

Immunofluorescence of Green Monkey Kidney Cells Infected with Adenovirus 12 and with Adenovirus 12 Plus Simian Virus 40

RICHARD A. MALMGREN, ALAN S. RABSON, PAULA G. CARNEY, AND
FRANCES J. PAUL

Pathologic Anatomy Branch and Laboratory of Pathology, National Cancer Institute, U.S. Public Health Service, Bethesda, Maryland

Received for publication 25 August 1965

ABSTRACT

MALMGREN, RICHARD A. (National Cancer Institute, Bethesda, Md.), ALAN S. RABSON, PAULA G. CARNEY, AND FRANCES J. PAUL. Immunofluorescence of green monkey kidney cells infected with adenovirus 12 and with adenovirus 12 plus simian virus 40. *J. Bacteriol.* 91:262-265. 1966.—Immunofluorescence studies of the viral antigens and tumor (T) antigens of adenovirus 12 and simian virus 40 (SV40) in green monkey kidney (GMK) cells infected with adenovirus 12 alone or in combination with the SV40 virus showed that the adenovirus 12 viral antigen was produced in detectable amounts only in the cells infected with both viruses. The adenovirus 12 T antigen, on the other hand, was formed in the GMK cells infected with the adenovirus 12 only. This antigen was formed as early as 18 hr after viral infection, and persisted for at least 48 hr after virus infection. There was a correlation between the appearance of the immunofluorescent T antigen in the nucleus and the electron microscope appearance of "nuclear stippling," which developed in the nuclei of GMK cells after infection with adenovirus 12 only, as well as after infection with both viruses.

Simian virus 40 (SV40) has been shown to enhance the growth of human adenovirus 12 in cultures of African green monkey kidney (GMK) cells (6). Observations of the morphological changes produced by infection of these cells with adenovirus 12 alone and with adenovirus 12 plus SV40 have been reported (4). In the absence of SV40, adenovirus 12 produced cytopathic effects (CPE); however, electron microscopically, after 72 hr, less than 1% of the cells contained adenovirus particles, and titrations showed no increase in virus. Most of the cells of both the singly and doubly infected cultures developed a characteristic "nuclear stippling," characterized by the appearance of clumps of intranuclear basophilic electron-dense material. Immunofluorescence studies with antisera prepared in rabbits against adenovirus 12 showed no viral antigen in the nuclei of the cells of cultures infected with adenovirus 12 only, in spite of the development of nuclear stippling (4). It was suggested that the stippling might represent accumulations of some newly synthesized material such as an "early protein," directed by the viral genome but not in-

corporated into the infectious particles. Recent studies by Huebner et al. (2) and Pope and Rowe (5) have shown that adenovirus 12 hamster tumors contain a viral specific antigen which is not present in the virion but which does appear early in the lytic cycle. They originally called this the "T" antigen but subsequently have referred to it as the "neoantigen." The present study was carried out to determine whether T antigen was formed in GMK cells infected with adenovirus 12 only and whether the nuclear stippling represents an accumulation of T antigen.

MATERIALS AND METHODS

Cell cultures. Primary GMK cell cultures and primary human embryo kidney cell cultures were obtained from Microbiological Associates, Inc., Bethesda, Md. The GMK cell cultures used for immunofluorescence studies were grown on cover slips in Leighton tubes. After virus infection, the cultures were maintained in a medium composed of 2% fetal bovine serum and 98% mixture 199, incubated at 36.5 C.

Viruses. SV40 strain Vac 777L1, obtained from Paul Gerber, Division of Biologics Standards, National Institutes of Health, and the prototype strain

of adenovirus 12, obtained from the American Type Culture Collection, were used. SV40 was grown and titered in GMK cell cultures; adenovirus 12 was grown in a human epidermoid carcinoma cell line maintained in our laboratory, and was titered in human embryo kidney cell cultures by serial 10-fold dilutions with three to five tubes per dilution. Titrations were observed for 21 days, and end points were calculated by the Reed-Muench method.

Virus-infected cultures for immunofluorescence study. The methods for single and double virus infection have been previously described (3). Leighton tube cultures of GMK cells infected with adenovirus 12 only received $10^{6.2}$ TCID₅₀/tube. The GMK cell cultures infected with both viruses received $10^{6.3}$ TCID₅₀ of adenovirus 12 and $10^{8.9}$ TCID₅₀ of SV40. At 18, 30, and 48 hr after virus infection, the cover slips were removed from the Leighton tubes, air-dried, and fixed in acetone at room temperature for 10 min.

Virus for production of antiviral sera. GMK cell culture infected with SV40, and HEp 2 cell cultures infected with adenovirus 12, were allowed to proceed to complete CPE, and were frozen and thawed three times; the undiluted material was used for rabbit immunization.

Antisera. Antiviral sera were obtained from two rabbits for each virus by injecting, subcutaneously, 2 ml of a homogenate consisting of 0.8 ml of mineral oil, 0.2 ml of Arlacel A, and 1 ml of the tissue culture-virus preparation. At the same time, 0.5 ml of the tissue culture-virus preparation was injected intravenously and 0.25 ml was injected intracutaneously. At 1-week intervals thereafter for 5 weeks, the rabbits received 1.0 ml of the virus intraperitoneally and 0.25 ml intracutaneously. Blood was collected from the ear vein 10 days after the last injection. The sera were removed, and the globulin fraction was precipitated with half-saturated ammonium sulfate. After dialysis against phosphate-buffered saline (PBS; pH 7.4) to remove the ammonium sulfate, the globulin fraction was labeled with 0.02 mg of fluorescein isothiocyanate per mg of protein for 18 hr at pH 9.5 at 4 C. It was then dialyzed against PBS (pH 7.4) until the dialysate no longer contained fluorescent dye. The fluorescein-labeled globulin fraction was then absorbed with 0.1 ml per ml of globulin of the same (noninfected) tissue culture cells which had been used for growth of the viruses. This adsorption procedure was repeated three times, to remove antibodies to the nonviral antigens in the tissue culture preparations. The precipitate was removed after each absorption by centrifugation at $18,000 \times g$ for 20 min. The serum was then further absorbed twice with 50 mg per ml of globulin, with bovine liver-acetone powder to reduce nonspecific fluorescence.

The SV40 antiviral serum was tested by immunofluorescence on GMK cells infected with SV40 and on uninfected cells. The SV40-infected cells were also treated with unlabeled immune serum, followed by labeled immune serum. The same procedures were carried out with the adenovirus 12 antisera tested against adenovirus 12-infected and SV40-infected tissue culture cells. The lack of cross-reactivity between the sera and the virus-infected cells, the absence of reaction with uninfected cells, and the inter-

ference with the reaction by pretreatment of the cells with unlabeled immune sera established the reactivity and specificity of the antiviral sera.

Anti-T sera were obtained from hamsters bearing large, transplanted tumors which had been induced by SV40 or adenovirus 12. Similarly, preliminary immunofluorescence tests of the hamster anti-T sera on virus-free SV40 and adenovirus 12 tumors in tissue culture established the reactivity and specificity of the hamster anti-T sera.

Fluorescent-antibody technique. The acetone-fixed tissue culture cells infected with adenovirus 12 or adenovirus 12 plus SV40 were placed in a petri dish containing a piece of filter paper moistened with PBS. Rhodamine-labeled bovine albumin (RBA) was placed on the cells for 20 min. This was washed off by three changes of PBS at 5-min intervals. The fluorescein-labeled antiserum was then applied for 20 min, followed by three more PBS washes. The cover slips were mounted on slides in 25% glycerol in PBS, and were examined with a Leitz ultraviolet microscope with the UG 12 and OG 1 filter system.

The indirect technique was used for the hamster anti-T sera. For these studies, the cover slips were first covered with the hamster serum for 20 min. This was washed three times in PBS, followed by exposure to RBA for 20 min. After three 5-min PBS washes, fluorescein-labeled rabbit anti-hamster globulin (BBL) was applied for 20 min. This was then washed in PBS as before and mounted on a slide for microscopic examination.

RESULTS

GMK tissue culture cells infected with adenovirus 12 only, or with adenovirus 12 plus SV40 simultaneously, were examined 18, 30, and 48 hr after virus inoculation for the presence of SV40 and adenovirus 12 viral antigens and T antigens (Table 1). As previously observed (4), adenovirus 12 viral antigen was not demonstrable at 30 hr in the cells infected with adenovirus 12 only, and at 48 hr was found only in a rare cell. A weak antiviral reaction, however, did occur at 30 hr in the GMK cell nuclei infected with both viruses, and, by 48 hr, fluorescence was well developed and strong in about 40% of the cells. In contrast, nuclear fluorescence with antisera against adenovirus 12 T antigen was observed after 18 hr in most cells infected with adenovirus 12 only or with adenovirus 12 plus SV40, and this reactivity persisted through the 48-hr study. In addition, the cultures infected with adenovirus 12 only showed a "flecklike" cytoplasmic fluorescence, which was much less evident in the doubly infected cultures.

SV40 viral antigen was detectable in the doubly infected cultures at 30 and 48 hr, but the reaction was not strong. The reaction for SV40 T antigen was strongly positive at 18 hr in the doubly infected cultures, but the intensity of fluorescence and the number of cells reacting decreased with time from 18 to 48 hr.

TABLE 1. Immunofluorescent localization of viral and T antigens of adenovirus 12 and SV40 in GMK cells infected with adenovirus 12 or adenovirus 12 mixed with SV40

Antiserum	Adenovirus 12 only (18 hr)	Adenovirus 12 plus SV40 (18 hr)	Adenovirus 12 only (30 hr)	Adenovirus 12 plus SV40 (30 hr)	Adenovirus 12 only (48 hr)	Adenovirus 12 plus SV40 (48 hr)
Adenovirus 12 (antiviral rabbit serum)	Not done	Not done	Negative	+	++++	++++
Adenovirus 12 (anti-T antigen, hamster serum)	++++*	+++ (most cells)	++++	++++	++++	++++
SV40 (antiviral rabbit serum)	Not done	Not done	Cytoplasm "fleck" (most cells) Negative	Some cytoplasm "fleck" (most cells) +	Negative	+
SV40 (anti-T antigen, hamster serum)	Negative	+++ (most cells)	Negative	++++ (40% of cells)	Negative	+++ (most cells) ++ (20% of cells)

* Plus signs indicate degree of nuclear fluorescence.

DISCUSSION

The results of this study are consistent with the hypothesis previously suggested (4) that adenovirus 12 is able to infect the GMK cells but requires a factor provided by SV40 to progress to the formation of complete virus. The presence of the T antigen of the adenovirus 12 in the infected cells indicates that the interference with complete virus formation occurs at a stage after the T antigen is formed.

The appearance of the nuclear "flecks" of T antigen with immunofluorescence suggests that the nuclear stippling observed by O'Connor et al. (4) in preparations stained by hematoxylin-eosin, and by electron microscopy, represents accumulations of T antigen. It is possible, however, that the nuclei contain multiple flecks of T antigen as well as accumulations of some other material, and that the two are not identical. Electron microscopic studies with ferritin-labeled antisera to adenovirus 12 T antigen should provide conclusive evidence of the relationship of the nuclear stippling to T antigen.

The fluorescent cytoplasmic "flecks" present in the GMK cells infected with adenovirus 12 when these cells were tested with the hamster anti-adenovirus 12 T antigen sera were particularly interesting because these "flecks" were much less evident when the GMK cells were infected with both adenovirus 12 and SV40. Since the presence of SV40 plus adenovirus 12 does lead to the formation of complete adenovirus 12, the cytoplasmic flecks may be related to the interruption of adenovirus 12 production after the formation of T antigen.

The significance of the continued presence of the adenovirus 12 T antigen at 48 hr, in contrast to a gradual decrease in the SV40 T antigen with time, is not known. In the hamster embryo kidney cell studies of Pope and Rowe (5), the T

antigen of adenovirus 12 began to disappear after 48 hr. Interpretation of these findings will probably have to await elucidation of the nature and function of the adenovirus 12 T antigen.

Evidence related to these findings has recently been reported by Gilead and Ginsberg (1). Using complement-fixation and fractionation procedures, they demonstrated that the T antigen of adenovirus 12 is distinct from the viral antigen, produced earlier, and that it is not dependent upon deoxyribonucleic acid synthesis, as is the viral antigen.

LITERATURE CITED

1. GILEAD, Z., AND H. S. GINSBERG. 1965. Characterization of a tumorlike antigen in type 12 and type 18 adenovirus-infected cells. *J. Bacteriol.* **90**:120-125.
2. HUEBNER, R. J., W. P. ROWE, H. C. TURNER, AND W. T. LANE. 1963. Specific adenovirus complement fixing antigens in virus-free hamsters and rat tumors. *Proc. Natl. Acad. Sci. U.S.* **50**:379-398.
3. O'CONNOR, G. T., A. S. RABSON, I. K. BEREZESKY, AND F. J. PAUL. 1963. Mixed infections with simian virus 40 and adenovirus 12. *J. Natl. Cancer Inst.* **31**:903-917.
4. O'CONNOR, G. T., A. S. RABSON, R. A. MALMGREN, I. K. BEREZESKY, AND F. J. PAUL. 1965. Morphologic observations of green monkey kidney cells after single and double infection with adenovirus 12 and simian virus 40. *J. Natl. Cancer Inst.* **34**:679-693.
5. POPE, J. H., AND W. P. ROWE. 1964. Immunofluorescent studies of adenovirus 12 tumors and of cells transformed or infected by adenoviruses. *J. Exptl. Med.* **120**:577-588.
6. RABSON, A. S., G. T. O'CONNOR, I. K. BEREZESKY, AND F. J. PAUL. 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV-40. *Proc. Soc. Exptl. Biol. Med.* **116**:187-190.