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Neoplastic transformation of chimpanzee cells induced by adenovirus type 12–simian virus 40 hybrid virus

(chimpanzee fibroblasts)

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ABSTRACT **The adenovirus 12–simian virus 40 hybrid virus produced neoplastic transformation of chimpanzee skin fibroblasts *in vitro*. The transformed fibroblasts showed morphological alteration and became permanent lines. The transformed cells contained both adenovirus 12 and simian virus 40 large tumor antigens and were virus producers. However at passage 9, one line (WES) was found to be a nonproducer, producing neither infectious virus nor virus-specific antigen detectable by the complement fixation test. Virus particles were not detected nor could infectious hybrid virus be rescued from this line by cocultivation with Vero cells. The transformed cells formed large cell aggregates and grew in liquid growth medium above an agar base, formed colonies in soft agar, and grew to high saturation densities; the normal chimpanzee skin fibroblasts did not. One transformed WES line produced tumors when transplanted subcutaneously into newborn *nude* mice, thus providing an important tool for studying tumor immunity in the chimpanzee.**

The adenovirus 12–simian virus 40 hybrid (Ad12–SV40) virions consist of recombinant DNA containing all or part of the SV40 genome and part or all of the adenovirus genome enclosed in adenovirus capsids. They were originally isolated either from stocks of adenoviruses (types 5 and 7) that had been adapted to grow in rhesus monkey kidney cells for vaccine production (1) or from propagation of adenovirus (type 12) and SV40 together in African green monkey kidney culture (2). The Ad12–SV40 virus was found to be highly oncogenic in newborn hamsters (1). *In vitro* transformation of hamster cells by the Ad12–SV40 virus has also been described (3).

Chimpanzee (*Pan troglodytes*) is one of the anthropoid apes that had been used for experimental purposes because of its susceptibility to some of the diseases of man (4). However, no viral transformation study has been reported in the chimpanzee. This communication describes (*a*) productive and nonproductive transformation of chimpanzee skin fibroblasts by Ad12–SV40 virus; (*b*) establishment of these transformed skin fibroblasts cultures as permanent lines; and (*c*) production of sarcomas in *nude* mice by a subline of the nonproductive transformed cells. The derivation of a virus-free (nonproducer) subline is of importance because it is now possible to use this line for production of anti-cancer tumor cell vaccines in the chimpanzee, the host closest to the human, a follow-up to cancer prevention in lower species (5).

MATERIALS AND METHODS

Virus. Ad12–SV40 virus stock (T-12017) (2) was grown in Vero cells (6). Freshly harvested supernatant fluids of Ad12–SV40 virus-infected Vero cells were centrifuged in a Beckman 21 rotor at 21,000 rpm for 90 min, and the virus pellet was resuspended in Eagle's minimal essential medium with 10% fetal bovine serum at a concentration of 100×. Its infectivity titer was 10^9 TCD_{50}/ml in Vero cells (see below).

Cell Culture and Media. Chimpanzee skin fibroblasts were derived from subepidermal biopsy samples of normal-appearing flat skin grown as explants. The samples were minced into 7–10 fragments and transferred to a culture vessel that contained 4 ml of culture medium supplemented with 20% fetal bovine serum. The vessel was incubated for 3 weeks, with medium changes twice weekly. During the incubation period, a large number of dermal fibroblasts grew out as a monolayer around the explant, and the outgrowth was subcultured within 2–3 weeks and again a week later when the primary subcultures had become confluent. After processing of the subepidermal outgrowths, skin fibroblasts were passed at a subculture ratio of 1:2 and used from stocks between the 7th and 15th passages. These cell strains were grown and maintained in Dulbecco's modified Eagle's medium (DME medium) with 10% fetal calf serum, 1% nonessential amino acid, and 100 units of penicillin and 100 μg of streptomycin per ml.

Virus Assays. The replication of Ad12–SV40 virus in cultures was determined by (*a*) examination for the presence of cytopathic effect, (*b*) assay for complement-fixing (CF) antigen reactive with Ad12 and SV40 virus antisera, and (*c*) examination by electron microscopy for the presence of virus particles. Virus infectivity for Ad12–SV40 virus was assayed in Vero cells. The infectivity titers were expressed as 50% infective tissue culture doses per ml (TCD_{50}/ml).

CF Test. Cell pack preparations for CF testing were made as described (7). CF tests were performed by the microtiter technique described for tumor antigen studies (7). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement.

Antisera. Ad type 12 large tumor antiserum was obtained from rats bearing Ad type 12 tumors. SV40 tumor antiserum was obtained from hamsters bearing SV40 tumors. In tests for Ad12 virus antigen, adenovirus group reactive reference antisera were used. The SV40 antiserum was obtained from hyperimmune guinea pig serum.

Transformation Studies. One-day-old cultures of chimpanzee skin fibroblasts were infected with Ad12–SV40 virus at an input multiplicity of 100. The infected cultures were incubated at 37°C under a 5% CO_2/95% air atmosphere with medium changes twice weekly. Starting 21 days later the cultures were passaged by trypsin treatment every 7 days and propagated as continuous lines.

Cell Aggregation Assay. Formation of cellular aggregates by normal and transformed cells was tested by the method described by Steuer *et al.* (8). Freshly trypsinized viable cells (2

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Abbreviations: Ad12–SV40, adenovirus 12–simian virus 40 hybrid; DME medium, Dulbecco's modified Eagle's medium; CF, complement fixing.

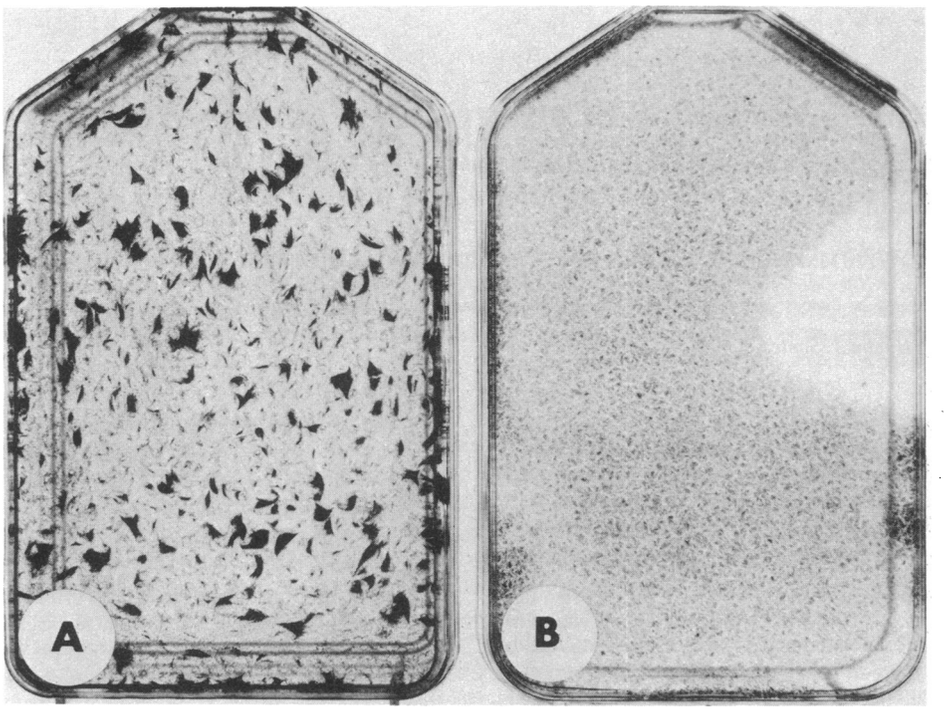

FIG. 1. Chimpanzee fibroblasts. (Giemsa stain.) (*A*) Foci of Ad12–SV40-transformed cells 4 weeks after virus infection. (*B*) Uninfected cells.

$\times 10^5$) were seeded into 35-mm plastic dishes containing a 2-ml agar base layer (0.9% Difco agar in growth medium with 20% fetal calf serum). The dishes were incubated undisturbed at 37°C under 5% CO_2/95% air. Viable cells were counted daily for 4 days.

Soft Agar Assays. Cells were suspended in 0.36% agar medium (DME medium containing 20% fetal calf serum and antibiotics). This suspension was layered onto a 0.9% agar base layer at concentration of 10^4–10^5 cells per 35-mm dish. Dishes were incubated in a humidified atmosphere (5% CO_2) at 37°C. After 2 weeks, colonies greater than 0.125 mm in diameter were counted. Results are expressed as percentage plating efficiency [%PE = (number of colonies × 100)/number of cells plated].

Rescue of Virus. A genome-rescue experiment was done by cocultivation of equal numbers of Ad12–SV40-transformed chimpanzee skin cells and Vero cells. Supernatants from these cultures taken from the 14th to 21st days after infection were assayed for virus on Vero cells.

Oncogenicity of Transformed Cells. Newborn 129J *nude* mice were inoculated subcutaneously with 5×10^6 freshly trypsinized cells in order to determine cell tumorigenicity.

RESULTS

Transformation of Chimpanzee Skin Fibroblasts by Ad12–SV40 Virus. In the first 2 weeks after exposure of chimpanzee skin fibroblasts to the virus, no differences between infected and control (uninfected) cultures could be seen. In the third week, a self-limiting, focal cytopathic effect was seen on the infected cells. Foci of rounded, granular cells were seen; these cells ultimately detached from the monolayer and left plaque-like regions. Approximately 10–30% of the cells from the infected cultures were involved. The degree of cytopathic effect observed was different in individual fibroblast strains. During the third and fourth weeks, distinct transformed foci were seen. These foci of highly proliferative activity bore no relation to the foci of degenerating cells also present in the cultures. The foci seen stained darkly with Giemsa and could be recognized readily (Fig. 1). Two distinct types of transformed foci were observed. The predominant type consisted of cuboidal cells (Fig. 2*A*). This type has been described for other SV40-transformed cells (3, 9, 10). Dispensed among the cuboidal cells in this type of transformed focus were numerous single and multinucleated giant cells. A second type of transformed focus contained small, rounded cells which were densely packed into multilayers (Fig. 2*B*). In cultures that were transferred after the third week and then once a week thereafter, a marked increase in cell number in the infected cultures was evident by the fourth week. However, uninfected cultures could not be carried through more than five to seven additional cell generations. They underwent nonspecific, progressive deterioration. Five transformed cell lines were established. At present they have survived more than 15 serial passages. All except one (WES) were found to be virus producers (Table 1). Two transformed lines, designated Ad12–SV40 WES and Ad12–SV40 Dagwood, were further characterized.

Table 1. *In vitro* transformation of chimpanzee skin fibroblasts by Ad12–SV40 virus

Cell strain	Subculture at infection*	First observed transformation, day	CPE†	Virus yield,‡ TCD_{50}/ml P-2	P-5	P-9
Lavinda	12	24	++	10^7	10^5	$>10^2$
George	14	24	+	10^5	10^4	$>10^2$
WES	7	21	+	10^5	10^2	0
Dagwood	8	28	++	10^7	10^3	$>10^2$
Sherlock	15	22	++	10^6	10^3	$>10^2$

* Uninfected cultures died at 21–25 subcultures.
† Cytopathic effects: +, 11–25% of cells affected; ++, 26–50%.
‡ Infectious hybrid virus in Vero cells from supernatant fluids of the infected chimpanzee skin cells at second, fifth, and ninth passages.

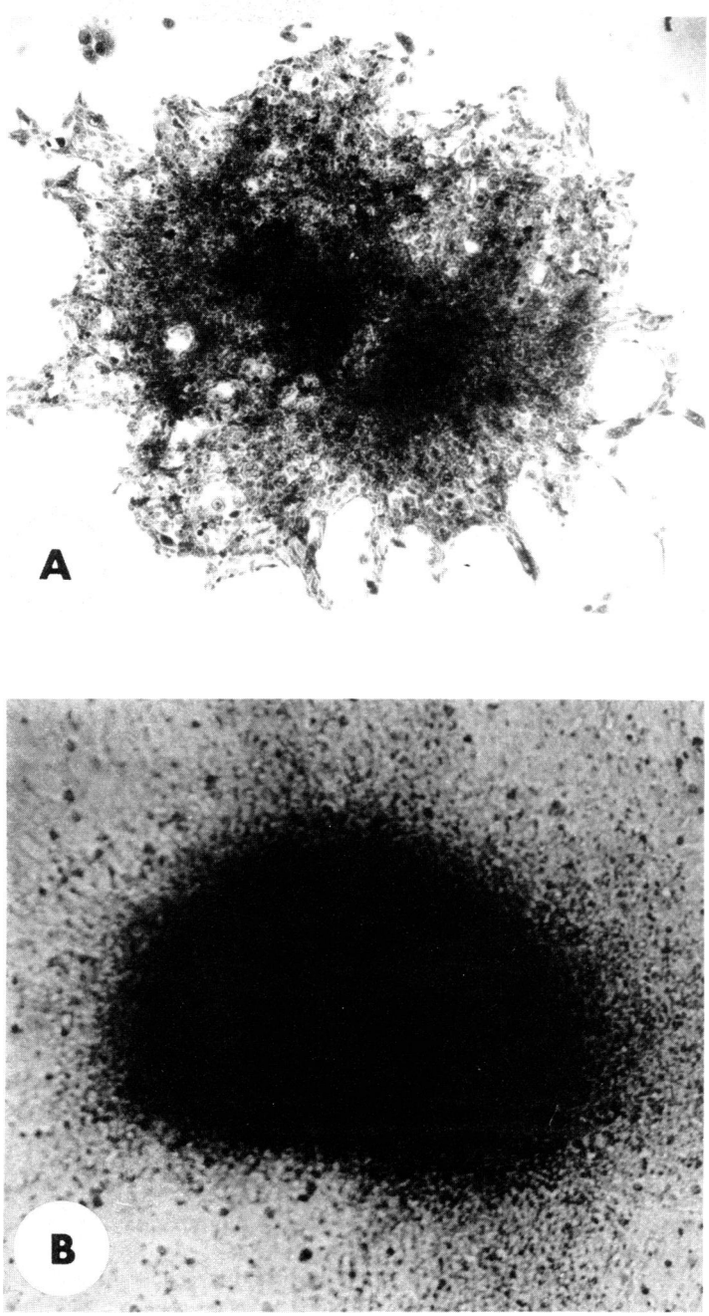

FIG. 2. Transformed foci induced by Ad12–SV40 virus after exposure of chimpanzee skin fibroblasts (Giemsa stain; ×50.) (*A*) Cuboidal or SV40-type transformed focus. (*B*) Epithelial or Ad-type transformed focus.

Characteristics of Ad12–SV40-Transformed Chimpanzee Skin Cell Lines. Transformed cells in tissue culture generally can be distinguished from their normal counterparts by quantitative differences in growth properties such as contact inhibition, saturation density, soft agar colony-forming ability, and cell aggregation properties (Table 2). Saturation densities of transformed lines WES and Dagwood were 6.8 and 2.5×10^5 cells per cm^2, respectively, which are 2- to 5-fold more than for the control untransformed cells. Ad12–SV40 WES lines formed larger aggregates than did the uninfected control or Ad12–SV40 Dagwood lines. Cell counts in trypsinized aggregates at 4 days after plating of 2×10^5 cells per plate indicated that control and Ad12–SV40 Dagwood cells underwent a significant decline in number of viable cells, whereas the Ad12–SV40 WES cells exhibited growth in the aggregate form. The AD12–SV40 WES cells formed colonies in soft agar, whereas the control and the Ad12–SV40 Dagwood cells did not.

Twenty percent suspensions, containing 1×10^7 cells per ml, from both WES and Dagwood transformed cells repeatedly gave positive CF reactions with a specific Ad12 tumor serum and SV40 tumor serum; >32 units of antigen were present in such preparations. However, both transformed cells were negative for Ad12 virus or SV40 virus CF antigens.

No infectious hybrid virus was found in the supernatant fluid of a culture of transformed WES at passage 9 or in higher passages. However, infectious hybrid virus was found in the supernatant fluids of transformed Dagwood cells at passage 9 or in higher passages. The virus titers, determined in Vero cell cultures, varied from 10^3 to 10^4 TCD_{50}/ml. In an attempt to rescue infectious hybrid virus, transformed WES lines at various pas-

Table 2. Properties of Ad12–SV40-transformed chimpanzee skin cell lines

Property	Uninfected WES cells	Ad12–SV40	
		WES cells	Dagwood cells
Morphology	Normal	Transformed	Transformed
Saturation density*	1.2	6.8	2.5
Cell aggregates†			
Size	Small	Medium	Small
Viability of cells, no. $\times 10^{-5}$	0.6	5.6	0.7
Colony formation in soft agar	Neg.	Pos.	Neg.
CF titers of			
Ad12 T antigen	<2	>32	>32
SV40 T antigen	<2	>32	>32
Ad12 and SV40 V antigens	<2	<2	>32
Infectious virus	Neg.	Neg.	Pos.
Virus particles	Neg.	Neg.	Not done
Tumorigenicity in *nude* mice‡	Neg.	Neg.	Neg.
Life-span	Limited	Permanent	Permanent

* Maximum number of cells, shown as no. $\times 10^{-5}/cm^2$, obtained after initial plating of 5×10^3 cells per cm^2.

† Size and viability of cell aggregates were determined on day 4 after initial plating of 2×10^5 cells per plate, with the use of an agar static assay (7).

‡ Cells (5×10^6) were inoculated subcutaneously into each newborn 129J *nude* mouse.

sage levels were cocultivated with Vero cells. Supernatants and cell extracts from these cultures taken at 21 days were inoculated into Vero cells and examined for cytopathic effects. All rescue experiments were negative. Neither Ad12 nor SV40 virus particles were found in WES transformed cells examined by electron microscopy.

Tumorigenicity of Transformed Cells. The transformed cells were tested for their ability to produce tumors in *nude* mice. Cells were trypsinized, centrifuged, suspended in growth medium, and inoculated subcutaneously. Neither the WES nor Dagwood transformed line was tumorigenic; however, the WES 1 line, derived from cell aggregates of nonproducer Ad12–SV40-transformed WES cells growing in liquid growth medium above an agar layer, was tumorigenic in *nude* mice (Table 3). The tumors were characterized pathologically as poorly differentiated sarcomas. Cells established from the tumors resembled the WES transformed cells and were shown to be chimpanzee by karyological analysis (kindly performed by W. A. Nelson-Rees, Oakland, CA).

DISCUSSION

From the data presented, it is concluded that the Ad12–SV40 virus is capable of inducing neoplastic transformation of chimpanzee fibroblasts *in vitro*. These findings provide further confirmation of the highly oncogenic potential of this virus *in vivo* previously described by Huebner and his associates (2). RNA tumor viruses such as Kirsten mouse sarcoma virus and Rous sarcoma virus also induced morphological transformation of chimpanzee skin fibroblasts. However, the RNA virus-transformed fibroblasts had a limited life-span, failed to become established lines, and were unable to produce tumors when transplanted into *nude* mice (unpublished data). Thus, we have demonstrated (*a*) productive and nonproductive transformation of chimpanzee skin fibroblasts by Ad12–SV40 virus; (*b*) establishment of these transformed skin fibroblast cultures as permanent lines; and (*c*) production of sarcomas in *nude* mice by the nonproductive transformed cells.

Table 3. Tumors produced in 129J *nude* mice by Ad12–SV40-transformed chimpanzee skin cells

Ad12–SV40 cell line inoculum	Subculture level tested	No. of tumors / No. mice inoculated	Day under observation
Dagwood	5	0/7	120
WES (parent line)	5	0/8	120
WES 1*	4	3/5 (60)†	
WES 1*	7	5/5 (30)†	

Cells (5×10^6 per mouse) were inoculated subcutaneously. Tumors, poorly differentiated sarcomas, were sectioned and diagnosed by Dr. N. Taylor. Tumors also were reestablished in tissue cultures.

* Ad12–SV40 WES 1 subline was derived from cell aggregates of Ad12–SV40 WES cells growing above an agar layer.

† Day tumors first noted.

Ad12–SV40-transformed chimpanzee fibroblasts had the following properties generally associated with viral transformation; (*a*) altered appearance; (*b*) increased growth rate; (*c*) colony formation in soft agar medium; (*d*) formation of large cell aggregates and growth in this aggregate form above an agar base; (*e*) establishment of immortal lines; and (*f*) tumorigenicity in *nude* mice. The transformed cells contained both Ad12 and SV40 large tumor antigens. With one exception, they all were virus producers. However, a nonproducer line negative for viral antigen and rescuable infectious virus was established. It had previously been reported that the Ad12–SV40 virus is highly oncogenic in newborn hamsters; the resulting tumors phenotypically resembled those induced by Ad alone but contained both Ad12 and SV40 tumor antigens (2). Black and Todaro (10) successfully used the Ad7–SV40 virus to transform cultures of hamster kidney and of diploid human skin fibroblasts. The transformation observed resembled that induced by SV40, and almost all of the cells in the transformed culture demonstrated SV40 tumor antigen but not Ad7 tumor antigen. No infectious virus was found in the supernatant fluid of Ad7–SV40-transformed hamster kidney cells; however, infectious hybrid virus was always found in the supernatant fluid of Ad7–SV40-transformed human skin fibroblasts.

Evidence is presented to show that one of the Ad12–SV40-transformed but virus-free WES lines derived from cell aggregates growing in liquid medium above an agar layer was tumorigenic in *nude* mice, whereas the parent Ad12–SV40 WES line was not (Table 3). A cell aggregation assay for evaluating *in vitro* transformation has been described by Steuer *et al.* (8). Transformed cells formed larger aggregates than did normal counter-

part cells when suspended in liquid medium above an agar base. The transformed cell aggregates were further characterized by an increase in growth and viability over untransformed cells. Recent studies indicated that certain aggregation properties (size, viability, and proliferation of cell aggregates) of transformed cells were correlated with tumorigenicity (8). As shown in our study, a cell aggregation assay can be utilized not only to evaluate the tumorigenic potential of transformed cells but also for selection of the neoplastic altered cells.

These observations in the chimpanzee model are directly applicable to the eventual development of human anticancer vaccines. The experiments described were predicated on earlier studies in which spontaneous and induced cancers of mice and rats were prevented by the use of allogeneic and syngeneic tumor cell vaccines (5). Such a cancer immunoprevention program in humans would require safety and efficacy trials in primates, preferably chimpanzees, the primate most analogous to man. The development of a nonproducer transformed chimpanzee cell line now makes it possible to induce protective allogeneic immunity in chimpanzees which can then be challenged with syngeneic transformed cells.

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