Ultrastructural Localization of SV40 T Antigen with Enzyme-labelled Antibody

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SUMMARY

T antigen (neoantigen) of Simian Virus 40 has been localized at the ultrastructural level in infected MA 104 cells in culture. The cells were fixed 15 min. to 1 hr in 1% formaldehyde, freshly prepared from paraformaldehyde, in 0.2 M cacodylate buffer, pH 7.3, + 0.25 M sucrose. This allowed adequate conservation of ultrastructure while permitting subsequent penetration of reagents into the cells. The antigen was revealed by successive exposure to unlabelled hamster serum containing antibody against the T antigen and to labelled rabbit antibody against hamster immunoglobulin. The latter conjugate of rabbit antibody was labelled with peroxidase for which the cytochemical reaction results in an osmiophilic, electron dense reaction product which can be visualized by electron microscopy. The T antigen of SV40 first appeared in a reticular network throughout the nucleus, except the nucleolus, in a position which seems to correspond with that of the chromatin network.

INTRODUCTION

Viral antigens have been detected routinely by fluorescence microscopy with conjugates of their specific antibodies coupled to fluorescein. Wicker & Avrameas (1969) have recently employed conjugates labelled with enzymes to localize viral antigens by light microscopy, both in the lytic cycle of infected cells and in virus-free transformed cells. Coloured reaction products, produced by cytochemical reactions for the enzymes used as markers, are deposited at the sites of the antigens. Wicker & Avrameas (1969) also found that not only the classical fixatives for light microscopy but also some fixatives used for electron microscopy allowed the reaction to take place between the antigen in the cells and the antibody of the conjugate. Since the reaction products of some of the cytochemical methods for enzyme activity are electron dense (Nakane & Pierce, 1967), it has been possible to adapt this method for electron microscopy. In this paper we describe a method for and the results of the detection of T antigen (neoantigen) in the lytic cycle of SV40 infected cells at the ultrastructural level.

METHODS

Monkey (Rhesus) kidney cells of the strain MA104 were cultivated in Leighton tubes without coverslips in Eagle’s minimum essential medium with fourfold amino acids and vitamins and 10% calf serum without antibiotics. Approximately 24 hr after subculture, before the cells were confluent, they were infected with SV40 at an infection
multiplicity of 10 p.f.u./cell. The cells were fixed 24 hr later when T antigen was abundant and usually just before or at the time that virus particles first became visible.

Cells were fixed in situ in the Leighton tubes for 5 and 15 min. and 1 hr in 1% formaldehyde, freshly prepared from paraformaldehyde (Karnovsky, 1965), in 0.2 M cacodylate buffer + 0.25 M sucrose at pH 7.3. This fixative was chosen because it had been used successfully with rabbit spleens for the detection of immunoglobulins with enzyme-labelled antibodies (Leduc, Scott & Avrameas, 1968). Furthermore, at the light microscope level it was superior to formaldehyde solutions prepared from formalin and to glutaraldehyde solutions for the detection of viral antigens with enzyme-labelled antibodies (Wicker & Avrameas, 1969). The cells were then washed at least 30 min. up to 3 days in a solution of 0.2 M cacodylate buffer and 0.25 M sucrose at pH 7.3 at 3\°.

The antigen was revealed by an indirect, sandwich method as was employed by Levinthal, Wicker & Cerottini (1967). The fixed infected cells first were exposed to unlabelled whole serum diluted 1:8 times with phosphate-buffered saline from hamsters bearing grafted tumours induced by SV40. This serum contains antibodies against the viral T antigen titrating at 1:640 when tested by complement fixation. Exposures of 1 to 4 hr at 20\° or 37\° were tried. The optimum length of exposure to the serum varies with the degree of fixation, longer times being needed after 1 hr fixation than after 15 min. fixation, and, a factor of great importance, with the titre of the serum itself. In general, we obtained repeatable results with 15 min. fixation and 2 hr exposure to a serum of high titre at 20\°. Freezing and thawing the cells one to four times while they are in the serum enhances somewhat the penetration of the antibody, although it ruins the ultrastructure of cells fixed only 5 min. During the course of our experiments it became apparent that some of our sera and conjugates had been diluted too much. Antisera and conjugates of higher protein concentration would undoubtedly provide electron micrographs of higher contrast, but we were limited by small quantities of sera.

Excess, unbound antibody was removed by three brief changes of sucrose-buffer solution for a total of 5 to 10 min., and then the cells were refixed for 15 min. with the 1% formaldehyde–sucrose–cacodylate fixative. This postfixation seems necessary for electron microscopy but is not essential for light microscopy (Wicker & Avrameas, 1968). The cells were then washed in the sucrose–buffer solution 30 min. or longer.

The cells were then exposed to a conjugate consisting of rabbit antibody anti-hamster immunoglobulin coupled to horseradish peroxidase. Rabbit anti-hamster immunoglobulin antibody was isolated by passage of the whole antiserum on polymerized hamster immunoglobulins (Avrameas & Ternynck, 1969). The isolated antibody was labelled with peroxidase using glutaraldehyde as the coupling agent by procedure described in detail elsewhere (Avrameas, 1968; Avrameas, 1969). A stock of conjugate with a protein concentration of 0.5% was diluted 10 times with phosphate buffered saline before use. Exposure to the conjugate is usually longer than for the first serum, and, for convenience, usually an overnight bath, 18 to 26 hr, at 20\° was employed, but positive reactions were also obtained with incubations of 3 and 6 hr. During exposure of the cells to the serum and the conjugate the Leighton tubes were continuously and gently rocked, 10 times per min. After brief rinses in three changes of sucrose–cacodylate solution to remove unbound conjugate, the cells were again postfixed with an aldehyde, usually 2.5% glutaraldehyde, because subsequent reactions are not inhibited
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by this fixative and preservation of ultrastructure is better than that with formaldehyde. Another wash in buffer for 30 min. or longer is required to remove excess fixative.

The site of the peroxidase label was revealed by the method of Graham & Karnovsky (1966) in which the cells were exposed 30 min. to an incubation mixture of diaminobenzidine and hydrogen peroxide in tris + HCl buffer at pH 7.6. A brown precipitate is deposited at the site of enzyme activity, and the cells can be examined by light microscopy while still in the Leighton tubes. Parallel immunofluorescent preparations were always made in which the relative number of positive cells was determined (40 to 80% in these experiments).

Finally, the cells were post-fixed in 2% osmium tetroxide in cacodylate buffer for 1 hr. The osmium accentuated the electron density of the enzyme reaction product and imparted sufficient electron density to the cells so that they could be photographed without further staining. The cells were then gently scraped off the surface of the Leighton tube with a rubber policeman and centrifuged into a pellet. Because the cells were few in number, they were then handled like a pellet of bacteria (Ryter & Kellenberger, 1958), that is, a drop of agar was added to the pellet so that small blocks of cells suspended in agar could be made. This greatly facilitates subsequent dehydration in alcohol and propylene oxide and subsequent embedding in Epon. Ultrathin sections were cut with diamond knives and examined without post-staining.

Two kinds of controls were employed. In one, uninfected cells were processed exactly as above. In the other, infected cells were treated with normal hamster serum instead of serum containing antibody anti-SV40 T antigen. A third type of control was carried out by fluorescence microscopy only, namely, normal cells treated with normal serum.

The cells were photographed with a Siemens Elmiskop I at 80 kv with 30 and 50 μm. objective apertures.

OBSERVATIONS

As seen by immunofluorescence or by light microscopy with enzyme labels (Wicker & Avrameas, 1968) not all cells of an infected culture were infected simultaneously. Twenty-four hr after exposure to SV40, there were still cells with negative nuclei, strongly positive cells in which the viral neoantigen filled all of the nucleus except the nucleolus, and intermediate stages in which the antigen formed a reticular pattern in the nucleus. In the control preparations the nuclei were negative but there were varying degrees of non-specific background staining of the cytoplasm because whole hamster serum had been used instead of purified immunoglobulin.

In electron microscope preparations of the controls, that is, either uninfected cells treated with specific serum or infected cells treated with normal serum, there was no internal structure visible in the nucleus except for the reticular portion of the nucleolus which was slightlydenser than the background (Pl. 4b), because no counterstain was applied to the sections. The use of a uranyl or lead counterstain obscures the reaction product of the enzyme marker by overstaining all cell structures. In the method described here, there were two types of non-specific staining which occurred in both types of controls as well as the experimental cultures. One occurred among some but not all of the cells of each culture and consisted of the staining of all elements in the cytoplasm (Pls. 2b, 3b, 4a). The other occurred regularly and resulted in the deposition of a thin layer of the enzyme reaction product on the superficial surface, that not in contact with the culture tube, of all cells (Pl. 1).
In infected cultures treated with the specific antisera, the nuclei of cells that were not yet making viral T antigens, often adjacent to infected cells (Pl. 1), had the same unstructured appearance as above and were, in fact, good additional controls. Among the positive nuclei, those that were most heavily stained exhibited a fairly uniform distribution of stain throughout the nucleoplasm except the nucleolus (Pl. 2a, b). There often appeared to be a dense layer of reaction product encircling the nucleolus (Pl. 3a), as if a barrier existed to the penetration of T antigen into the nucleolus, but this could not be associated with any particular nucleolar structure. In some apparently overstained preparations, presumably exposed too long to the serum and the conjugate, even the nucleolus acquired some additional density (Pl. 3b), but this might be interpreted as an artifact since nucleolar staining was not seen at the light microscopic level. Intermediate between the unreactive nuclei and heavily reactive nuclei, there were large numbers of cells in which the enzyme reaction product, indicating the site of the viral T antigen, was distributed in the form of a reticular network dispersed throughout the nucleus (Pls. 1, 2a). This pattern was found both in cells that were still free of virus particles (Pl. 2a) and in cells in which the virus was already detectable (Pl. 1). This reticulum is usually distinctive, although the intensity of the reaction product at the site of the antigen varied from one experiment to another, depending on the length of fixation, the sample of antiserum and conjugate used and the duration of the exposure of the cells to the antisera.

The same non-specific staining of the cytoplasm described in the control preparations was also seen in some of the infected cells treated with the specific antisera (Pl. 2b). The non-specific deposition of conjugate on the free surface of all cells is evident in the illustrations (Pls. 1, 2a). Preservation of the cytoplasm was poor after fixation for 15 min. or less in 1% formaldehyde, in that mitochondria and ergastoplasmic cisternae were swollen, but the nuclei appeared to be well preserved. Longer fixation results in better ultrastructure but poorer penetration of the antisera.

**DISCUSSION**

Ferritin has already been used successfully as a marker in localizing viral antigens (Singer, 1959), including those of structural and T antigens of SV40 (Oshiro et al. 1967a, b; Levinthal et al. 1967), but penetration of cells with ferritin-labelled conjugates is difficult to achieve because of the large size of the ferritin molecule (650,000 M.w.). The use of an enzyme marker, peroxidase, of smaller molecular size (40,000 M.w.) has diminished this problem, but there is still a compromise between the degree of preservation of cell ultrastructure and the extent of penetration of labelled immunoglobulins. In the procedure used here, since a nuclear antigen was involved, a poor conservation of the cytoplasm was accepted. Preliminary trials with other viral antigens suggest that the methods of cell fixation and exposure to immunosera may have to be worked out individually for each viral antigen and, in particular, for each sample of serum and conjugate.

In this study an indirect or sandwich method was used like that of Levinthal et al. (1967) in which the antigen is reacted first with whole serum containing antibody and then with anti-immunoglobulin conjugated with a marker. It would be simpler to use a single labelled antibody, but the amount of hamster antiserum available was very small and this made it impossible for us to isolate and label this antibody directly.
Cultured monkey kidney cells, strain MA104, 24 hr after infection with SV40, fixed in 1% formaldehyde 1 hr, exposed 3 hr to unlabelled antiserum and 6 hr to a conjugate of anti-IgG labelled with peroxidase. The cell on the left is uninfected, that on the right is infected and virus particles are beginning to appear in the nucleoplasm. The uninfected nucleus gives a negative reaction for viral antigen and, because no counter-stain was used, no internal structure is visible. SV40T antigen (small arrows) is revealed in the infected nucleus by the reaction product of the enzyme label as a moderately dense network. The surfaces of both cells are non-specifically stained by adsorbed conjugate (large arrows), but there is little non-specific staining of the cytoplasm in the infected cell and none in the uninfected cell.

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(a) An infected cell in which virus particles have not yet appeared, fixed 15 min. and exposed to the sera 3 hr and 24 hr, respectively. The neoantigen is distributed in irregular masses in the form of a dense network (arrows).

(b) An infected cell fixed 15 min., exposed to the antiserum 1 hr, during which the cells were frozen and thawed once and exposed to the conjugate 3 hr. All the nucleoplasm is heavily stained except the nucleolus. The cytoplasm is poorly fixed and stained non-specifically.

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(a) A higher magnification of an infected cell, fixed and treated as that in Pl. 2b, to illustrate the sharp boundary between the negative nucleolus and the highly positive nucleoplasm.

(b) An overstained infected cell, fixed 1 hr and exposed to the antiserum and the conjugate 24 hr and 62 hr, respectively. The network of neoantigen is still detectable in the densely stained nucleoplasm and even the nucleolus has acquired additional density.

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(a) An uninfected control, fixed 15 min., exposed to the antiserum 4 hr with 1 freeze-thaw cycle and treated with the conjugate for 28 hr. The nucleus is unstained. There is strong non-specific staining of the cytoplasm, but that of an adjacent cell (top) is not stained.

(b) An uninfected cell, fixed 1 hr and treated 3 and 24 hr respectively, with the specific antiserum and conjugate. The nucleus is unstained except for the nucleolus which exhibits a slight density presumably imparted by osmium. The cytoplasm is non-specifically stained.

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A conjugate could be employed more successfully (Avrameas, 1968) if purified immunoglobulin rather than whole serum were labelled. The advantages, as described by Levinthal et al. (1967), of using a sandwich method with labelled immunoglobulins instead of a direct method with a labelled antiserum, namely, the ready availability of antiglobulins and the possibility of having stocks of conjugates of known reactivity, are applicable to all immunocytochemical investigations.

The non-specific staining of the cytoplasm of some cells in both infected and uninfected cultures was probably due to the fact that whole serum rather than purified antibody was used. It is not clear why the cytoplasm of some cells but not of others was stained. In immunofluorescent preparations of the same cultures, the intensity of non-specific cytoplasmic staining also varied from one cell to another and differed with different samples of antiserum. The second type of non-specific staining, which appears to be an adsorption of the conjugate on to the free surfaces of all cells, could probably be eliminated by more extensive washing of the cells after removing them from the antiserum. This staining did not present a serious problem in this study but it would be unacceptable in studies of surface antigens.

The distribution of SV40 T antigen throughout the nucleoplasm except the nucleolus in cells that reacted heavily corresponded with the pattern commonly seen in immunofluorescent preparations (Wicker & Avrameas, 1969). On the other hand, in cells in which there apparently was less T antigen, presumably in an earlier stage of formation, we observed a loose, irregular network of reactive material of moderate density. In two studies of SV40 antigens with ferritin-labelled conjugates, one a direct method (Oshiro et al. 1967a) and the other an indirect method (Levinthal et al. 1967), the T antigen was described as being in irregular aggregates, few in number, randomly dispersed in the nucleus. It is possible that the ‘aggregates’ seen with the ferritin label may correspond with small portions of the ‘network’ seen with the peroxidase label, particularly if we assume that peroxidase-labelled conjugates can penetrate more readily into the cells than ferritin-labelled conjugates.

We have interpreted the distribution of T antigen in the pattern of a loose network to represent an association of the antigen with the nuclear chromatin. This interpretation is based particularly on the fact that the network includes a layer on the inner surface of the nuclear envelope, a position always occupied by chromatin. It is to be expected that the early appearing viral neoantigen might be found in association with newly formed RNA, synthesized under the direction of viral DNA. If this is the case, it would appear that both the viral DNA and the RNA directing the synthesis of the antigen are associated with the cell chromatin. Indeed, the first virions are thought to assemble in close contact with chromatin (Gaylord & Hsiung, 1961; Granboulan et al. 1963). However, we do not know if T antigen or structural proteins of SV40 are in fact synthesized in the nucleus or, as in the case of adenoviruses, are synthesized on cytoplasmic polysomes (Thomas & Green, 1966) and subsequently transported into the nucleus. The frequent non-specific reaction in the cytoplasm of our cells does not yet allow us to examine this problem.

The procedure described here for the demonstration of SV40 antigen was repeated five times with cells fixed for 1 hr, six times with cells fixed for 15 min. and twice after 5 min. fixation. In the course of these experiments two samples of antisera and two of conjugate were used. The localizations of antigen in all these tests were the same; the reaction varied only in intensity. Hence, this is a reproducible method for this
viral antigen. It is perhaps too early for the use of enzyme-labelled antibodies for the ultrastructural localization of antigens to be considered as a routine method, but its potential seems as great as the immunofluorescence method for optical microscopy.

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