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Interaction of a Simian Papovavirus and Adenoviruses

I. Induction of Adenovirus Tumor Antigen During Abortive Infection of Simian Cells

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ABSTRACT

FELDMAN, LAWRENCE A. (Baylor University College of Medicine, Houston, Tex.), JANET S. BUTEL, AND FRED RAPP. Interaction of a simian papovavirus and adenoviruses. I. Induction of adenovirus tumor antigen during abortive infection of simian cells. J. Bacteriol. 91:813–818. 1966.—Adenovirus types 2, 7, and 12 undergo an abortive growth cycle in green monkey kidney cells; they induce the formation of adenovirus tumor antigen, but synthesis of adeno capsid antigen and infectious adenovirus was observed only when cultures were concomitantly infected with a simian papovavirus (SV40). Several other viruses, including herpes simplex and measles which replicate in monkey cells, and rabbit papilloma and human wart papovaviruses which do not, failed to stimulate adenovirus replication in the monkey cells. Adenovirus tumor antigen was detected 8 to 10 hr postinfection by immunofluorescent techniques. The antigen induced by adenovirus types 2 and 7 appeared as intranuclear masses; adenovirus type 12 tumor antigen also appeared as cytoplasmic and nuclear flecks. Sera from hamsters bearing tumors induced by adenovirus type 12 cross-reacted with tumor antigens induced by types 2 and 7 but not with antigens induced by SV40.

Previous reports have indicated that a simian papovavirus (SV40) enhances the replication of adenovirus types 5, 12, and 7 (3, 14, 17) in African green monkey kidney (GMK) cells. In the absence of SV40, nuclear stippling was observed by electron microscopy, but this was unaccompanied by the production of any particles resembling adenovirus (15). The purpose of the present investigation was to study in greater depth the effect of SV40 and other viruses on the replication of adenoviruses in simian cells. One particular aim was to determine whether adenoviruses which do not multiply in simian cells can induce the synthesis of new cellular antigens related to those found in adenovirus-induced tumors (9).

MATERIALS AND METHODS

Cell cultures. Primary GMK cells were grown in 1-oz (ca. 30-ml) bottles as previously described (13) in Melnick-Hanks lactalbumin hydrolysate (M-H) medium containing 2% calf serum. Secondary cultures grown on 15-mm cover glasses in plastic petri dishes were utilized for the detection of antigens by immunofluorescence (20). Primary human embryonic kidney (HEK) cells were grown in 35-mm plastic petri dishes or in 1-oz bottles in M-H medium with 10% fetal calf serum. BSC-1 cells, a continuous line of green monkey kidney cells (7), were grown in 1-oz bottles in Eagle's medium supplemented with 10% calf serum.

Viruses. The SV40 virus used in these studies was the Baylor reference strain described in detail elsewhere (12, 19); the virus had been passaged six times in GMK cells in this laboratory. Type 7 adenovirus was derived from an adenovirus-SV40 "hybrid" population (8, 22, 24), and was plaque-purified three times in HEK cells. This purified strain replicates in HEK cells, but does not induce the synthesis of SV40 tumor antigen (2). Similar results were obtained with a strain of adenovirus type 7 isolated and propagated exclusively in HEK cells (1).

The parent strain of the "hybrid" virus contained small 20-mμ particles (11), but after the plaque-purification the adenovirus progeny were free from these particles. The adenovirus type 2 (a fresh Baylor isolate) and type 12 (obtained from Robert J. Huebner) were propagated in KB cells. Although these virus strains had previously been shown to be devoid of the 20-mμ particles (11), all adenovirus stocks used in this study were re-examined extensively by electron micro-

scope techniques and were shown to be free from the small particles.

Assays. SV40 was assayed in GMK cells by the plaque technique (25), and adenovirus plaque titers were obtained in HEK cells (2, 3). Adenovirus yields from double infections were obtained by direct plating of the virus on HEK cells, since SV40 did not interfere with the assay. SV40 in mixed harvests was titrated after the virus suspension was heated at 50 C for 30 min to inactivate the adenovirus (26).

Immunofluorescent techniques. Procedures used in this laboratory for the immunofluorescent detection of SV40 tumor (T) and viral (V) antigens have been described in detail (12, 19, 20). Adenovirus antigens were detected by similar techniques. To detect the adenovirus T antigen, infected cells on cover slips were reacted with sera from hamsters bearing tumors induced either by the adenovirus-SV40 "hybrid" or by adenovirus type 12 followed by fluorescein-labeled antihamster globulin. Sera from hamsters with primary tumors, as well as from hamsters carrying transplanted tumors, were used. The reactions seen with both types of sera were similar. The adenovirus V antigen was detected by reacting infected GMK cultures with antiadenovirus sera prepared in rabbits. Tests for SV40 and adenovirus T antigens were carried out 24 hr after infection except where otherwise noted; those for the respective V antigens were performed 48 hr postinfection. All tests included noninfected cells which failed to react with the positive sera and infected cells which failed to react with proven negative sera. Preparations were examined with a Zeiss fluorescence microscope equipped with a dark-field condenser and with an Osram HBO 200 mercury arc vapor lamp for illumination.

RESULTS

Enhancement experiments were carried out by simultaneously infecting GMK cells (about 10^6 cells in 1-oz bottles) with SV40 and with adenovirus. Each culture was inoculated with a multiplicity of 4 plaque-forming units (PFU) of adenovirus per cell (representing $10^{5.8}$ PFU per ml) and with 2 PFU of SV40 per cell. The viruses were allowed to adsorb for 1 hr at 37 C, and the monolayers were then washed twice with warm tris(hydroxymethyl)aminomethane (Tris)-saline buffer (*p*H 7.4) to remove unadsorbed virus. Cultures were harvested by three cycles of quick freezing and thawing at various intervals after infection, and total adenovirus yields (intra- and extracellular) were determined by titrating the suspensions in HEK cells. The results of a typical experiment, in which the two viruses were inoculated simultaneously, are presented in Table 1. A rapid decrease in infectious adenovirus occurred during the 1st hr after inoculation. In cultures infected only with adenovirus, a gradual decrease in titer continued; at no time (throughout the 72-hr observation period) was an increase in infectious virus detected. This decrease in infectious virus in the cultures infected only with adenovirus contrasted sharply with the amount of adenovirus recovered from cultures simultaneously inoculated with SV40. In such mixed infections, the adenovirus titers after 72 hr were 1,000- to 1,800-fold higher than the corresponding titers from cells infected only with adenovirus. Cultures infected with adenovirus and SV40 showed a progressive cytopathic effect (CPE) typical of adenovirus, whereas only about 10% of the cells in the cultures infected singly with adenovirus showed cytopathic changes, and the CPE in such cultures failed to progress. Even when cultures were held for 120 hr, adenovirus failed to replicate.

TABLE 1. *Effect of SV40 on replication of adenovirus types 2 and 7 in green monkey kidney cells*

"Enhancing" virus	Adenovirus	Titer of adenovirus ($\log_{10}$ PFU/ml)			Enhancement at 72 hr
		1 hr*	24 hr	72 hr	
None	Type 7	4.7	3.9	3.8	—
SV40	Type 7	4.5	4.7	6.8	1,000-fold
None	Type 2	4.9	4.5	4.0	—
SV40	Type 2	5.0	5.6	7.3	1,800-fold

* Time postinfection.

Enhancement studies with other viruses were carried out to determine whether enhancement of adenoviruses in GMK cells was specific for SV40. The results of these experiments (Table 2) indicate the specificity of the enhancement phenomenon. Neither herpes simplex virus, an intranuclear deoxyribonucleic acid (DNA) virus, nor measles virus, a cytoplasmic ribonucleic acid (RNA) virus, enhanced replication of adenovirus type 7, although the "enhancing" viruses were capable of replicating in GMK cultures (18). The two papovaviruses (rabbit papilloma and human wart virus) did not cause CPE in these cultures and did not enhance replication of the adenovirus. Conversely, replication of herpes simplex or of measles virus was not enhanced by the co-infecting adenovirus (Table 2).

Enhancement experiments in BSC-1 cells in which SV40 was inoculated 24 hr prior to adenovirus type 7 yielded results similar to those obtained in GMK cultures. SV40 did not enhance the replication of adenovirus type 7 in HEK cultures, and the replication of SV40 in GMK cultures was not affected by the presence of adenovirus, confirming earlier results (3).

Induction of virus-specific antigens, detectable by immunofluorescence, was compared in singly

TABLE 2. *Failure of other viruses to enhance the replication of adenovirus in green monkey kidney cells*

Virus tested for enhancing activity	Adenovirus*	Yield of adenovirus (log_{10} PFU/culture) 72 hr postinfection	Yield of "enhancing" virus (log_{10} PFU/culture)	Enhancement of adenovirus 72 hr postinfection
None	Type 7	5.7	—	
Herpes simplex	Type 7	6.0	7.0†	Twofold
Herpes simplex	—	—	6.6	
None	Type 7	5.6	—	
Measles	Type 7	5.5	6.0‡	None
Measles	—	—	6.3	
None	Type 7	5.2	Not done	
Rabbit papilloma	Type 7	5.0	Not done	None
None	Type 7	5.2	Not done	
Human wart	Type 7	5.0	Not done	None

* Adenovirus input per culture was $10^{7.0}$ PFU.
† Herpes simplex titers 72 hr postinoculation of virus; input per culture was $10^{5.3}$ PFU.
‡ Measles titers 96 hr postinoculation of virus; input per culture was $10^{4.0}$ PFU.

TABLE 3. *Formation of antigens in GMK cells infected with adenovirus in the presence and absence of SV40**

Virus	Adenovirus-induced antigens		SV40-induced antigens	
	Tumor	Viral	Tumor	Viral
Adenovirus type 2	+	0	0	0
Adenovirus type 7	+	0	0	0
Adenovirus type 12	+	0	0	0
SV40	0	0	+	+
SV40 + adenovirus types 2, 7, or 12	+	+	+	+

* Symbols: + = presence of antigen detectable by immunofluorescence; 0 = absence of antigen detectable by immunofluorescence.

and doubly infected cultures of GMK cells (Table 3). In cells infected only with type 2, 7, or 12 adenovirus, T antigen was synthesized, but the corresponding V antigen was not detected. T antigen was detected in 75 to 100% of the cells when the multiplicity of adenovirus was greater than 3 PFU per cell. GMK cultures infected with SV40 and adenoviruses produced adenovirus T and V (in 100% of the cells) antigens in addition to SV40 T and V antigens.

The adenovirus T antigens induced by adenovirus types 2 and 12 in GMK cultures are shown in Fig. 1 and 2. The type 2 T antigen appeared as large, intranuclear masses with little cytoplasmic involvement (Fig. 1); localization and morphology of type 7 T antigen was similar to that of type 2. The type 12 T antigen (Fig. 2) also appeared as intranuclear masses but intranuclear and cytoplasmic "flecks" were also commonly observed. When high multiplicities of infection were used, the adenovirus T antigens could be detected 8 to 10 hr postinfection. The appearance of the T antigens agreed with the previous observations of Pope and Rowe (16), and differed from SV40 T antigen; the latter antigen is also intranuclear but is generally represented by fine, granular bodies. Furthermore, the SV40 T antigen could not be detected in GMK cells prior to 18 hr postinfection and, therefore, both its appearance and time of synthesis serve to differentiate it from the adenovirus T antigen.

The specificity of the positive sera used to detect the different tumor antigens is illustrated in Table 4. Sera from animals bearing adenovirus type 12-induced tumors reacted with T antigens in GMK cells infected with adenovirus types 2, 7, and 12 but not with SV40 T antigen. Serum from an animal bearing an adenovirus-SV40 "hybrid"-induced tumor reacted with both the adenovirus and SV40 T antigens, whereas sera from animals bearing tumors induced by SV40-transformed cells reacted with T antigen induced by SV40 but did not react with adenovirus antigens.

DISCUSSION

The results of these experiments indicate that adenovirus types 2, 7, and 12 fail to replicate in GMK cultures in the absence of SV40. Since the adenoviruses were able to induce the synthesis of adenovirus T antigen, the inability of the viruses to replicate does not appear to be due to a failure of the virus to penetrate or become uncoated. It is

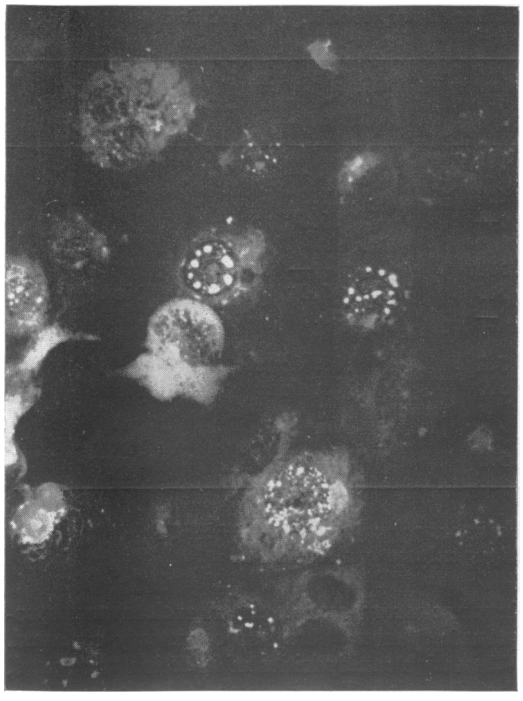

FIG. 1. *Immunofluorescent photomicrograph of green monkey kidney cells inoculated 24 hr previously with adenovirus type 2. Reacted with serum from hamster bearing tumor induced by adenovirus type 12 and with fluorescein-labeled anti-hamster globulin* × *330.*

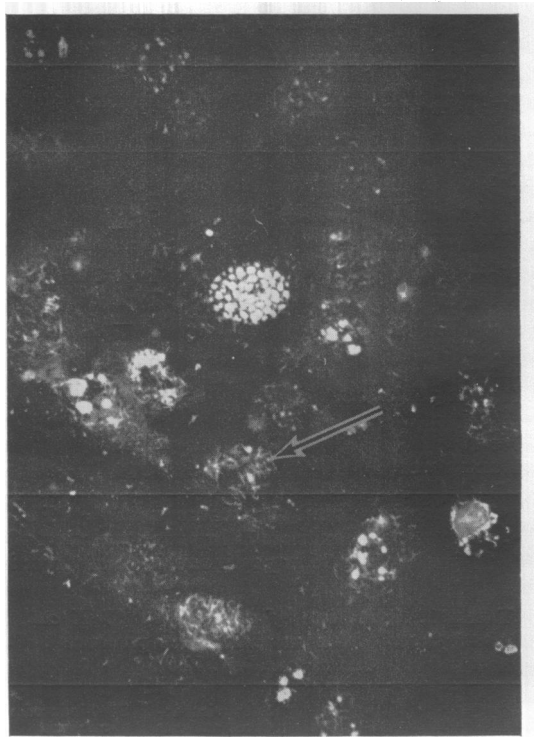

FIG. 2. *Immunofluorescent photomicrograph of green monkey kidney cells inoculated 24 hr previously with adenovirus type 12. Reacted with serum from hamster bearing tumor induced by adenovirus type 12 and with fluorescein-labeled anti-hamster globulin. Arrow points to a cell containing flecks of adenovirus type 12 tumor antigen.* × *330.*

noteworthy that the adenovirus T antigens can be detected as early as 8 to 10 hr after infection of GMK cells. The early appearance of the adenovirus T antigen was recently reported by Gilead and Ginsberg (4). The T antigens induced by adenoviruses appear earlier than those induced by SV40 in GMK cells. In GMK cells infected with adenovirus, O'Conor et al. (15) observed an intranuclear stippling that may represent accumulation of adenovirus T antigen. Since viral DNA synthesis is not required for the induction of SV40 or adenovirus T antigens (4, 19), the abortive cycle of adenoviruses in GMK cells may be due to a lack of replication of viral DNA. This would be reflected by failure of the cells to synthesize viral capsid antigen, a process requiring DNA synthesis (4).

The helper virus activity of SV40 for adenovirus replication, first observed by O'Conor et al. (14), appears to be virus-specific, since measles, herpes simplex, human wart, and rabbit papilloma viruses failed to enhance replication. The enhancement phenomenon, therefore, does not appear to be a generalized effect attributable to the papovavirus group, but only to that papova-

TABLE 4. *Specificity of sera used for the detection of tumor antigens in simian cells*

Serum	GMK cells infected with			
	Adenovirus 2	Adenovirus 7	Adenovirus 12	SV40
SV40-tumor hamster serum*	0	0	0	+
SV40-adenovirus 7 hybrid tumor hamster serum†	+	+	+	+
Adenovirus type 12 tumor hamster serum‡	+	+	+	0

* Sera from animals bearing tumors induced by hamster cells transformed by SV40, diluted 1:10.

† Sera from hamsters bearing tumors induced by the adenovirus-SV40 hybrid, diluted 1:2.

‡ Sera from hamsters bearing tumors induced by adenovirus type 12, diluted 1:4.

virus which replicates in GMK cells. Extracts of GMK cells infected with SV40 and filtered through a 10-mμ Millipore filter were also unable to initiate adenovirus replication.

The failure of the adenoviruses tested to replicate by themselves in monkey kidney cells is in accord with recent findings that certain adenoviruses capable of replicating in simian cells were contaminated with SV40 or with particles containing SV40 genetic material (8, 22, 24). It is the presence of such particles, named PARA (21), that enables the adenovirus particles in the population to replicate (21, 23). Similar situations of helper virus activity have also been described among the RNA viruses: RAV as a helper for Rous sarcoma virus (5) and hog cholera (10) and parainfluenza (6) for Newcastle disease virus.

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LITERATURE CITED

1. BENYESH-MELNICK, M., AND H. S. ROSENBERG. 1964. The isolation of adenovirus type 7 from a fatal case of pneumonia and disseminated disease. J. Pediat. **64**:83–87.
2. BOEYÉ, A., J. L. MELNICK, AND F. RAPP. 1965. Adenovirus-SV40 "hybrid": plaque purification into lines in which the determinant for the SV40 tumor antigen is lost or retained. Virology **26**:511–512.
3. FELDMAN, L. A., J. L. MELNICK, AND F. RAPP. 1965. Influence of SV40 genome on the replication of an adenovirus-SV40 "hybrid" population. J. Bacteriol. **90**:778–782.
4. 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.
5. HANAFUSA, H., T. HANAFUSA, AND H. RUBIN. 1963. The defectiveness of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S. **49**:572–580.
6. HERMODSSON, S. 1963. Inhibition of interferon by an infection with parainfluenza virus type 3 (PIV-3). Virology **20**:333–343.
7. HOPPS, H. E., B. C. BERNHEIM, A. NISALAK, J. H. TJIO, AND J. E. SMADEL. 1963. Biologic characteristics of a continuous kidney cell line derived from the African green monkey. J. Immunol. **91**:416–424.
8. HUEBNER, R. J., R. M. CHANOCK, B. A. RUBIN, AND M. J. CASEY. 1964. Induction by adenovirus type 7 of tumors in hamsters having the antigenic characteristics of SV40 virus. Proc. Natl. Acad. Sci. U.S. **52**:1333–1340.
9. HUEBNER, R. J., W. P. ROWE, H. C. TURNER, AND W. T. LANE. 1963. Specific adenovirus complement-fixing antigens in virus-free hamster and rat tumors. Proc. Natl. Acad. Sci. U.S. **50**: 379–389.
10. MATUMOTO, M., T. KUMAGAI, T. SHIMIZU, AND S. IKEDA. 1961. A new *in vitro* method (END) for detection and measurement of hog cholera virus and its antibody by means of effect of HC virus on Newcastle Disease Virus in swine tissue culture. J. Immunol. **87**:257–268.
11. MELNICK, J. L., H. D. MAYOR, K. O. SMITH, AND F. RAPP. 1965. Association of 20-millimicron particles with adenoviruses. J. Bacteriol. **90**: 271–274.
12. MELNICK, J. L., S. E. STINEBAUGH, AND F. RAPP. 1964. Incomplete simian papovavirus SV40. Formation of non-infectious viral antigen in the presence of fluorouracil. J. Exptl. Med. **119**: 313–326.
13. MELNICK, J. L., H. A. WENNER, AND L. ROSEN. 1964. The enteroviruses, p. 194–242. *In* E. H. Lennette and N. J. Schmidt [ed.], Diagnostic procedures for viral and rickettsial diseases, 3rd ed. American Public Health Association, New York.
14. O'CONOR, G. T., A. S. RABSON, I. K. BEREZESKY, AND F. J. PAUL. 1963. Mixed infection with simian virus 40 and adenovirus 12. J. Natl. Cancer Inst. **31**:903–917.
15. O'CONOR, 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.
16. 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.
17. RABSON, A. S., G. T. O'CONOR, I. K. BEREZESKY, AND F. J. PAUL. 1964. Enhancement of adenovirus growth in African green monkey kidney cell cultures by SV40. Proc. Soc. Exptl. Biol. Med. **116**:187–190.
18. RAPP, F. 1964. Plaque differentiation and replication of virulent and attenuated strains of measles virus. J. Bacteriol. **88**:1448–1458.
19. RAPP, F., J. S. BUTEL, L. A. FELDMAN, T. KITAHARA, AND J. L. MELNICK. 1965. Differential effects of inhibitors on the steps leading to the formation of SV40 tumor and virus antigens. J. Exptl. Med. **121**:935–944.
20. RAPP, F., J. S. BUTEL, AND J. L. MELNICK. 1964. Virus-induced intranuclear antigen in cells transformed by papovavirus SV40. Proc. Soc. Exptl. Biol. Med. **116**:1131–1135.
21. RAPP, F., J. S. BUTEL, AND J. L. MELNICK. 1965. SV40-adenovirus "hybrid" populations: transfer of SV40 determinants from one type of adenovirus to another. Proc. Natl. Acad. Sci. U.S. **54**:717–724.
22. RAPP, F., J. L. MELNICK, J. S. BUTEL, AND T. KITAHARA. 1964. The incorporation of SV40

genetic material into adenovirus 7 as measured by intranuclear synthesis of SV40 tumor antigen. Proc. Natl. Acad. Sci. U.S. **52**:1348–1352.

23. ROWE, W. P. 1965. Studies of adenovirus-SV40 hybrid viruses. III. Transfer of SV40 gene between adenovirus types. Proc. Natl. Acad. Sci. U.S. **54**:711–717.
24. ROWE, W. P., AND S. G. BAUM. 1964. Evidence for a possible genetic hybrid between adenovirus type 7 and SV40 viruses. Proc. Natl. Acad. Sci. U.S. **52**:1340–1347.
25. STINEBAUGH, S., AND J. L. MELNICK. 1962. Plaque formation by vacuolating virus, SV_{40}. Virology **16**:348–350.
26. YANG, C., AND J. L. MELNICK. 1963. Contamination of adenovirus stocks with SV40 (papovavirus group). Proc. Soc. Exptl. Biol. Med. **113**:339–343.