DETECTION OF SPECIFIC ANTIGEN IN SV40-TRANSFORMED CELLS BY IMMUNOFLUORESCENCE

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Plates 7 and 8

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Immunofluorescent techniques have been used by several investigators to study the replication of SV40 virus (1–3); in all cases, antiviral immune serum was used, which would detect antigens on the surface of the viral particle. In African green monkey kidney cell cultures (AGMK) infected with SV40 virus, viral antigen appeared in the nucleus at 18 hours and reached a peak at 38 hours, after which nuclear antigen decreased and a cytoplasmic stage occurred (1). Nuclear fluorescence was also observed 10 days after infection of cells of grivet monkey kidney, rhesus monkey kidney, human embryo kidney, and Syrian hamster kidney (2). The same techniques were applied to cells transformed by SV40 virus in a search for evidence of viral antigen. Faint nuclear fluorescence, interpreted as non-specific, was observed in transformed Syrian hamster kidney cells (2). In cultures of transformed human embryo kidney cells, which were producing virus, less than 0.1 per cent of cells showed nuclear fluorescence (3) and it seems probable that these were the cells responsible for virus replication.

It has recently been demonstrated that SV40 tumors and transformed cells contain a specific complement-fixing (CF) antigen, distinct from that of the virus particle, which reacts with sera of hamsters bearing SV40 tumors (4). Based on this information, an indirect immunofluorescent test for SV40-transformed cells was developed using the serum of hamsters bearing SV40 tumors. It was hoped that such a technique would prove effective in detection of transformed cells and that information would be gained on the location of the antigen and the percentage of cells elaborating antigen.

Materials and Methods

Tissue Cultures.—All immunofluorescent studies were done with cells grown in tissue culture, on 11 x 22 mm coverslips in 60 x 15 mm plastic Petri dishes. The growth medium was

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Eagle's basal medium 2 (BME) containing 5 or 10 per cent heated (56°C/30 minutes) newborn agamma calf serum (Hyland Laboratories, Los Angeles), 100 units of penicillin and 100 μg of streptomycin per ml. Plates were incubated at 37°C in an atmosphere of 5 per cent CO_2.

SV40 transformed cell lines included the transformed hamster kidney line THK-1 (5); transformed kidney cells of C3H mouse, rabbit, and porcine origin (6); and a transformed hamster embryo cell line (H50-1) (7) received from Dr. J. L. Melnick. Control cell lines included lines from 2 hamster tumors induced by adenovirus type 12, polyoma-transformed hamster embryo cells (received from Dr. R. Dulbecco), and the normal hamster cell BHK-21 (8).

Primary cultures of hamster tumors induced by SV40, polyoma, or adenovirus 12 and 18 were established by the trypsinization procedure. When possible, coverslips of cultures of tumors and cell lines were harvested before a complete cell sheet was formed.

Secondary cultures of AGMK were inoculated with $10^{6.7}$ ID_{50} of SV 40 virus strain 777 and harvested at 53 hours. BSC-1 cells (9) were similarly infected, and harvested at 38 hours. Maintenance was on 2 per cent heated agamma calf serum in BME in an atmosphere of 5 per cent CO_2.

Antisera.—H50-1 cell-induced tumors were used for production of hamster antitumor serum. This tumor line was maintained by serial transplantation in weanling hamsters by subcutaneous inoculation of tumor fragments. Serum was collected as late as possible after transplantation, and tested for CF antibody to tumor antigen. Two pools of CF-positive sera were prepared. Pools 1 and 2 titered 1:40 and 1:10, respectively, against SV40 tumor antigen, were negative for antiviral CF antibody at 1:10 dilution, and had no virus-neutralizing activity at 1:5 dilution. Control sera were pooled sera from normal hamsters and from hamsters bearing tumors induced by adenovirus type 12 (Ad. 12); the latter serum pool contained CF antibodies to both Ad. 12 tumor and viral antigen (10).

Conjugates.—For the indirect test, fluorescein-labeled goat antiserum to hamster serum globulin was used; this conjugate was kindly supplied by Dr. Roger Wilsnack. The conjugate was diluted 1:10 in a normal hamster brain extract (20 per cent in phosphate-buffered saline pH 7.2 (PBS) clarified at 2000 RPM/15 minutes), held at 4°C for 1 hour, and then centrifuged at 15,000 RPM for 15 minutes in a Spinco 40 rotor. The direct test was conducted with the conjugated serum of a Cercopithecus monkey which had been inoculated with SV40 virus; this serum had a CF titer of 1:320 to virus, a neutralizing antibody titer of 1:1280, and <1:10 CF titer to SV40 tumor antigen.

Immunofluorescent Tests.—Coverslips were washed twice in PBS, fixed for 10 minutes in acetone at −60°C, air-dried, and stored in stoppered vials at 4°C. In the indirect test, cells were treated with a 1:5 dilution of unheated hamster serum at 37°C for 30 minutes in a humid atmosphere, washed twice in PBS for 5 minutes, and rinsed in distilled water. Anti-hamster conjugate was then added for 30 minutes at 37°C, the washing process was repeated, and the coverslip mounted in buffered glycerol (pH 8.5). Similar incubation and washing procedures were used with the direct method. The specificity of reactions in the indirect test was checked repeatedly by testing uninfected cells treated with the SV40 hamster antiserum, and infected cells treated with control serum. The direct test was controlled by testing the conjugated antiserum against uninfected cells.

RESULTS

Table I summarizes the results obtained with various combinations of antisera and cells. Specific immunofluorescence was readily demonstrated when the hamster tumor sera were tested with SV40 tumor and transformed cells. In all instances, the fluorescence of cells in interphase was confined to the nucleus,

TABLE I

Immunofluorescent Staining Reactions of SV40 Antitumor and Antiviral Sera

	Tissue culture passage level*	Hamster tumor serum (indirect test)			Anti-SV40 monkey serum (direct test)
		Anti-SV40		Anti-Ad. 12	
		Pool 1	Pool 2		
		per cent	*per cent*		*per cent*
SV40-transformed cell lines					
Hamster (H50-1)	>10	++++‡ 100		−	−
Hamster (THK-1)	76	++++ 100	++++ 100	−	−
C3H mouse kidney (TCMK-1)	21	++++ 100	++++ 100	−	−
Rabbit kidney (TRK-1)	20	++++ 100	++++ 100	−	−
Porcine kidney (TPK-1)	11	++++ 100	++++ 100	−	++ 0.01
Primary SV-40-induced hamster tumor	1		++++ 30	−	−
Ad. 12-induced hamster tumors					
Prototype (Huie)	1	−	−	++++	−
Field strain 10534	1	−		++++	
Ad. 12-induced mouse tumor	1 and 2	−	−	++++	
Ad. 18-induced hamster tumor	1	−			
Polyoma hamster tumor	1	−			
BHK-21 hamster cell line	37	−			
Normal hamster embryo	1	−	−		
SV40-infected					
AGMK (53 hrs.)			++++ 95		++ 90
BSC-1 (38 hrs.)		++++ 95			++ 90
Controls, uninfected					
AGMK		−	−		−
BSC-1		−	−		±

* Number of tissue culture passages since removal from hamsters. The H50-1 and polyoma cells were from tumors produced by inoculation of transformed tissue culture cells; the other cell lines had not been passed in animals.

‡ The − to ++++ scale refers to the intensity in individual cells, and the percentage refers to the estimated proportion of cells showing specific fluorescence.

the nuclei appearing finely granular and of uniform brightness throughout, except for small unstained areas the size and shape of nucleoli. However, cells in mitosis showed uniform cytoplasmic staining, the chromosomal material being completely negative (Fig. 1).

In the continuous transformed cell lines of all four host species, essentially every cell demonstrated strong fluorescence. Multinucleated giant cells were

evident in the H 50-1 cell line; in these cells, every nucleus was brightly fluorescent (Fig. 2). First passage tissue cultures from tumors showed fluorescence in a smaller proportion of cells, presumably because of admixture of tumor cells with normal cells; large fibroblast cells, which were clearly distinct from tumor cells, were invariably negative.

In contrast, the antiviral serum gave no fluorescence with the SV40-transformed cells of hamster, rabbit, and mouse origin; these cells do not contain extractable virus, although the THK-1 cells yield virus when overlaid on AGMK monolayers (5).

AGMK and BSC-1 cell cultures acutely infected with SV40 virus reacted with both the SV40 antiviral and antitumor sera. With both sera, the fluorescence was confined to the nucleus.

The various control preparations, listed in Table I, gave no staining, with the exception of faint nuclear fluorescence of uninfected BSC-1 cells treated with the monkey antiviral serum.

Hamster tumor serum pool 2, with CF antibody titer of 1:10, was found to produce nuclear fluorescence of full intensity up to a dilution of 1:20. Nuclear fluorescence was markedly reduced, both in incidence of fluorescent nuclei and in intensity of staining, by absorption of serum pool 2, at a final dilution of 1:10, with SV40 tumor extract, while absorption with Ad. 12 tumor extract or normal hamster brain had no apparent effect.

An attempt was made to compare reactivity of individual hamster sera when tested in CF against SV40 tumor antigen and in the fluorescent antibody test against SV40-transformed cells of the THK-1 line. Nine hamsters were inoculated subcutaneously with THK-1 cells, and serum was obtained 10 weeks later, at which time seven bore tumors. The two animals without tumors, and one tumorous animal, were negative in both the CF and fluorescent antibody tests. Five tumorous animals were negative for CF antibody, but gave faint (1 to 2 plus) nuclear fluorescence; the other tumorous hamster had a CF antibody titer of 1:40 and gave 4 plus nuclear staining. Thus, there appeared to be a parallel between antitumor CF antibody and ability to stain transformed cells, with the latter test somewhat more sensitive.

DISCUSSION

The results of the immunofluorescent studies confirm the findings of Black *et al.* (4, 5, 11) in several important respects. First, they confirm that cells of various species altered by SV40 virus continue to elaborate a specific antigen(s) which reacts with sera of hamsters carrying SV40 tumors. Secondly, they confirm that the antigen is serologically distinct from that reactive in neutralization and antiviral CF tests. Most important, they indicate that continued production of the antigen is an integral property of essentially every transformed cell. The studies of Sabin and Koch (12) and of Black *et al.* (11) indicated that

a high proportion of transformed hamster cells carry the viral genome. The present studies indicate that this proportion is of the order of 100 per cent, and also that the same holds true for transformed rabbit, mouse, and porcine cells.

The validity of these conclusions is entirely dependent on the specificity of the fluorescent reactions observed. Three lines of evidence indicate that the reactions are specific for antigen(s) formed from viral genetic information. First, the lack of reaction in the various control preparations, which included tumors induced by other viruses, strongly indicates that the antigen occurs only in response to SV40 infection. Secondly, as pointed out by Black *et al.* (4), the presence of the antigen in each of the various species transformed cells indicates that the antigen is under viral genetic control. And thirdly, the formation of the antigen in acutely infected AGMK and BSC-1 cultures undergoing cytolytic response indicates its relationship to the virus; in this connection it should be stressed that hamster tumor sera reacted with the acutely infected cells even though devoid of virus-neutralizing and antiviral CF antibodies. This finding confirms the results of Hoggan, Black, and Rowe (in preparation) which showed the development during the cytolytic infection of AGMK cells of a CF antigen reacting with sera of hamsters bearing SV40 tumors.

It is not possible at present to say that the fluorescent antigen observed in infected AGMK is identical with the tumor CF antigen, although this seems probable. It may be that in both infected AGMK cells and transformed cells a specific antigen associated with an early stage of viral synthesis but distinct from that of the intact virus, is produced in the nucleus. In infected AGMK and BSC-1 cells, but not in transformed cells, the synthetic processes continue further and normally result in production of intact virus.

Melnick *et al.* (13) have shown that in the presence of 5-fluorouracil, infected AGMK cells produce large amounts of viral protein which is not incorporated into complete viral particles, but it is detectable by fluorescent antibody tests with antiviral immune serum. While it is conceivable that their test was detecting the "tumor antigen," it is more likely that it was revealing the viral surface antigens. Thus, the arrest of viral protein production in the transformed hamster, rabbit, and mouse cells appears to be more complete than that produced by 5-fluorouracil.

The nuclear localization of the fluorescent-stainable tumor antigen suggests that it is distinct from the SV40-specific transplantation antigen (14–16), which is presumably located in the cell membrane.

SUMMARY

With an immunofluorescent technique involving the use of serum of hamsters with SV40 tumors, nuclear fluorescence was detected in each of five cell lines, derived from four mammalian species, transformed by SV40 virus. Essentially all nuclei, including those of multinuclear cells, were fluorescent-stainable. Serum

of hamsters bearing SV40 tumors was also found to give nuclear fluorescence in susceptible cells (AGMK or BSC-1) acutely infected with SV40 virus.

These findings provide further evidence that cellular incorporation of the SV40 viral genome, with partial expression of the genome by synthesis of at least one virus-specific antigen, is an integral property of all SV40 transformed cells.

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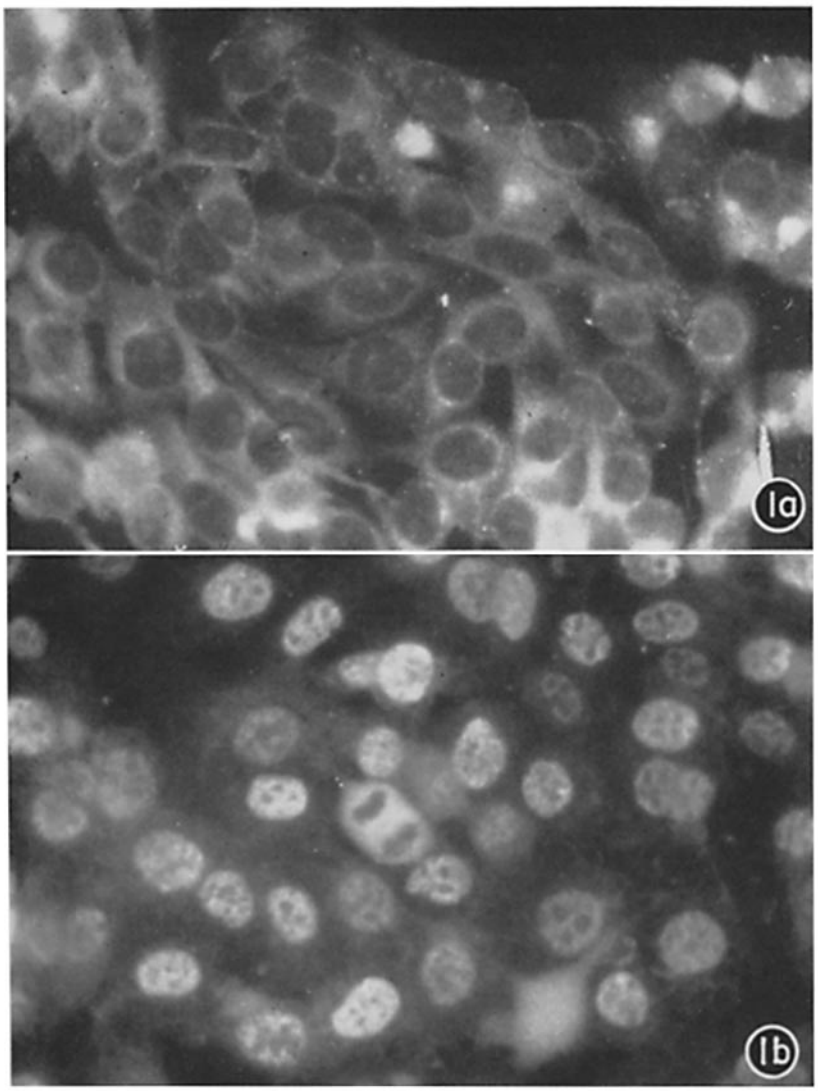
EXPLANATION OF PLATES

PLATE 7

FIGS. 1 *a* and 1 *b*. Fluorescent staining of H50-1 tumor cells, in first tissue culture passage, with sera of tumorous hamsters (indirect test). × 450.

FIG. 1 *a*. Stained with negative serum (from hamster with Ad. 12 tumor). This picture was overexposed relative to Fig. 1 *b* to show cellular outlines. Nuclei are completely unstained.

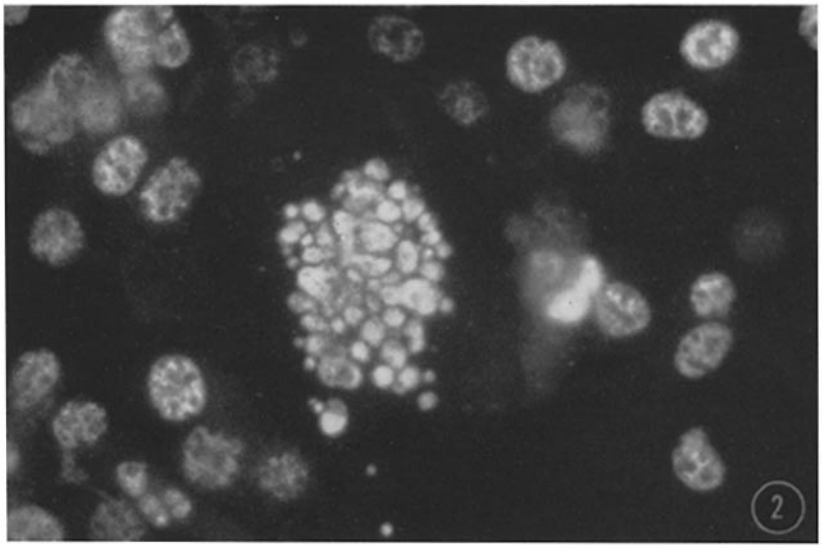
FIG. 1 *b*. Stained with SV40 hamster tumor serum pool 1. Every nucleus is stained. A cell in mitosis, in center of figure, shows diffuse staining of cytoplasm.



(Pope and Rowe: Specific antigen in SV40-transformed cells)

PLATE 8

FIG. 2. Fluorescent staining of SV40-transformed hamster kidney cells (THK-1 line) with serum pool 2. All nuclei but one are stained. Multinucleated giant cell (center) with every nucleus brightly fluorescent. × 450.



(Pope and Rowe: Specific antigen in SV40-transformed cells)