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An Improved Immunoperoxidase Technique for Identifying SV40 V and T Antigens by Light Microscopy[1] (38120)

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The highly valuable immunofluorescence technique developed by Coons and coworkers (1) has been widely used to detect viral and virus-related cellular antigens in a variety of cells. Although by means of this procedure it is possible to localize low levels of specific antigens, the following limitations (2) are also apparent: First, darkfield microscopy, usually in combination with an ultraviolet light source, is required to examine the preparations. Second, during conjugation of the fluorescent dye to the antibody, the reactivity of both compounds may be altered. Third, the intensity of fluorescence varies with the pH and the ratio of fluorescent dye to antibody. Fourth, the preparations are not permanent, since they must be mounted in buffered glycerol, a temporary medium, and also because their fluorescence fades when they are exposed to intense light over long periods, such as during photography.

Many of these problems can be avoided by replacing the fluorescent dye with various enzymes, such as horseradish peroxidase, which can be localized histochemically at both the light and electron microscope levels (2). Although Wicker and Avrameas (3) have reported the identification of simian virus 40 (SV40) V (virion) (4, 5) and T (tumor) (6, 7) antigens by means of an immunoperoxidase procedure, the results presented in this communication are based on a gentle yet highly efficient conjugation procedure slightly modified from that recently developed by Kawaoi and Nakane (8), which links horseradish peroxidase to immunoglobulin. This conjugate is then utilized in an indirect immunoperoxidase procedure that identifies SV40 V and T antigens.

Cells. The following 4 cell lines were used: (a) green monkey kidney (GMK) cells, line AH-1 (9) producing both SV40 V and T (10, 11) antigens when infected with SV40 *in vitro*; (b) line 2K (12) consisting of SV40-transformed hamster embryo cells carrying SV40 T (6, 7) but not V antigen; (c) line Sk/B (13) consisting of SV40-transformed hamster embryo skin and subcutaneous tissue cells carrying SV40 T but not V antigen; (d) line I (12) consisting of hamster embryo cells shown by immunofluorescence to be negative for SV40-related antigens.

Sera. Normal serum was derived by pooling the blood of a number of 3-month-old male Syrian golden hamsters. Serum containing antibody against SV40 V antigen (anti-V serum) but not against SV40 T antigen was prepared by inoculating a number of 3-week-old hamsters iv with SV40 and pooling their blood 2 weeks later. Serum containing antibody against SV40 T antigen (anti-T serum) was derived by pooling the blood of a number of adult hamsters bearing large intramuscular tumors which were induced by *in vitro* SV40-transformed homologous embryonic skin and subcutaneous tissue cells (13) (line Sk/B) known to contain SV40 T but not V antigen. Goat serum containing antibody against hamster gamma globulins was obtained commercially.[3] The gamma globulin fraction of the goat serum was isolated as the first protein band eluted from DEAE cellulose by 0.01 *M* phosphate buffer, pH 7.0, a modification of the procedure by Levy and Sober (14). The fluo-

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[3] From Antibodies Incorporated, Davis, California.

rescein-labeled gamma globulin fraction of goat anti-hamster-globulin serum used as conjugate in the immunofluorescence test was obtained commercially.[3] All sera were stored at −20° to prevent loss of activity.

Conjugation. The conjugation procedure (8) took place in 2 phases: first, the "activation" of peroxidase to the aldehyde form; second, its "conjugation" to the immunoglobulin followed by borohydride reduction. The latter step was introduced in order to stabilize the Schiff's base formed by conjugation, so that dissociation of the conjugate would be avoided.

In the "activation" phase of the procedure, 5 mg of horseradish peroxidase type VI[4] was dissolved in 1.0 ml of 0.3 *M* sodium bicarbonate. The free amino groups of the peroxidase were blocked by stirring it with 0.1 ml of 1% fluorodinitrobenzene[5] (v/v in absolute ethanol), for 1 hr at room temperature. Glassware that had come in contact with fluorodinitrobenzene was detoxified by rinsing it with 1 *M* NaOH. The sugar residues of the peroxidase were oxidized to aldehyde form by stirring it with 1.0 ml of 0.08 *M* sodium periodate for 30 min at room temperature. The periodate was inactivated by adding 1.0 ml of 0.16 *M* ethylene glycol while continuing the incubation as before for 1 hr. The resultant peroxidase aldehyde was dialyzed against 0.01 *M* carbonate buffer, pH 9.5 at 4°. When desirable, the peroxidase aldehyde was stored at 4° for up to one month.

In the "conjugation" phase of the procedure, the 3.3 ml of peroxidase aldehyde prepared above was added to 5 mg of the gamma globulin fraction of goat anti-hamster-globulin serum and stirred at room temperature for 2–3 hr. The peroxidase-antibody conjugate was dialyzed against phosphate buffered saline (PBS) pH 7.1–7.2 at 4° and then against PBS containing 4 m*M* sodium borohydride for 8 hr at 4°. Finally, the reduced conjugate was extensively dialyzed against PBS at 4°. In general, the peroxidase-antibody conjugate was used in the immunoperoxidase technique without further modification. The conjugate was at times partially purified by precipitation in 50% saturated ammonium sulfate to remove unbound peroxidase (15).

Adsorption of sera and conjugate. Aliquots of normal and anti-T serum as well as of the conjugate were at times adsorbed with rabbit liver powder[6] (16) or with a 1:4 dilution of 50% normal hamster brain homogenate (6) in order to remove nonspecific activity.

Indirect immunoperoxidase procedure. To detect SV40 V and T antigens, cells grown on cover glasses were rinsed 3 times in PBS inside Columbia staining jars.[7] These preparations were then air dried for 30 min and placed in acetone for 5 min at room temperature. Individual cover glasses were overlaid with several drops of normal, anti-V, or anti-T serum (diluted 1:4 with PBS) and were incubated in a humidified chamber at 37° for 1 hr. The various sera were removed from the cover glasses by touching them to absorbent paper and rinsing them twice in PBS. Several drops of undiluted conjugate were spread on each cover glass. The cover glasses were then incubated in the same humidified chamber at 37° for 1 hr. The conjugate was removed and the cover glasses were rinsed as before. For development of the peroxidase reaction (17), the cover glasses were placed for 10 min in a freshly prepared, filtered solution of 0.1 *M* phosphate–0.1 *M* sucrose buffer (pH 7.4) containing 0.05% diaminobenzidine tetrahydrochloride[4] (w/v) and 0.01% hydrogen peroxide (v/v). The diaminobenzidine acts as an electron donor in the peroxidase reaction, yielding a brown, insoluble reaction product at the site of peroxidase activity (17). At the end of this period, the cover glasses were rinsed 3 times in phosphate-sucrose buffer which was added to the development jars by means of a wash bottle to float off any precipitate that formed on the surface of the developing solution. The cover glasses were dehydrated through graded alcohols, cleared in xylene and mounted in a permanent mounting medium (Permount[8]) on microscope slides.

[4] From Sigma Chemical Company, St. Louis, Missouri.

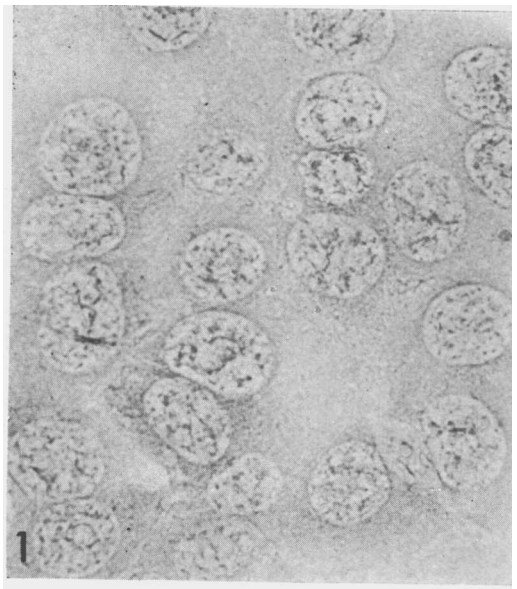
[5] From Eastman Organic Company, Rochester, New York.

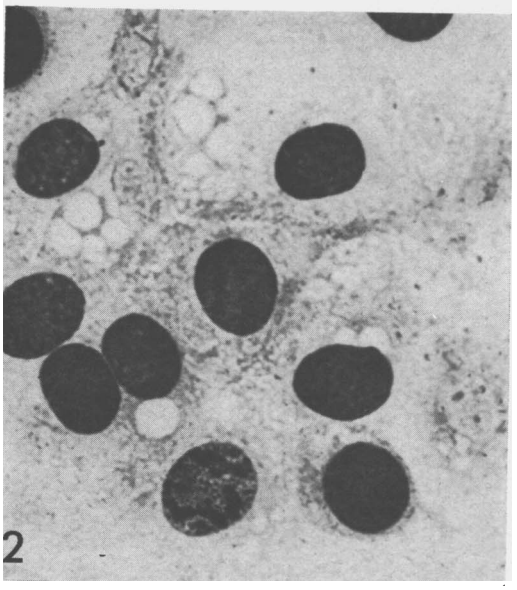
[6] From Grand Island Biological Company, Grand Island, New York.

[7] From Arthur Thomas Company, Philadelphia, Pennsylvania.

[8] From Fisher Scientific Company, Pittsburgh, Pennsylvania.

Comparison of the sensitivities of the immunoperoxidase and immunofluorescence techniques. The sensitivities of the 2 techniques were compared by quantitating the antibody titer to SV40 V and T antigens using serial two-fold dilutions of antiserum. Positive control serum possessing anti-V activity up to a dilution of 1:40 was defined as producing 4+ intensity of staining against V antigen when undiluted. Positive control serum possessing anti-T activity up to a dilution of 1:640 was defined as producing 4+ intensity against the T antigen in lytically infected cells (10, 11) and 3+ against the T antigen in transformed cells (6, 7) when used undiluted.





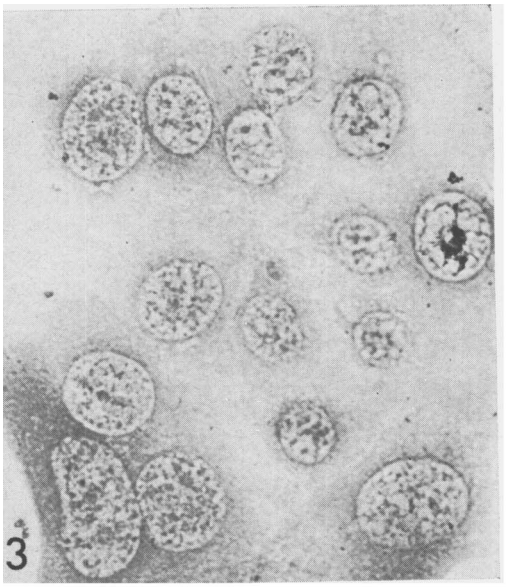
FIGS. 1 and 2. SV40-infected green monkey kidney cells stained by the indirect immunoperoxidase technique for the intranuclear SV40 V (virion) antigen. Fig. 1, negative (normal serum). Fig. 2, positive (anti-V serum). ×550.

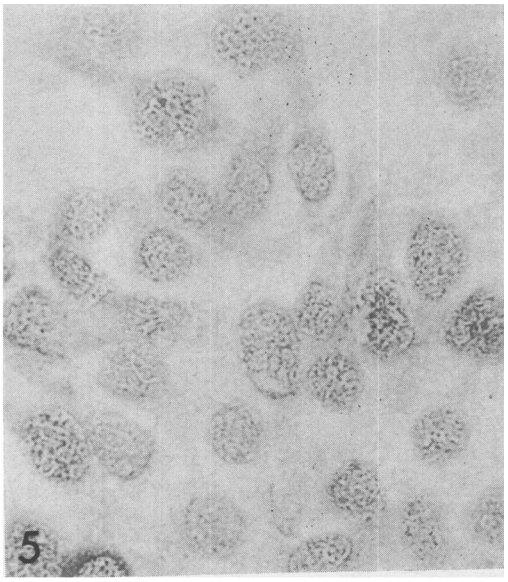
Results. Figures 1 and 2 demonstrate preparations of SV40-infected GMK cells stained by the indirect immunoperoxidase procedure for SV40 V antigen. Cells of Fig. 1 have been exposed to normal serum and are negative, while cells of Fig. 2 have been incubated with serum containing antibody against V antigen and are SV40 V positive. It should be pointed out that SV40 V antigen is finely granular, resulting in a ground glass appearance in contrast to the coarsely granular SV40 T antigen (see below). These differences in texture have also been demonstrated by the immunofluorescence test. A recently infected cell has a partially stained nucleus while 3 uninfected cells have negatively stained nuclei (Fig. 2).

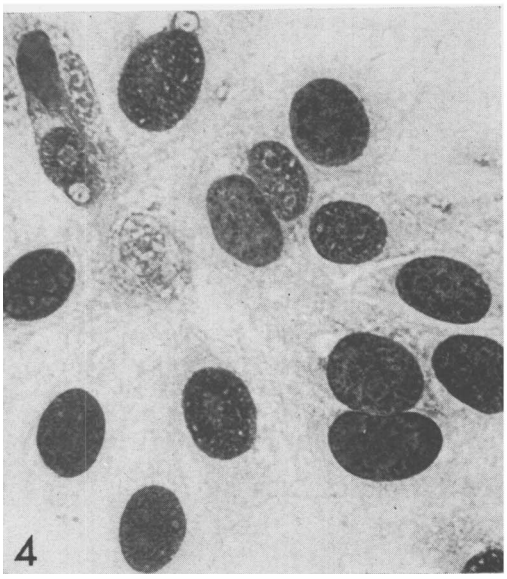
Figures 3–6 demonstrate preparations of an SV40-infected GMK cell line (Figs. 3 and 4) and of an SV40-transformed hamster embryo cell line (Figs. 5 and 6) stained by the indirect immunoperoxidase procedure for SV40 T antigen. Figs. 3 and 5 exhibit cells incubated with normal serum and are negative; Figs. 4 and 6 are comparable preparations that have been exposed to anti-T serum and are positive. It can be noted that the SV40 T antigen found in lytically infected GMK cells (10, 11) (Fig. 4) stains slightly more intensely than the T antigen present in SV40-transformed cells (6, 7) (Fig. 6). This phenomenon has also been observed following immunofluorescent staining, although its significance has not been elucidated. The intranuclear SV40 T antigen in transformed cells (Fig. 6) appears as coarse granules; the nucleoli do not stain. Similar results were obtained with SV40-transformed hamster embryo skin and subcutaneous tissue cells (line Sk/B). Hamster embryo control cells (line I) which were SV40 T negative by immunofluorescence were also negative by the immunoperoxidase technique when incubated with either normal or anti-T serum.

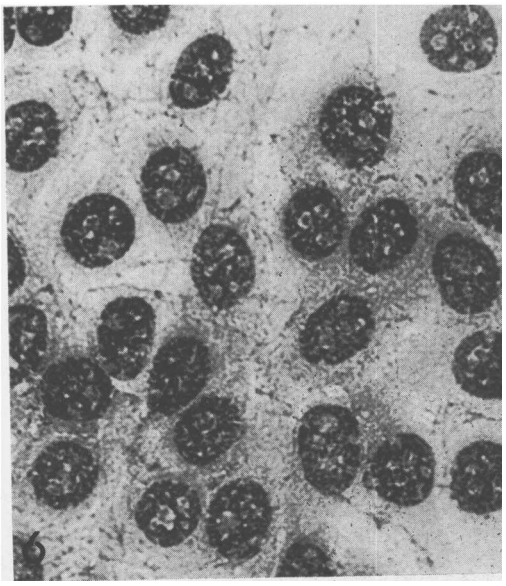
Since the intensity of staining as revealed by the immunoperoxidase and the immuno-

fluorescence tests depends on the amount of specific antibody present in the serum as well as on the antigen concentration, the sensitivity of the immunoperoxidase procedure was compared with that of the immunofluorescence procedure by determining the end-point of positive staining with serial two-fold dilutions of antiserum. The end-points of positive staining with the immunoperoxidase procedure occurred at four-fold higher dilutions of antiserum than with the immunofluorescence procedure for all three antigens, indicating that the immunoperoxidase technique is slightly more sensitive than the immunofluorescence









FIGS. 3 and 4. SV40-infected green monkey kidney cells stained by the indirect immunoperoxidase technique for the intranuclear SV40 T (tumor) antigen. Fig. 3, negative (normal serum). Fig. 4, positive (anti-T serum). ×550.

FIGS. 5 and 6. SV40-transformed hamster embryo cells stained by the indirect immunoperoxidase technique for the intranuclear SV40 T (tumor) antigen. Fig. 5, negative (normal serum). Fig. 6, positive (anti-T serum). ×550.

technique.

Discussion and Summary. Development of the peroxidase reaction for a period of 10 min has consistently resulted in strong nuclear staining of SV40 V and T positive cells. These cells are easily distinguished from antigen negative cells except for some finely granular cytoplasmic staining present in the latter cells. Attempts to eliminate this nonspecific staining by adsorbing either the serum or the conjugate with liver powder or with brain homogenate were unsuccessful. On the contrary, such adsorption weakened the activity of the serum. Incubation in absolute methanol or in 0.5% H_2O_2 (18) before exposure to serum did not eliminate the nonspecific staining but resulted in some nuclear damage and in a general decrease in staining intensity. Lowering the concentration of H_2O_2 (0.1%, 0.01%, 0.001%) for this pretreatment did not improve the overall staining reaction and also resulted in occasional nuclear damage. Finally, partial purification of the conjugate by removing unbound peroxidase did not improve the staining effect as compared to that of the crude preparation due both to the efficiency of the peroxidase conjugation and to the lack of affinity of free peroxidase for the cells employed.

Throughout the immunoperoxidase test individual staining jars and separate forceps should be used for each cover glass in order to avoid transferring antigen-positive cells or antiserum to control preparations. In addition, three separate sets of staining jars should be used for each cover glass: one for the predevelopment part of the test, another for the development, and a third for the final rinses and dehydration, to avoid formation and deposition on the cover glasses of an intractable dark brown precipitate. (Flecks of dark precipitate may be noted on the nucleus and cytoplasm of some cells in Fig. 3.)

On the basis of our own experience and that of others (19, 20), it can be stated that, in addition to its slightly greater sensitivity, the indirect immunoperoxidase method offers the following major advantages when it is compared to the immunofluorescence technique. First, it allows examination of the preparations under brightfield light microscopy, thus eliminating the requirement for darkfield equipment and an ultraviolet light source. Second, since the procedure is based on a gentle although highly efficient conjugation reaction, neither the antibody nor the enzyme activity appears to be reduced to any appreciable degree while, at the same time, the conjugate remains stable. Third, the color intensity of the staining reaction is not dependent upon narrow pH changes or upon the ratio of peroxidase to antibody in the conjugation reaction, since it depends upon the accumulation of a reaction product. Admittedly, the contrast of a dark deposit against a light background, characteristic of the immunoperoxidase method, is not so marked as that of a few photons of fluorescent light against a black background characteristic of the immunofluorescence technique. Finally and most importantly, since the preparations are permanently mounted, no alterations of the label can occur. As a result, the preparations can be examined repeatedly over prolonged periods for the purpose of differential cell counts, for demonstration, and for photomicrography. In light of these advantages, it appears that the immunoperoxidase procedure is a simple, sensitive technique that can be a useful adjunct to the immunofluorescence procedure in identifying viral and virus-related cellular antigens.

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