and enzyme activity of the label. Three fixatives employed in cytochemistry and electron microscopy were also studied: 10% formalin in PBS, 2% paraformaldehyde in phosphate buffer, pH 7.3, and 2.5% glutaraldehyde in phosphate buffer, pH 7.3. Paraformaldehyde (20 min. at 4°) was the best of these. Cell structure was well conserved and a positive reaction was always obtained, but after this fixation the cells readily became detached from the coverslip and special precautions were necessary to avoid losing them.

Production of antisera. The sera were prepared as follows: first, for antibodies against the structural antigens of SV40 and adenovirus 12, rabbits were inoculated intradermally with a mixture of equal volumes of crude virus suspensions and complete Freund's adjuvant. After 6 weeks of rest, the animals received weekly intravenous injections of the viruses without the Freund's adjuvant for 5 to 7 weeks. The sera were then taken and absorbed with an insolubilized mixture of calf serum, homogenized uninfected cells and culture medium (Avrameas & Ternynck, 1969). Secondly, for antibodies against K virus, Syrian hamsters were immunized in the same way with a crude suspension of the virus grown in hamster embryonic cells. Thirdly, the T antigens of SV40 and adenovirus 12 were localized with sera of hamsters that were hosts to transplanted tumours induced by these viruses. The hamster sera were not pretreated with insolubilized cells.

Sheep antibodies prepared against rabbit immunoglobulins and rabbit antibodies prepared against hamster immunoglobulins were specifically isolated by passage of the corresponding whole antisera on immuno-adsorbents prepared from rabbit or hamster gamma-globulin fractions. Preparations of the immuno-absorbent, using either ethylchloroformate or glutaraldehyde as an insolubilization agent, and isolation of antibodies were done using procedures described in detail elsewhere (Avrameas & Ternynck, 1969).

Preparation of conjugates. The coupling of the rabbit antibody anti-hamster immunoglobulin with peroxidase is given as an example. Twelve mg. of peroxidase (RZ3; C. F. Boehringer, Mannheim, France) were dissolved in 1 ml. of 0.1M phosphate buffer pH 6.8, containing 5 mg. of antibody. While the solution was gently stirred, 0.05 ml. of 1% aqueous solution of glutaraldehyde was added drop by drop. The reaction mixture was allowed to stand for 2 hr at room temperature, then dialysed overnight against PBS at 4°. This stock solution of peroxidase-labelled antibody was stored at 4° and diluted 1/10 before use. Glucose oxidase (90 units/mg.; C. F. Boehringer, Mannheim, France) and alkaline phosphatase (BAPSF 26 units/mg.; Worthington Biochemical Corporation, U.S.A.) were coupled with the antibody by the same general method using conditions and amounts of reagents described elsewhere (Avrameas, 1969).

Reaction of antigen and antibody. The reaction for localizing antigens was done by the indirect method of Coons, Leduc & Conolly (1955). First, the fixed cells on coverslips were exposed to an antiserum against the virus antigen to be detected. They were then washed in several changes of PBS for 10 min. and treated with a second antigen conjugated with an enzyme. The latter serum was directed against the immunoglobulin of the animal species which furnished the first serum. Thus, for example, T antigen of SV40 in the cells was first coupled with unlabelled hamster antisera against SV40
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T antigen, then the latter was coupled with labelled rabbit antibody anti-hamster immunoglobulin. A detailed list of the antigens sought, the virus + cell systems employed, the two antibody systems used which permitted detection of the antigens and the enzymes used as labels is presented in Table 1. The time and temperature of incubation for each serum could be varied widely. For the first serum a standard was adopted of 1 hr at 37°, but 15 hr at 4° gave comparable results. The second, conjugated serum, needed more time and 2 to 3 hr at 37° or 15 hr at room temperature was used. After incubation in the conjugated serum, the cells were again washed in several changes of PBS.

Table 1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Virus + cell system</th>
<th>First antiserum</th>
<th>Enzyme label</th>
<th>Conjugated antibodies</th>
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<tbody>
<tr>
<td>Structural antigens of</td>
<td>Lytic cycle of SV40 in MA104 Rhesus monkey cells</td>
<td>Rabbit anti-SV40</td>
<td>Peroxidase</td>
<td>Sheep anti-rabbit immunoglobulins</td>
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<tr>
<td>SV40 and adenovirus 12</td>
<td>Lytic cycle of adenovirus 12 in (1) KB cells and (2) human renal cells</td>
<td>Rabbit anti-adenovirus 12</td>
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<td></td>
<td>Lytic cycle of SV40 virus in MA104 Rhesus monkey cells</td>
<td>Rabbit anti-SV40</td>
<td>Alkaline phosphatase</td>
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<td></td>
<td>Lytic cycle of adenovirus 12 in KB cells</td>
<td>Rabbit anti-adenovirus 12</td>
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<td></td>
<td>Lytic cycle of SV40 virus in MA104 Rhesus monkey cells</td>
<td>Rabbit anti-SV40</td>
<td>Glucose oxidase</td>
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<td>T antigens of SV40 and</td>
<td>Lytic cycle of SV40 virus in MA104 Rhesus monkey cells</td>
<td>Hamster anti-SV40 virus-</td>
<td>Peroxidase</td>
<td>Rabbit anti-hamster</td>
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<td>adenovirus 12</td>
<td>Hamster cells transformed by SV40 virus</td>
<td>induced tumour</td>
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<td>immunoglobulins</td>
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<td>Rat cells transformed by SV40 virus</td>
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<td>Lytic cycle of adenovirus 12 in human renal cells</td>
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<td>Abortive cycle of adenovirus 12 in hamster embryonic cells</td>
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<td>Structural antigens of</td>
<td>Lytic cycle of K virus in rat embryonic cells</td>
<td>Hamster anti-K virus</td>
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<td>K virus</td>
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The reproducibility of results with each antigen was controlled by at least three experiments, and each of these was compared with a parallel immunofluorescent staining procedure. In all experiments two controls were employed, namely uninfected cells treated with positive serum and the conjugate, and infected cells treated with normal serum or a serum directed against a different virus antigen followed by the conjugate.

Staining procedure. The detection of the immuno-complex was achieved by incubating the cells in the presence of a substrate and capture reagent appropriate to the enzyme marker so that a coloured reaction product visible by light microscopy was precipitated at the site of the antigen under study. Three enzymes were used as markers in this study—horseradish peroxidase, alkaline phosphatase and glucose oxidase—
whose activities are revealed by classical histochemical reactions (Graham & Karnovsky, 1966; Burstone, 1962; Pearse, 1960). Peroxidase activity was revealed by a brown deposit after incubation with 3,3'-diamino-benzidine in the presence of hydrogen peroxide. Alkaline phosphatase activity was revealed, in this study, by incubation with naphthol AS-MX phosphate plus a diazonium salt, Fast Garnet, giving a red stain. For glucose oxidase activity, a blue compound was formed by incubation with D-glucose, phenazine methosulphate and thiazolyl blue (MTT). In general, 5 min. incubation at room temperature in freshly prepared substrate mixtures sufficed. The reaction could be stopped by transferring the coverslips into distilled water, the cells examined, and the reaction reinitiated, if necessary, to produce a heavier reaction product. The coverslips were mounted on slides in buffered glycerine or examined directly in distilled water. The preparations utilizing peroxidase and alkaline phosphatase could be kept for several days.

RESULTS

Localization of antigens

The comparison of these procedures using enzymes as markers with the immunofluorescence technique showed that the specificity of the reactions, localization of the antigens and morphology of the cells were remarkably similar. The localization of structural antigens of SV40 in the nuclei of infected MA104 cells is illustrated in Pl. 1a, b, d. They differ from one another only in the nature of the marker. Plate 1a represents the classical picture obtained by immunofluorescence. The same localization is revealed by peroxidase (Pl. 1b) and alkaline phosphatase (Pl. 1d). In all three some nuclei were strongly positive and others completely negative, because even with high multiplicities of infection virus multiplication is not synchronous. Plate 1c is a peroxidase control in which infected cells were treated with normal rabbit serum. Certain nuclei exhibit some background staining, a phenomenon which always occurs in infected cells treated with any normal rabbit serum. This non-specific staining may be related to nuclear modifications caused by virus multiplication. Plate 1e is an alkaline phosphatase control. These were uninfected cells treated with anti-SV40 antiserum. Background staining of nuclei was very faint, even weaker than that of the cytoplasm. The background staining of the cytoplasm occurred with all enzyme markers employed.

PLATE I

Detection of SV40 structural antigens in MA104 cells, fixed with acetone 48 hr after infection, treated with rabbit anti-SV40 antibody and then with conjugates of sheep antibody anti-rabbit IgG plus various labels. In each picture positive nuclei are indicated by small arrows and negative nuclei by large arrow heads.

(a) Conjugate labelled with fluorescein.
(b) Conjugate labelled with peroxidase.
(c) Conjugate labelled with peroxidase. A control preparation in which infected cells were treated with normal serum instead of antibody to SV40. Most nuclei are negative but an occasional non-specific reaction (arrow) suggests that these may be modifications of the nucleus in an advanced state of infection.
(d) Conjugate labelled with alkaline phosphatase.
(e) Conjugate labelled with alkaline phosphatase. A control preparation of non-infected cells treated with the anti-SV40 serum. The nuclei are negative, and the background staining of the cytoplasm is found not only with the conjugate labelled with alkaline phosphatase, but may occur with any label.
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as well as with fluorescein, and it could not be confused with the specific staining of the SV40 antigens.

Examples of other virus antigens localized by antisera labelled with enzymes are given (Pl. 2). The cells were fixed with acetone in all except Pl. 2e in which paraformaldehyde was used. Plate 2a, b represent antigens of adenovirus 12. The T antigens (Pl. 2a) were in the form of intranuclear fibres (Pope & Rowe, 1964b), revealed by the peroxidase label in abortively infected hamster cells. The structural antigens (Pl. 2b) occurred as small intranuclear masses, revealed by the alkaline phosphatase label. The T antigens of SV40 in transformed hamster cells were detected by a peroxidase-labelled conjugate and occurred as an intranuclear network (Pl. 2c). These same cells treated with normal hamster serum instead of serum from a tumour-bearing hamster were completely negative (Pl. 2d). T antigens of SV40 (Pl. 2e) also appeared during the lytic cycle in MA104 monkey kidney cells where they preceded the appearance of structural antigens (cf. Rapp, 1964). In this example, fixation in paraformaldehyde gave results comparable with those after acetone fixation.

Two different antigens may be detected in the same preparation. In MA104 cells infected with SV40, T antigens were stained brown by the activity of a peroxidase-labelled conjugate and structural antigens were stained red by the activity of an alkaline-phosphatase-labelled conjugate (Pl. 2f).

As in immunofluorescence, there is a background staining of the cells but this is faint and is probably related to the fact that the antibodies against the various virus antigens were whole sera and not purified globulins.

DISCUSSION

By using several enzymes as markers in antibody conjugates in comparison with a fluorescent dye we confirmed the reproducibility of the immunocytochemical procedures of localizing antigens. The enzyme conjugates present certain advantages over the fluorochrome conjugates. Although the number of fluorescent dyes that may be employed in immunocytochemical reactions is rather limited, there is potentially a

PLATE 2

(a) Hamster cells fixed with acetone 18 hr after infection with adenovirus 12, treated with hamster serum of hosts bearing tumours induced by adenovirus 12 and with a conjugate of rabbit anti-hamster immunoglobulin labelled with peroxidase. The T antigens appear as dense fibres in the nucleus of one cell.

(b) KB cells fixed with acetone 36 hr after infection with adenovirus 12, treated with rabbit antiserum to adenovirus 12 structural antigen and a conjugate labelled with alkaline phosphatase. The antigen appears as small round masses in the nuclei of infected cells.

(c) Hamster cells transformed by SV40 fixed with acetone, treated with hamster serum of host bearing tumours induced by SV40 and a conjugate labelled with peroxidase. The T antigens of SV40 appear in all nuclei in the form of a dense network.

(d) Control for (c). Same cells and same conjugate but with normal hamster serum. The nuclei are entirely negative.

(e) T antigens of SV40 virus observed during the lytic cycle of the virus in MA104 cells fixed with paraformaldehyde 24 hr after infection; positive nuclei (arrows) and negative nuclei (arrow heads).

(f) Double reaction. MA104 cells fixed with acetone 48 hr after infection with SV40 virus. T antigens were revealed by a conjugate labelled with peroxidase and were stained deep brown; structural antigens were revealed by a conjugate labelled with alkaline phosphatase and were stained red. A negative nucleus is indicated by an arrow head.
large number of enzymes that can be used as markers (Burstone, 1962). Although at least two fluorescent dyes of different colours can be used simultaneously on the same section (Bernier & Cebra, 1964), the possibility of revealing more than one antigen at a time with enzyme markers is potentially greater because reaction products of several different colours are available. As pointed out by Graham & Karnovsky (1966) and Nakane & Pierce (1967), exceedingly small amounts of enzymes in tissues can be detected by prolongation of incubation in the substrate medium for the enzyme, until sufficient enzyme reaction product has accumulated. Another advantage is that permanent preparations can be made of some enzyme cytochemical reactions. Finally, the reaction products of some enzyme markers, including peroxidase and alkaline phosphatase, are electron dense and may be employed directly in electron microscopy (Ram, Nakane & Rawlinson, 1966; Nakane & Pierce, 1967; Leduc, Avrameas & Bouteille, 1967; Scott, Avrameas & Bernhard, 1968).

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REFERENCES


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