HUMAN CELL STRAINS SUSCEPTIBLE TO FOCUS FORMATION BY HUMAN ADENOVIRUS TYPE 12

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Adenovirus type 12 has been shown to produce tumors when inoculated into newborn animals of various species.[1-4] Primarily for this reason it has also been suspected of being involved in human neoplasia.[5] In cell culture, adenoviruses have been shown to transform hamster, rat, and rabbit embryo cells.[6-9] The transformants are recognized by their altered morphology and by their ability to grow on top of one another. They form multilayered foci and can be readily distinguished from the background of untransformed cells. The adenovirus type 12 tumors and transformed lines contain a virus-specific new intranuclear tumor (T) antigen[10] and virus-specific messenger RNA.[11] The cells transformed in culture produce tumors when inoculated into the appropriate host, and these tumors continue to show the presence of virus-specific genetic and antigenic material.[5]

The present experiments demonstrate that adenovirus type 12 is able to produce altered foci in certain "susceptible" human cell cultures. Discrete adenovirus-induced foci are first seen 10–15 days after infection. The system, using human diploid cells, provides a method of quantitating the focus-forming ability of at least one type of human adenovirus.

Materials and Methods.—Cells: Human fibroblast strains were derived from embryo lung, newborn foreskin, and adult skin. The last were initiated from 4-mm skin-punch biopsies. The tissue was finely minced and inoculated into 20 cm^2 plastic Petri dishes containing Dulbecco and Vogt's modification of Eagle's medium supplemented with 10% calf serum. After approximately 2 weeks there generally was sufficient growth to allow subculture. The cells were then passaged at a 1:2 to 1:10 dilution every 4–5 days and were used in the assays before the tenth transfer generation.

Skin fibroblast cultures have been obtained from normal individuals and from individuals with genetic diseases associated with an increased risk of tumor formation. In particular, cell strains have been initiated from individuals with Fanconi's anemia, an autosomal recessive disease associated with a high risk of leukemia.[12, 13] These strains have previously been shown to be particularly susceptible to transformation in culture by another oncogenic DNA virus, SV40.[14]

Virus: Adenovirus type 12 (Huie strain), titering $10^{6.5}$ PFU/0.1 ml on human embryonic kidney (HEK) cells, was obtained from Dr. W. Rowe (NIH), and one pool was used in all experiments.

Experimental: Previous studies with SV40 transformation of the mouse line 3T3[15] and human diploid strains[14] have shown that the efficiency of transformation is increased if rapidly dividing cultures rather than stationary, nonproliferating cultures are infected, and also if the cells are allowed several divisions subsequent to infection before they become arrested by contact inhibition of cell division. A similar requirement for several cell divisions for the expression of transformation has been shown for adenovirus type 12 focus formation in hamster embryo cells.[16] For this reason rapidly dividing cultures were inoculated at 1×10^5 cells per 50-mm plastic Petri dish. On the following day, the cells were washed twice with serum-free medium and then infected with 1.0 ml of a 1:10 dilution of adenovirus 12 ($10^{6.5}$ PFU) at an infectious virus:cell ratio of roughly 50:1.

The cultures were incubated with the virus for 3 hr at 37°C, with gentle agitation every 15 min. After the adsorption period, the cell layer was washed twice, and new medium was added.

For the detection of adenovirus 12 nonvirion T antigen, the fluorescent antibody technique described by Pope and Rowe[10] was used. The cells were inoculated onto glass cover slips, fixed in cold acetone, and stained by the direct method, using FITC-conjugated hamster antiadenovirus type 12 T antisera (Flow Laboratories). FITC-conjugated hamster antiadenovirus group-specific hexon (V) antibody was kindly supplied by Dr. R. Gilden (Flow Laboratories). The source and methods used for detection of SV40 T and V antigens have been described elsewhere.[17]

Results.—Embryo lung, newborn foreskin, and adult strains were tested. These strains can be separated into three fairly distinct groups—susceptible, normal, and resistant—on the basis of their efficiency of transformation by SV40.[17] Two days after infection with adenovirus type 12, large ballooned cells typical of adenovirus cytopathic effect were seen in all cultures. At this time representative strains were tested for both T and V antigen. Table 1 shows that between 2.3 and 4.7 per cent of the cells stained for adenovirus type 12 T antigen and a comparable number stained for the hexon antigen at this time. No difference was seen between the various cell strains tested. This result is in striking contrast to the behavior of the cells following SV40 infection. In the latter case there are marked differences between the strains. Those that are susceptible to SV40 transformation also show an increased fraction of SV40 T antigen- and V antigen-positive cells, and those that are particularly resistant to SV40 transformation also are resistant to SV40 T antigen induction. The relationship between SV40 T antigen induction and the probability of transformation for human cell strains has been described in detail elsewhere.[17]

With the adenovirus 12-infected cultures, the large, ballooned "adeno" cells continued to be present but in diminishing numbers over the course of the next two weeks; they never comprised more than 1–5 per cent of the population.

TABLE 1. *Acute SV40 and adenovirus 12 T and V antigen production in human cell strains.*

Cell strain	Adenovirus 12[a] (% positive cells)		SV40[b] (% positive cells)	
	T antigen	V antigen	T antigen	V antigen
Susceptible				
L. S.[c]	3.3	5.7	23.5	19.4
B. L.[c]	2.7	—[d]	19.7	26.0
J. M.[c]	4.7	—	20.8	—
Normal				
C. M.[e]	3.2	3.8	2.3	3.1
B. D.[c]	2.6	—	2.6	2.9
Resistant				
M. B.[c]	3.1	4.0	0.3	0.2
C172[f]	2.3	—	0.4	—
M413[f]	3.0	—	0.1	0.1
M439[f]	2.8	—	0.1	—

[a] Infected with adenovirus type 12 at a multiplicity of 50:1.
[b] Infected with SV40 small-plaque virus at a multiplicity of 400:1.
[c] Adult skin fibroblasts.
[d] Not tested.
[e] Newborn foreskin fibroblasts.
[f] Embryo lung fibroblasts.

None of the cultures went on to show extensive cell destruction; the cells continued to divide in all of them and within five to seven days had reached confluence. Rabbit adenovirus 12 virion antiserum was added after the adsorption period in some experiments but was not found to be necessary for cell survival.

Altered foci were first visualized at 10–15 days as areas where the cells were more refractile. Within the next week or two the cells in these areas grew over one another and formed multiple cell layers. The number of foci that could be counted increased until the fourth week; therefore, the foci were routinely scored 28 days after infection. Early in the development of a colony the individual cells were fibroblastic and piled over one another in random fashion. Later on, as the colony got denser, the cells became much more epithelioid in appearance. Degenerating and dying cells could be seen in the center of the focus at the latter time.

"SV40-susceptible" skin fibroblast cultures derived from two patients with Fanconi's anemia (L. S. and J. M.) and one from a "transformation-prone" family (B. L.)[18] showed many discrete adenovirus-induced foci. Under the same conditions, only a rare focus was seen in the normal strains; no transformed foci were seen in the "resistant" cultures (Table 2). Susceptibility of the various strains to SV40 transformation is seen to parallel closely the susceptibility to adenovirus 12 focus formation.

The adenovirus type 12 foci can be readily distinguished from SV40-transformed human colonies. SV40-transformed colonies, although they form multiple cell layers, tend to follow the orientation of the surrounding normal fibroblasts and, consequently, are frequently irregular in outline. The adenovirus 12-altered cells are rounder and more refractile, and the whole focus is rounder with more well-circumscribed edges (see Fig. 1).

In those cultures that showed adenovirus 12-induced foci, several focal areas were isolated and inoculated onto cover slips. In each case a large fraction of the cells showed the specific intranuclear adenovirus 12 T antigen. Some also

TABLE 2. *Transformed foci produced on various human cell strains with SV40 and adenovirus 12.*

Cell strain	Focus-Forming Units/10^5 Cells[a]	
	Adenovirus 12[b]	SV40[c]
Susceptible		
L. S.	146	90
B. L.	50	75
J. M.	65	52
Normal		
C. M.	2.5	8
B. D.	5.3	10
Resistant		
M. B.	0	0.3
C172	0	1
M413	0	0.3

[a] Pooled data from at least three separate experiments where in each experiment 1×10^5 cells were infected.

[b] Infected with adenovirus type 12 at a multiplicity of 50:1.

[c] Infected with SV40 small-plaque virus at a multiplicity of 400:1.

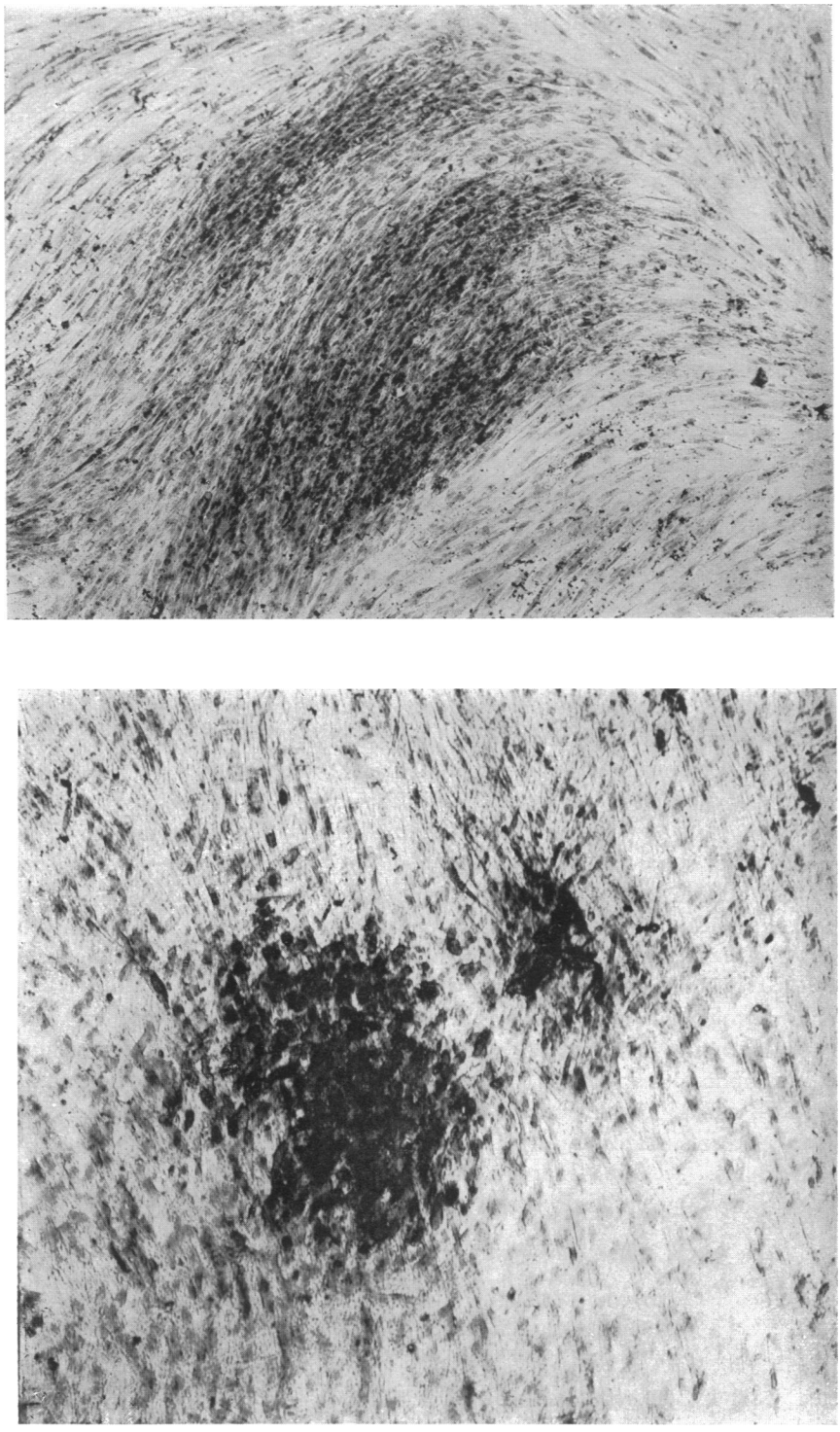

FIG. 1. *Left:* Adenovirus type 12 focus, 18 days after infection, ×60. *Right:* An early SV40-transformed colony, 14 days after infection, ×30.

stained for the adenovirus virion antigen. When adenovirus 12-infected cells were inoculated directly into Petri dishes containing cover slips and fixed and stained two to four weeks later, discrete focal areas of T antigen-positive cells were seen. These areas contained from 50 to 500 cells. Such areas were frequently seen in the "susceptible" cultures but were rare or absent in the normal and the resistant strains. It is assumed that these areas are the adenovirus 12-induced foci seen in the unstained cultures. The foci also contained cells that stained for adenovirus hexon antigen. In general, the hexon antigen-positive cells were found in the center of the foci and became more numerous the longer the culture was maintained. The continued production of virion antigen (and presumably infectious virus) by cells in a developing focus has also been seen with SV40 in human cells.[17] In the latter case, however, there is little problem in obtaining pure cultures of focal lines of transformed cells; this has not been possible as yet with cells picked from the adenovirus-induced human foci.

One of the susceptible strains (L. S.) was used with various media and culture conditions in an attempt to favor the detection of transformed foci. The standard medium, Dulbecco and Vogt's modification of Eagle's medium supplemented with 10 per cent calf serum, gave reproducible numbers of transformed foci. The addition of human serum to the medium increased the number of foci seen at two weeks, though there was no difference at four weeks. The number of foci was not decreased when the cells were grown continuously in the presence of adenovirus 12 antiserum. However, it was found that the low calcium medium of Freeman *et al.*[19] retarded the appearance of transformed foci (Table 3). A lower multiplicity of infection also lowered the number of foci obtained.

Discussion.—Clearly identifiable altered foci can be seen within two weeks after infection of certain human cell strains with adenovirus 12. Several factors probably are responsible for the ability to detect adenovirus focus formation in the systems described above. The most important factor appears to be the choice of human cells. While genetic factors are well known to be crucial in Rous sarcoma virus focus formation in chick cells and tumor production in the chicken,[20, 21] the influence of the cell genotype on transformation of mammalian cells by oncogenic viruses has received little attention. Reed[22] noted that different batches of hamster embryo cultures could differ greatly in their sensitivity to adenovirus 12 transformation. In the human system there is a vast body of clinical genetic data to draw upon. In studying the factors influencing transformation of human cells by SV40, we had found that cell strains derived from certain individuals were considerably more susceptible to transformation than were strains derived from other individuals. Multiple biopsies from the same person, however, led to cell strains that were very similar in their transformation susceptibility with SV40.

TABLE 3. *Some factors affecting the appearance of adenovirus 12-induced foci.*

Multiplicity	Medium	Foci/10^5 Cells[a]	
		Two weeks	Four weeks
50:1	Standard medium (SM)	15	146
50:1	SM + 2% human serum	62	135
10:1	SM + 2% human serum	18	66
50:1	Low calcium medium	3	23

[a] Pooled data from two separate experiments where in each experiment 1×10^5 cells were infected.

Certain genetic diseases of man are well known to be associated with an unusually high risk of developing tumors.[13] It was reasonable to assume that if increased tumor susceptibility had a genetic basis, it might be possible to demonstrate the altered susceptibility in a cell culture system. This became possible when a quantitative system for transformation of human cells by SV40 became available. While cell strains from normal individuals showed very similar transformation frequencies, those strains derived from persons with Fanconi's anemig and from persons carrying the Fanconi's anemia gene were found to have a markedly increased transformation susceptibility. In addition, in screening normal adult populations for susceptibility to SV40 transformation, we found three members of one family whose skin fibroblasts showed unusual susceptibility when tested with SV40.[18] Strains from both these groups when tested with adenovirus 12 have shown a greatly heightened susceptibility as compared to other normal adult fibroblast strains.

In the present experiments, susceptible, normal, and resistant human fibroblast strains defined by their transformation frequency with SV40 were tested. The results indicate that the susceptible strains also have a much greater focus-forming frequency with adenovirus type 12. Such foci were occasionally present with normal adult skin fibroblast cultures, while no foci have been seen among the resistant strains studied, nor have they ever been seen in control cultures. With SV40 infection, embryonic lung fibroblasts, in general, were found to be relatively insensitive to transformation;[17] the same relative resistance is also seen with adenovirus 12 focus formation. Human embryonic lung strains thus appear to be much less sensitive than at least certain strains of adult fibroblasts to the action of two different oncogenic DNA viruses. In contrast, all the cell strains tested are equally susceptible to lytic infection by viruses such as polio and vesicular stomatitis (unpublished observations).

The basis for the differences between human strains is still not known. The greatly enhanced susceptibility of the Fanconi's anemia homozygous and heterozygous cultures, however, indicates that alteration of a single autosomal gene can have a pronounced effect on the outcome of at least certain virus-cell interactions, those involving oncogenic DNA-containing viruses.

With the susceptible strains the efficiency of focus formation by adenovirus 12, while still low compared to other DNA-containing tumor viruses, is higher than the efficiency previously reported for hamster and rat cells. At a multiplicity of 50–80 PFU/cell, McAllister and MacPherson found that 0.001 per cent rat embryo cells and 0.002 per cent hamster NIL-2 cells were transformed.[9] They calculate that roughly 4×10^6 infectious units were required per transformation. In hamster embryo cells Casto[16] also has found that one transforming unit corresponds to $1–2 \times 10^6$ PFU. Higher than optimal multiplicities (>120 PFU/cell) resulted in a decline in transformed colonies, presumably because of the cell-killing effect of the virus. At a multiplicity of 50 PFU/cell with "susceptible" human cells, the value is around 0.1 per cent. Thus 5×10^4 PFU of adenovirus 12 correspond to one focus-forming unit. It is worth noting that the foci appear relatively early compared to the time of appearance reported for adenovirus focus formation in certain other systems. The foci can first be seen 10–15 days after

infection. Adenovirus 12-transformed foci have also been seen at 15 days by Yamane and Kusano,[23] who used hamster brain cells, and Casto observed transformed foci in hamster embryo cultures within two weeks.[16]

Attempts to establish continuous lines from human cells containing adenovirus-transformed foci so far have not been successful. In the present studies virion antigen and infectious virus continue to be produced in a portion of the cells obtained from the altered foci. With SV40, a less cytopathic virus for human fibroblasts, transformants are also found to contain virion antigen for many generations.[17] In the latter case there is seemingly little ill effect on the processes of cellular replication. It is possible, therefore, that in the interaction between virus and cell in adenovirus-induced foci, cell destruction is favored over cell proliferation. Various conditions that might favor the latter are currently being studied.

Summary.—Human fibroblast cell strains that are highly susceptible to transformation by one oncogenic DNA virus, SV40, have been tested with human adenovirus type 12. The susceptible strains develop foci of altered cells with much greater frequency than do normal human cell cultures. These susceptible cell strains would appear to offer the best possibility for the detection of hitherto undiscovered focus-forming viruses that may be involved in human neoplasia.

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