THE PRESENCE OF ADENOVIRUS-SPECIFIC ANTIGENS IN HAMSTER CELLS RENDERED NEOPLASTIC BY ADENOVIRUS 1-SV40 AND ADENOVIRUS 2-SV40 HYBRID VIRUSES*

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Extensive studies concerning the oncogenicity of human adenoviruses (Ad.) in newborn hamsters have demonstrated a lack of oncogenic potential in the strains of Ad. 1, 2, and 5 tested thus far. Such studies have resulted in the classification of these serotypes in the nononcogenic subgroup (group C) which differs from the mildly oncogenic subgroup (group B) (including Ad. 3, 7, 14, 16, and 21) and the highly oncogenic subgroup (group A) (including Ad. 12, 18, and 31) with respect to the guanine plus cytosine (G-C) content of the viral DNA, oncogenic potential for hamsters, and hemagglutination characteristics.1

Studies of T antigens have demonstrated that virus-free cells rendered neoplastic by oncogenic adenoviruses as well as Simian virus 40 (SV40) and polyoma viruses contain specific viral genetic determinants which are integrated within these neoplastic cells.2-4 By "integration" we mean that viral genetic determinants have become heritable cell factors. In addition, studies of hybrid-virus populations obtained by mixed infection of monkey kidney cells with SV40 and oncogenic adenoviruses types 7 and 12 demonstrated marked oncogenicity and ability to transform cells in vitro, and suggested the integration in the tumor and transformed cells of both SV40 and adenovirus genetic material.5

We have recently found that the "hybrid" virus populations obtained by growing nononcogenic Ad. 1, 2, and 5 with SV40 are likewise capable of inducing tumors in hamsters in vivo,6 and that the Ad. 2 hybrid produces transformation of hamster cells in vitro.7 These tumors and transformed cells provided an approach to the question of whether genetic material from nononcogenic adenoviruses could be integrated into neoplastic cells.

This report describes studies utilizing serologic markers to show that both SV40 and adenovirus genetic material are integrated in tumor and transformed cells induced by populations of nononcogenic adenoviruses which are hybrids with SV40.

Materials and Methods—Tumor and transformed cell lines: Three lines of cells were studied. The Ad. 1++ hybrid virus strain previously described9 was inoculated into newborn hamsters. A tumor which developed 142 days later was carried through two transplant generations in newborn hamsters and thereafter as a cell line in tissue culture; the cell line is referred to here as Ad. 1++ TL-1.

The Ad. 2++ HK-1 and HK-2 cell lines,7 were obtained by transforming weanling hamster kidney (WHK) cells in vitro with the Ad. 2++ hybrid virus population.10 The derivation of these lines and the characteristics of the tumors produced on transplantation to hamsters have been described.7 11

The three cell lines, both as tumor and tissue culture cells, were repeatedly tested for virus. Culture fluids and 10% extracts of tissue culture cells and tumors were inoculated into human embryonic kidney (HEK) and African green monkey kidney (AGMK) cell cultures, with blind passage at 3-4 weeks; in 20 such tests no adenovirus was ever isolated, but two Ad. 2++ tumor extracts yielded small amounts of SV40.7 In addition, viable tumor or tissue culture cells were
tested by the overlay procedure on IIEK and AGMK cells, with blind passage after 2–3 weeks; of five tests with Ad. 1++ TL-1 and four tests with the Ad. 2++ cell lines, no infectious adenovirus was ever recovered, but the Ad. 2++ cell lines yielded SV40.

Clonal sublines of Ad. 1++ TL-1 and Ad. 2++ HK-1 lines were obtained by a modification of the agar suspension colony technique of MacPherson and Montagnier. Cell lines were maintained for three to five passages in medium containing 1% homologous adenovirus rabbit antiserum (neutralizing antibody titer 1:320 to 1:640) prior to cloning. The cells were dispersed with trypsin and 5–10^6 and 10^6 cells planted in 0.33% agar; in the cloning of the Ad. 2++ HK-1 line, the agar overlay also contained 1% antiserum. The cells were observed to be monodispersed at the time of counting, the clump rate being less than 1%. At 3–6 weeks well isolated colonies were picked with Pasteur pipettes and grown to mass cultures.

Serologic studies: The approach which we have utilized in previous studies of adenovirus and SV40 hamster tumors to demonstrate by means of serologic techniques that viral genetic material is integrated in tumor cells has been to: (1) detect new antigens in tumor cells; (2) show that these antigens are virus-specific and unlikely to be coded for by host cell genes; and (3) demonstrate that the antigen is produced by all the cells. The detection of virus-specific antigens in the tumor cells has been based almost exclusively on T antigens (those viral antigens which form early and presumably prior to viral DNA replication in the acute infectious cycle), but Ad. 12 tumors contain certain viral structural antigens as well. Two approaches can be used for detection of the antigens; directly by complement fixation (CF) or fluorescent antibody (FA) testing against a known antiserum, or indirectly by showing that the cells stimulate formation of specific antibodies. Demonstrating the antigen in all cells can be done by the direct approach, using the FA procedure, or by deriving clones and testing these for immunogenicity.

Because of the SV40 T antigen reactions, the indirect approach had to be used predominantly in the present studies. Sera from hamsters carrying the hybrid virus tumors uniformly contained antibody to the SV40 T antigen; consequently, serologic tests of these sera against the tumors or cells infected with the hybrid viruses could not give definitive information about adenovirus antigens because they also contained SV40 T antigen.

Consequently, emphasis was placed on testing the ability of the cell lines to stimulate antibodies in hamsters against nonhybrid Ad. 1 and 2. Since initial studies using the CF test gave only minimal evidence of antibody, all studies reported here were done by the FA procedure. Three types of antisera were used:

(1) Serum from tumor-bearing hamsters: Ad. 1++ tumor sera were obtained from weanling hamsters bearing first- to third-passage transplants of a tumor originally induced with the Ad. 1++ hybrid virus; Ad. 2++ tumor sera were obtained from weanling hamsters bearing first- to third-generation transplants of tumors originally induced with Ad. 2++ HK-1 and HK-2 cells. Hamsters were generally bled when their tumors reached a diameter of 3–7 cm.

(2) Ad. 2 T antigen-reactive immune serum: Detection of adenovirus antigens directly in the Ad. 2++ HK cell clones (see below) was carried out with an immune hamster serum prepared against an adenovirus T antigen preparation as follows. WHK cultures were infected with Ad. 2 at a virus:cell multiplicity of 10^4–10^5 in the presence of 10^-4 M 5-fluorodeoxyuridine (FUDR). A 10% cell pack was prepared 24–48 hr later, mixed with an equal volume of Freund’s complete adjuvant, and 0.5 ml was inoculated into each of the rear footpads of a hamster. The immune serum was obtained 14–16 days later. Although this serum stained Ad. 2 T antigen preparations predominantly, viral antigens were also stained. This serum did not stain control-hamster kidney cells; in addition, T and V antigen cover slips (see below) or Ad. 2++ HK tumor cells were not stained with a control hamster serum similarly prepared with uninfected hamster kidney cells.

(3) Hyperimmune ascites fluids: Hamsters were hyperimmunized for production of a hyperimmune ascitic exudate by a modification of a method recently described. A 20%-clarified extract prepared from a tumor induced with Ad. 2++ HK-1 cells, which was free of detectable infectious virus, was mixed with an equal volume of Freund’s adjuvant, and 1.0 ml of this mixture was inoculated into the peritoneal cavity of four hamsters every 5 days. After the fourth to the fifth inoculation, an accumulation of ascites fluid usually occurred. This fluid was harvested by paracentesis. These fluids stained “viral antigens” predominantly.

Antigens for FA tests consisted of cover slips containing HEK cells inoculated with the nonhybrid prototype Ad. 1 or 2 strains (virus:cell multiplicity 10^4 to 10^5) 16–48 hr prior to fixation.
“T antigen cover slips” and “V (viral) antigen cover slips” were prepared as described previously by maintaining the cultures with or without 10^{-4} M FUDR respectively. Staining of the T antigen cover slips with homologous adenovirus hyperimmune rabbit serum gave essentially negative results, less than 0.01% of the nuclei being stained; consequently, it is assumed that these cover slips contain primarily early antigens coded for by the input viral genome. V antigen cover slips, in contrast, were strikingly positive when stained with homologous adenovirus hyperimmune rabbit serum and contained antigens morphologically similar to those described by Boyer et al. For convenience these antigens will be referred to here as “viral antigens” but this does not imply that these antigens are part of the virion. V antigen cover slips contain T antigens as well. The adenovirus specificity of the antigens present in these cover slips was repeatedly tested by staining with normal hamster serum and with sera from hamsters bearing tumors induced with both SV40 and Schmidt-Ruppin viruses with uniformly negative results.

**Results.**—Presence of adenovirus antibodies in sera of tumor-bearing hamsters: The presence of adenovirus-specific antibodies in sera from hamsters bearing transplants of tumors induced with the Ad. 1++ virus or with Ad. 2++ HK cells was demonstrated by staining T and V antigen cover slips infected with the homotypic nonhybrid adenovirus.

Sixteen sera from hamsters with large tumors induced by the Ad. 1++ virus were screened at dilutions of 1:5-1:10; all stained both T and V antigen cover slips. Several of these sera were titrated and found to have FA-staining titers of 1:40-1:160. The T antigen staining was limited to the nuclei; these were generally filled with bright dots and ovoids or tiny, irregular particles distributed homogeneously throughout the nuclei imparting a bright fluorescent dust-like pattern. On V antigen cover slips, the dust-like pattern was seen with all 16 sera; nine of the sera also stained large, brilliant, irregular crystalline masses, as well as large balls and ring-like forms. Sera which stained the large balls and crystalline masses on V antigen cover slips generally presented both the dust-like and dot or ovoid-like stain on T antigen cover slips, while those which did not stain the larger masses imparted the dust-like pattern only. It is noteworthy that three of these sera were tested against T and V antigen cover slips infected with three different field isolates of Ad. 1 as well as the prototype strain, and all gave staining equivalent to that seen with the prototype. Also, these sera stained similar antigens in Ad. 1 T antigen cover slips made with African green monkey kidney and chick embryo fibroblast cells.

Sera from 23 hamsters bearing tumors induced with Ad. 2++ HK cells were screened at dilutions of 1:2-1:10 against Ad. 2 T and V antigen cover slips. Ten stained both T and V antigen cover slips, while 11 stained only V antigen cover slips. The titers of these sera were not as high as the Ad. 1++ hamster tumor sera, the sera that gave the brightest staining titering only 1:10-1:40. All staining was confined to the nucleus. In the T antigen cover slips the antigen was in the form of small dots and short flecks. Staining of V antigen cover slips was chiefly in the form of large, irregular, granular masses, frequently located at the nuclear membrane, with broad strands extending further into the nucleus. Sometimes ring-like masses, large balls, and a diffuse granular staining were also seen in the V antigen cover slips.

Further evidence of adenovirus antigens in Ad. 2++ HK-1 cells was obtained with the immune ascites fluids prepared by immunization with virus-free tumor extracts. All four immune ascites fluids stained Ad. 2 V, but not T antigen cover slips, the staining morphology being the same as seen with the tumor sera.

None of the Ad. 1++ or Ad. 2++ HK tumor sera or ascites fluids stained unimmunized HEK cells, and three sera from each group of hamsters did not stain HEK
cells infected with herpes simplex virus in the presence of 10^{-5} M FUDR. Moreover, absorption of two T-reactive Ad. 2^{++} HK tumor sera with an Ad. 2 WHK (FUDR) cell pack (see Materials and Methods), an Ad. 2^{++} HK-1 cell pack, and an Ad. 2^{++} HK-1 tumor extract resulted in a fourfold decrease in titer when these sera were reacted with Ad. 2 T antigen cover slips. Absorption with control preparations, consisting of an uninfected WHK cell pack, a 20 per cent extract of normal hamster brain and a Schmidt-Ruppin hamster tumor extract, resulted in little or no reduction in the percentage or intensity of staining.

Characterization of the Ad. 1 and 2 antigens in tumor and transformed cells: Studies were undertaken to further characterize the antigens present in the tumor or transformed cells by utilizing the reaction of the hamster tumor sera on T and V antigen cover slips. The T antigens of Ad. 1 were studied by testing with several individual T-reactive sera from Ad. 1^{++}-tumored hamsters, and those of Ad. 2 by tests with a pool of three T-reactive sera from hamsters with Ad. 2^{++} HK-1 tumors. Viral antigens were studied only with Ad. 2, using one of the immune ascites fluids.

Time-course studies with both Ad. 1 and Ad. 2 showed that the T antigens appeared at approximately eight hours, while viral antigens detected with homologous rabbit antiserum and with the Ad. 2^{++} immune ascites fluid appeared at 16-20 hours.

Heat-stability studies, carried out by heating fixed cover slips at 56\degree C for 30 minutes by the technique described previously, gave the same results as those obtained in studies of human antibodies. That is, T antigen reactivity was abolished, while viral antigens were only slightly affected by the heating.

The breadth of reactivity of the T and viral antibodies was tested with T and V antigen cover slips prepared with adenovirus serotypes representing the various subgroups. T-reactive Ad. 1^{++} and 2^{++} tumor sera cross-reacted completely, that is, the Ad. 1^{++} tumor sera always stained Ad. 2 T antigen cover slips and vice versa. Approximately 1/4 of these sera, however, stained dot- or fleck-shaped antigens in Ad. 7, and in the few cases tested, Ad. 3 T antigen cover slips as well. None of the sera tested stained T antigen cover slips prepared with Ad. 4, 8, or 12.

In contrast, the viral antibody in the ascites fluids was group reactive, in that all four fluids stained viral antigens of adenoviruses 1, 2, 7, and 12. About 25 per cent of the Ad. 2 viral antigen-reactive Ad. 2^{++} tumor sera stained Ad. 7 viral antigens; they were not tested against other serotypes. Moreover, absorption of an immune ascites fluid with standard viral stocks of Ad. 2 or 7 resulted in a loss of staining for both Ad. 2 and 7 V antigen cover slips. No Ad. 2-neutralizing antibody was found either in ten sera of hamsters bearing tumors induced with Ad. 2^{++} HK-1 and 2 cells or in the ascites fluid on which absorption studies were performed.

The above studies made it clear that the tumor and transformed cells contain adenovirus-specific antigens of both the early, FUDR resistant, and late, FUDR sensitive, varieties. The Ad. 1 and 2 T antigens are closely related to each other but have a distinctly different serologic reactivity from T antigens of the previously characterized adenovirus A and B subgroups. The occasional cross reactions with the B subgroup probably reflects the presence of a group-reactive T antigen in addition to the one which appears to be specific for the Ad. 1-Ad. 2 subgroup. Group reactivity of some of the T antigens detectable by FA is well established. The Ad. 1 T antigens detected with the Ad. 1 hamster tumor sera were morphologically
similar to those stained with human sera,\textsuperscript{18} but their relationship to these remains to be determined.

In contrast to the viral antigen present in Ad. 12 hamster tumors, which is the type-specific C\textsuperscript{18} or fiber antigen,\textsuperscript{18} the viral antigen in the Ad. 2\textsuperscript{++} -transformed cells is group reactive, and sera with this viral antibody did not neutralize Ad. 2. This may possibly represent the A or hexon antigen.\textsuperscript{18}

\textit{Studies of the proportion of cells containing adenovirus antigens:} Attempts to determine directly the percentage of cells containing adenovirus antigen(s) were made by staining the Ad. 2\textsuperscript{++} HK-1 and HK-2 cells with the Ad. 2 T-reactive immune serum, this being the only T-reactive hamster serum which would not stain the SV40 T antigen present in all these cells. With both cell lines, and with seven of the eight clonal lines from Ad. 2\textsuperscript{++} HK-1 described below, approximately 20–40 per cent of cells showed faint dot- and fleck-type staining in the nuclei. Although this was equivocal staining, it was not seen with SV40-transformed or control hamster kidney cells stained with the antiserum, and control antisera gave no staining of any of the Ad. 2\textsuperscript{++} cell lines. Attempts to stain Ad. 1\textsuperscript{++} TL-1 cells with Ad. 1\textsuperscript{++} hamster tumor sera absorbed with an SV40 hamster tumor extract or human sera containing high-titer Ad. 1 T antibody gave negative results.

On the other hand, studies of the antigenicity of the clonal lines gave clear-cut results. Ten clones of Ad. 1\textsuperscript{++} TL-1 and eight of Ad. 2\textsuperscript{++} HK-1 cells were carried as cell lines; as mentioned above, even though the parent cell lines were free of infectious adenovirus, they were carried for several passages in medium with neutralizing antiserum immediately prior to cloning to eliminate any possibility of spread of virus. Three to four passages after cloning, the ten clones derived from the Ad. 1\textsuperscript{++} TL-1 line were inoculated into hamsters. The cells were inoculated subcutaneously into weanling hamsters using 1 to 2 $\times$ 10\textsuperscript{6} cells per animal and sera were taken when tumors reached 3–5 cm in diameter. In addition, cell packs of the eight Ad. 2\textsuperscript{++} clonal lines were inoculated with adjuvant into the footpads of two hamsters, using the same immunization method as used for the Ad. 2 T antigenic immune serum. Two hamster sera per group were tested against T and V antigen cover slips of the homotypic adenovirus. Thirty-five of the 36 sera stained T antigens; about a third of the sera in each of the two groups also stained viral antigens. Thus, every one of the 18 clones contained adenovirus antigens.

It should be noted that all of the Ad. 2\textsuperscript{++} HK-1 clones also showed SV40 T antigen in essentially all nuclei when stained with a serum pool from hamsters bearing SV40 tumors. The Ad. 1\textsuperscript{++} TL-1 clones were not tested in this manner but all stimulated the production of SV40-specific T antibody in the tumor-bearing hamsters.

\textit{Discussion.—}From the studies presented, we may conclude that adenovirus-specific antigens are present in probably all cells rendered neoplastic by the Ad. 1\textsuperscript{++} and Ad. 2\textsuperscript{++} hybrid viruses. The presence of these antigens was not dependent on the spread of virus since the cell lines were repeatedly found to be free of infectious adenovirus and the cloning was done after prolonged passage with antiserum. Furthermore, we have demonstrated that it requires greater than 10\textsuperscript{5.5} infectious units of Ad. 1\textsuperscript{++} hybrid virus inoculated intraperitoneally or subcutaneously into weanling hamsters to produce an immune response detectable by FA staining of Ad. 1 T or V antigen cover slips;\textsuperscript{4} this quantity of virus would have readily been detected by
the methods of virus isolation utilized. Therefore, the presence of new, heritable, and specific adenovirus antigens in all clones examined, in the absence of infectious virus, indicates that the adenovirus genome, or part thereof, is integrated in these neoplastic cells. Additional evidence supporting this contention has been derived from nucleic acid hybridization studies in which the presence of Ad. 2-specific messenger RNA has been demonstrated in the Ad. 2++ HK-1 cell lines and in two of the clones derived therefrom.\textsuperscript{19}

It seems probable that the SV40 genetic material played an essential role in the integration of the Ad. 1 and 2 DNA, since the nonhybrid Ad. 1 and 2 show no oncogenic potential \textit{in vivo} or \textit{in vitro}.\textsuperscript{1,7} The most likely mechanism is that the adenovirus DNA is linked to the SV40-DNA and accompanies the latter as it becomes integrated. It has not yet been proved that the SV40-DNA in the Ad. 1++ and Ad. 2++ hybrid particles is linked to adenovirus DNA as it is in the Ad. 7+ hybrid,\textsuperscript{9} but interferon-sensitivity studies of the adenovirus and SV40 T antigens suggest that this is the case.\textsuperscript{21}

Since the hybrid populations contain nonhybrid adenovirus and the transformation tests were done with a relatively high virus:cell multiplicity, the integrated adenovirus genetic material could have been derived from the nonhybrid particles. Studies of the Ad. 2-SV40 "transfer hybrids,"\textsuperscript{22} in which the adenovirus DNA linked to the SV40 genome is different from that in the nonhybrid particles, should help clarify this point.

A less likely possibility is that the DNA of nononcogenic adenoviruses can itself become integrated into an occasional hamster cell, but without producing significant phenotypic alteration; the role of the SV40 would be to provide the growth stimulus which permits their emergence and recognition.

In studies reported elsewhere, it was found that tumors derived from transplantation of Ad. 2++ HK cells to hamsters had areas resembling adenovirus tumors, and some cells were intermediate in morphology between adenovirus and SV40 tumor cells.\textsuperscript{11} This would suggest that a nononcogenic adenovirus genome, if integrated, may supply the determinants for adenovirus morphogenesis and that this may not be an exclusive property of the oncogenic adenoviruses. This also suggests that if integrated, a nononcogenic adenovirus might supply the determinants for oncogenesis as well.

\textit{Summary.}—The presence of adenovirus antigens in adenovirus-free cells rendered neoplastic \textit{in vivo} and \textit{in vitro} by the Ad. 1-SV40 and Ad. 2-SV40 hybrid viruses, respectively, was examined by the fluorescent antibody technique. Antibodies present in sera of hamsters bearing tumors induced with the three cell lines examined and with 18 clonal sublines derived from two of these cell lines all reacted with early adenovirus T antigens in human cells infected with adenoviruses 1 or 2. Antibodies which reacted with late or viral, group-reactive antigens were also present in many of these hamster tumor sera and in hyperimmune ascites fluids prepared with extracts of tumor cells as well.

The presence of new, heritable adenovirus-specific antigens in essentially all cells transformed by the Ad. 1-SV40 and the Ad. 2-SV40 hybrid viruses indicates that the DNA of nononcogenic adenoviruses can become integrated in a host cell. The most likely mechanism is that the adenovirus and SV40 genetic materials are physically
linked and that the adenovirus genetic material accompanies the SV40 genome which readily integrates into hamster cells.

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1 Huebner, R. J., in *Perspectives in Virology*, ed. M. Pollard (New York: Academic Press, Inc.), volume 5, in press; Piña, M., and M. Green, these PROCEEDINGS, 54, 547 (1965). The lack of oncogenicity of the Ad. 1, 2, and 5 group is best documented by the unpublished observations of Huebner, R. J., R. V. Gilden, and T. G. Beddow that 362 hamsters inoculated as newborns with the prototype strains and surviving for 400 days were tumor-free; of 123 of these which survived for an additional 200 days only 1 developed a tumor which by serological studies contained no adenovirus-specific antigens.


4 Ibid., 120, 121 (1964).

5 Huebner, R. J., R. M. Chanoock, B. A. Rubin, and M. J. Casey, these PROCEEDINGS, 52, 1333 (1964); Schell, K., W. T. Lane, M. J. Casey, and R. J. Huebner, these PROCEEDINGS, 55, 81 (1966); Black, P. H., and G. J. Todaro, these PROCEEDINGS, 54, 374 (1965).

6 Lewis, A. M., Jr., unpublished data.


8 The superscript "++" indicates that the hybrid particles have the potential of yielding infectious SV40 virus. Under conditions of standard virus passage at high multiplicity, Ad. 1++ yields trace amounts of SV40, while the Ad. 2++ yields somewhat larger amounts. The virus inocula used for producing transformed cells contained SV40 antiserum. Both the Ad. 1++ and Ad. 2++ hybrid virus preparations used for production of tumors and transformation of hamster cells were found to be free of the aden-associated virus, types 1–4, by complement-fixation tests.


15 Lewis, A. M., Jr., W. H. Wiese, and W. P. Rowe, these PROCEEDINGS, 57, 622 (1967).


22 Rowe, W. P., these PROCEEDINGS, 54, 711 (1965); Rapp, F., J. S. Butel, and J. L. Melnick, these PROCEEDINGS, 54, 717 (1965).