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Studies on Nondefective Adenovirus-Simian Virus 40 Hybrid Viruses

I. A Newly Characterized Simian Virus 40 Antigen Induced by the Ad2+ND1 Virus

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The nondefective adenovirus 2 (Ad2)-simian virus 40 (SV40) hybrid virus, Ad2+ND1, does not induce heat-labile SV40 T antigen but does induce a previously uncharacterized heat-stable SV40 antigen—the SV40 "U" antigen. This antigen is detectable by both immunofluorescence and complement fixation by using sera from hamsters with SV40 tumors. Sera from hamsters bearing SV40 tumors can be divided into two groups, those that react with both SV40 T and U antigens (T^+U^+ sera) and those that react with SV40 T antigen only (T^+U^- sera). SV40 U-specific sera from monkeys immunized with Ad2+ND1-infected cells do not react with SV40 T antigen by immunofluorescence but do react with an antigen in the nucleus of SV40-transformed cells and with an early, cytosine arabinoside-resistant antigen present in the nucleus of SV40-infected cells. A heat-stable SV40 antigen detectable by complement fixation with T^+U^+ hamster sera is present in extracts of SV40-induced hamster tumors and in cell packs of SV40-infected or -transformed cells. SV40 U-antigen synthesis by Ad2+ND1 virus is partially sensitive to inhibitors of deoxyribonucleic acid synthesis, whereas U-antigen synthesis by SV40 virus is an early cytosine arabinoside-resistant event. As an early SV40 antigen differing from SV40 T antigen, U antigen may play a role in malignant transformation mediated by SV40.

The isolation of a nondefective adenovirus-simian virus 40 (SV40) hybrid virus from the adenovirus type 2 (Ad2)-SV40 ($Ad2^{++}$) hybrid population has been described (11). Designated Ad2+ND1, this virus replicates with one-hit efficiency in both human embryonic kidney (HEK) and African green monkey kidney (AGMK) cells.

The deoxyribonucleic acid (DNA) of the Ad2+ND1 virus contains nucleotide sequences complementary to Ad2 and SV40 ribonucleic acid (RNA), and these DNA segments are linked by alkali-resistant bonds (M. J. Levin et al., *in preparation*). This recombinant genome is a linear duplex DNA molecule with a guanine plus cytosine content of 56% and a molecular weight of 22×10^6 daltons; it is estimated that the Ad2+ND1 DNA molecule contains about 250,000 daltons of SV40 DNA (C. S. Crumpacker et al., *in preparation*).

During acute cytolytic infection, the Ad2+ND1 virus does not seem to induce SV40 T antigen but does induce both SV40 RNA and a previously uncharacterized SV40 antigen, the SV40 U antigen (11). Preliminary studies demonstrated that the SV40 U antigen reacted specifically with sera from hamsters bearing SV40 tumors and was detectable by both immunofluorescence (FA) and complement fixation (CF; 11). When cells infected with Ad2+ND1 virus were stained by FA with sera from SV40 tumor-bearing hamsters, SV40 U antigen was detected in the perinuclear region of the cell (11). This antigen was stable to heating at 50 C for 30 min, and its synthesis during Ad2+ND1 replication was partially sensitive to such inhibitors of DNA synthesis as cytosine arabinoside and 5-fluorodeoxyuridine (11).

This report discusses the distribution of SV40 U antibody in hamsters bearing SV40-induced tumors and presents a more detailed characterization of the U antigen as induced by both Ad2+ND1 and SV40 viruses.

MATERIALS AND METHODS

Virus. The pools of Ad2+ND1 virus used in these experiments represented the 13th to 16th HEK

passages; the titers of these pools ranged from $10^{8.4}$ to $10^{9.0}$ plaque-forming units (PFU)/ml. Each pool was shown to be free of AAV 1–4 by CF testing.

SV40 strain 777 (4) has been maintained by serial passage in BSC-1 cells (8) by techniques described elsewhere (M. J. Levin et al., *in preparation*).

Cell lines. The THK-1 line of SV40-transformed hamster cells was established by Black and Rowe from a culture of weanling hamster kidney cells transformed by SV40 strain 777 (3). The THK-3 line was established at the same time as the THK-1 line (P. H. Black, *personal communication*).

The W18 VA2 line of SV40-transformed human fibroblasts (obtained from R. V. Gilden), the line established from clone 479 of SV40-transformed 3T3 cells, and the T + S + nonyielding line of SV40-transformed hamster embryo cells (both obtained from M. N. Oxman) have been described (7, 10, 14).

Antigens. For the preparation of $Ad2^+ND_1$ cell pack antigens for CF testing, tube cultures of either HEK or AGMK cells were used. Cultures were fed every 3 to 4 days with Eagle's minimal essential medium containing 10% fetal bovine serum until the cell sheets were very dense. The medium was then removed, and each tube was inoculated with $Ad2^+$-ND_1 virus at a multiplicity of 200 to 600 PFU/cell. After a 4-hr adsorption period, each tube was washed twice and refed with Eagle's medium containing 2% agammaglobulinic calf serum. Tubes were harvested by scraping 24 hr after inoculation; cell suspensions from replicate tubes were pooled and concentrated by centrifugation at 1,000 × *g* for 10 min; the packed cells were resuspended in 1/20 of the culture fluid. To disrupt the cells and release intracellular antigens, the cell packs were sonically treated for 6 sec at 6 amp with a Branson Sonifier. Uninoculated tubes from the same cell lot harvested by these procedures served as controls.

SV40-infected AGMK cell packs were prepared by using the same procedures, except that these cultures were harvested 48 hr after inoculation.

Cell packs of lines transformed by SV40 were prepared by scraping and resuspending the cells in a 32-oz (ca. 960 ml) bottle culture in 1 ml of culture fluid.

After sonic treatment, all cell pack antigens were stored at −70 C until used.

SV40 virion (V) antigen was prepared from a pool of SV40 that was cushioned on saturated KBr, dialyzed overnight against phosphate-buffered saline at 4 C, banded once in CsCl, dialyzed again, and stored at −70 C. The resulting virus suspension had an antigen titer of 1:2,560 when tested by CF against 4 to 8 units of SV40 V antibody.

The techniques for preparing FA antigens in HEK and AGMK cells grown on cover slips have been described (13).

Immune sera and serologic tests. Techniques for preparing hyperimmune adenovirus and SV40 rabbit antisera used as FA reagents have been described (12).

The SV40 V-specific antiserum used in CF tests (provided by M. D. Hoggan) was prepared by inoculating the foot pad of a guinea pig with "empty" bands of SV40 strain 777 mixed with Freund's adjuvant; it had an antibody titer of 1:2,560 when tested by CF against purified SV40 virions.

To detect Ad2 group-specific hexon antigen by CF, a convalescent serum from a patient recovering from an Ad4 infection was obtained and used at a dilution giving 8 units of Ad antibody.

The microtiter CF test and the indirect FA procedure used for staining cover-slip preparations of $Ad2^+ND_1$ and SV40-infected cells have been described (9, 15).

In tests for SV40-neutralizing antibody, serial dilutions of serum were mixed with equal volumes of a virus dilution giving 35 to 75 PFU of SV40 strain 777 per dish and incubated at room temperature for 30 min. Virus-serum mixtures were then inoculated onto AGMK monolayers (two dishes per dilution) and allowed to absorb for 1 hr at 37 C before overlaying with 0.8% Noble agar in Eagle's medium containing 10% agammaglobulinic calf serum. Positive and negative sera were included in each test. All dishes were stained with neutral red at 12 to 13 days, and plaques were counted 24 and 48 hr later. The techniques for testing for adenovirus-neutralizing antibody have been described (16).

RESULTS

Distribution of SV40 U antibody in hamsters bearing tumors induced by SV40. Previous studies demonstrated that cells infected with $Ad2^+ND_1$ virus contained a heat-stable antigen which reacted specifically by CF and FA with a majority of sera from hamsters bearing SV40-induced tumors (11). To determine the distribution of SV40 U antibody in the sera of hamsters with various types of SV40 tumors and the appropriate conditions for selecting sera with high-titer SV40 U antibody, single sera and sera obtained from serial bleedings were tested by CF and FA for SV40 T, U, and V antibody. As seen in Table 1, 57 of 60 hamsters bearing large tumors induced by two lines of hamster cells transformed in vitro by SV40 strain 777 or primary tumors induced by three strains of SV40 developed antibodies detectable by CF with SV40 T antigen (titers ranged between 1:10 and $\geq$ 1:320). Forty-two of these sera contained antibodies to SV40 U antigen with titers of 1:10 to 1:160. Three sera from hamsters with primary tumors failed to react with either antigen. Antibodies to SV40 V antigen were present only in sera from hamsters bearing primary tumors. Although CF antibody titers to purified SV40 virions were low (1:10 to 1:20), each of these sera neutralized 30 to 75 PFU of SV40 in a plaque-neutralization test.

Serial bleedings at 1- to 2-week intervals from animals with tumors induced by the THK-1 cell line indicated that CF antibodies to SV40 T antigen first appeared when tumors were 10 to 25 mm in diameter. Antibody to SV40 U antigen de-

TABLE 1. *Distribution of SV40 T, U, and V antibodies in sera from hamsters bearing tumors induced by SV40 virus*

Tumors induced by	CF antibody response (no. positive/no. tested) to[a]		
	SV40 hamster tumor extract (SV40 T)	$Ad2^+ND_1$ cell pack (SV40 U)	SV40 virions (SV40 V)
THK-1 cell line[b]	19/19	13/19	0[c]/19
THK-3 cell line[b]	19/19	19/19	0/19
SV40 strain 777 (primary tumors)[d]	7/10	1/10	1/10[e,f]
SV40 strain 776 (primary tumors)[d]	8/8	6/8	3/8[e]
SV40 large-plaque mutant[g]	4/4	3/4	0/4

[a] Sera titrated against 4 to 8 units of: (i) SV40 T antigen in 20% extracts of hamster tumors induced by the H50-1 line of SV40-transformed hamster embryo cells (2); (ii) SV40 U antigen in $Ad2^+ND_1$-infected HEK cell packs; (iii) SV40 virions purified by banding in CsCl density gradient.

[b] Tumors were induced by injecting 2×10^6 to 3×10^6 cells subcutaneously in weanling hamsters. Animals were bled 90 days after inoculation when tumors were 20 to 40 mm in diameter.

[c] 0 = <1 to 10.

[d] Hamsters were bled when tumors were 30 to 40 mm in diameter. Sera from hamsters bearing tumors induced by strain 776 were provided by M. D. Hoggan.

[e] These sera neutralized 30 to 75 PFU of SV40 virus at 1:10 dilution.

[f] Serum from this hamster contained no SV40 U antibody at 1:5 dilution.

[g] This tumor has been maintained by serial passage in hamsters. Sera were provided by H. Ozer and K. Takemoto.

veloped 1 to 3 weeks later. Approximately 100 days after injection, when a majority of these animals had tumors 40 to 50 mm in diameter, SV40 T-antibody titers were between 1:40 and ≥ 1:320 whereas SV40 U-antibody titers ranged from less than 1:10 to 1:160. By selecting appropriate sera obtained during these serial bleedings, two pools of serum were prepared, one with high antibody titers in CF and FA to both SV40 T and U antigens (T^+U^+ pool), and one with high-titer SV40 T antibody and low-titer SV40 U antibody (Table 2). By using the latter pool at 1:40 dilution, a reagent specific for SV40 T antigen was obtained which is referred to as the T^+U^- pool.

A number of individual sera with SV40 T-antibody titers ranging between 1:40 and 1:320 failed to react in CF with SV40 U antigen in $Ad2^+ND_1$ cell packs (Table 2). Several of these sera also failed to react in FA (Fig. 1A) with the perinuclear SV40 antigen in $Ad2^+ND_1$-infected HEK cells (Fig. 1B), whereas others reacted weakly (± to 1+ intensity) with a small percentage of cells. As each animal in this test received portions (containing 2×10^6 cells) of the same cell suspension, the antibody response of certain tumor-bearing hamsters to SV40 T antigen is demonstrably different from the response to U antigen.

It should be emphasized that the T^+U^+ serum pool did not react by CF with intact purified SV40 virions or SV40 virions which had been disrupted by exposure to *p*H 10.5 (1; Ozer, Lewis, and Rowe, *unpublished data*).

Preparation of $Ad2^+ND_1$ immune monkey sera. Since sera containing high-titer SV40 U antibody were obtained only from hamsters with slow growing SV40 tumors developing over a 2- to 3-month period, such sera always had high-titer SV40 T antibodies. For definitive studies on the development of SV40 U antigen during the replication of SV40, an antiserum specific for SV40 U antigen was essential. To prepare such a reagent, African green monkeys were hyperimmunized with AGMK cells infected with $Ad2^+ND_1$ virus.

Two monkeys were injected intramuscularly at weekly intervals with 0.5 ml of a cell pack antigen containing 16 CF units of SV40 U antigen mixed with an equal volume of incomplete Freund's adjuvant. The animals were test bled at weekly intervals and bled out 1 week after the seventh inoculation. Controls consisted of monkeys immunized with adjuvant mixed with cell packs of the same lot of uninoculated AGMK cells or monkey kidney cells infected with Ad2.

The control monkeys failed to develop antibodies to antigens in SV40-infected cells (Table 3). The monkeys hyperimmunized with $Ad2^+ND_1$-infected cells developed low-titer FA antibody responses (1:20 to 1:40) to antigens present in SV40-infected cells and high-titer antibodies (≥ 1:160) to antigens in Ad2-infected cells (Table 3). To evaluate the FA reactions of the $Ad2^+ND_1$-immune monkey sera, each serum was used at a 1:5 dilution to provide 4 to 8 FA units of SV40 U antibody.

In contrast to the perinuclear accentuation noted in the FA staining reaction of SV40 hamster sera with $Ad2^+ND_1$-infected cells, the reaction of the $Ad2^+ND_1$ immune monkey sera with SV40-infected cells appeared as a diffuse nuclear staining which is generally impossible to distinguish from the morphology of SV40 T staining (7, 15). The same pattern was observed when cells infected with six strains or variants of SV40 were stained with the $Ad2^+ND_1$-immune monkey sera. These included SV40 strain 777, two minute-plaque viruses isolated from the $Ad2^{++}$HEY and

TABLE 2. *Serological reactions of selected sera from hamsters bearing THK-1 tumors*

Sera	Antibody titer by					
	Complement fixation[a]			Fluorescent-antibody[b]		SV40 neutralizing
	SV40 hamster tumor extract	$Ad2^+ND_1$ cell pack	SV40 virions	SV40-transformed human cells	$Ad2^+ND_1$ in HEK	
T^+U^+						
Pooled, pool 1	320	160	0[c]	320	160	0
Single C191 H_4	≥320	≥160	0	320	80	
C192 H_1	≥320	80	0	≥640	40	
C193 H_2	≥320	40	0	320	40	
C194 H_2	160	≥160	0	160	80	
T^+U^-						
Pooled, pool 2	160	10	0	80	10	0
Single C192 H_2	40	0	0	40	0	
C192 H_3	80	0	0	80	0	
C194 H_3	≥320	0		160	20	
C194 H_5	160	0	0	160	0	

[a] Pools were titrated against 4 to 8 units of the same antigens described in Table 1.

[b] Pools were titrated against SV40 T antigen present in W18 VA2 line of SV40-transformed human fibroblasts (7) and SV40 U antigen present in $Ad2^+ND_1$-infected HEK cells.

[c] 0 = <10.

LEY hybrids (12), and large-plaque, small-plaque, and minute-plaque virus (19) obtained from K. Takemoto.

Serum taken prior to immunization and during the first 2 weeks of immunization from the monkeys receiving $Ad2^+ND_1$ cell packs did not react with SV40-infected cells. The SV40-reactive $Ad2^+ND_1$-immune monkey sera failed to react with SV40 virions by neutralization (Table 3) or by immunofluorescence with either mouse embryo cells containing polyoma T antigen or many lots of uninfected monkey cells.

Due to anticomplementary activity at low dilutions and reactions with uninoculated AGMK cells and media containing calf serum, it was not possible to determine whether the $Ad2^+ND_1$-immune monkey sera reacted by CF with SV40 cell pack antigen.

It should be mentioned that several attempts to obtain U antibody by immunizing hamsters and rats with homologous cell packs infected with $Ad2^+ND_1$ virus were unsuccessful. These failures were attributed to the low titers (< 1:2 to 1:4) of SV40 U antigen produced in the hamster and rat cell packs used for immunization.

Presence of SV40 U antigen in extracts of SV40 tumors and in cells transformed or infected by SV40. Having selected reagents specific for SV40 U and T antigens, it was important to determine whether SV40 U antigen is present in detectable levels in tumors and cells transformed or infected by SV40. The presence of SV40 U antigen in these preparations was determined by CF testing for heat-stable antigens reactive with T^+U^+ hamster serum. Tumor extracts and cell packs of transformed cells contained 4 to 64 units of heat-stable SV40 antigen which reacted only with the T^+U^+ hamster pool (Table 4). These materials also contained 16 to 128 units of heat-labile SV40 T antigen which reacted with both T^+U^+ and T^+U^- serum pools.

Similarly, cell packs of SV40-infected AGMK cells contained 8 to 16 units of heat-stable SV40 antigen which reacted only with the T^+U^+ pool (Table 5).

Thus, by using sera from SV40 tumor-bearing hamsters selected on the basis of their reactivity with SV40 U antigen in $Ad2^+ND_1$-infected cells, we have shown that cells infected with, or transformed by, SV40 either in vitro or in vivo contain a heat-stable SV40 antigen that reacts only with T^+U^+ sera and presumably represents U antigen.

As described above, sera from monkeys immunized with SV40 U antigen in $Ad2^+ND_1$-infected cells reacted specifically by FA with cells infected with SV40. When these sera were tested on the SV40-transformed lines by the indirect FA procedure, three of five lines tested showed a high percentage of cells with intense nuclear stain (Table 4). The THK-3 and SV40-transformed 3T3 lines reacted weakly (± intensity), however. Both of these lines stained brilliantly (3 to 4+ intensity) with 4 to 8 units of SV40 T^+U^- hamster serum. We interpret this marked difference in

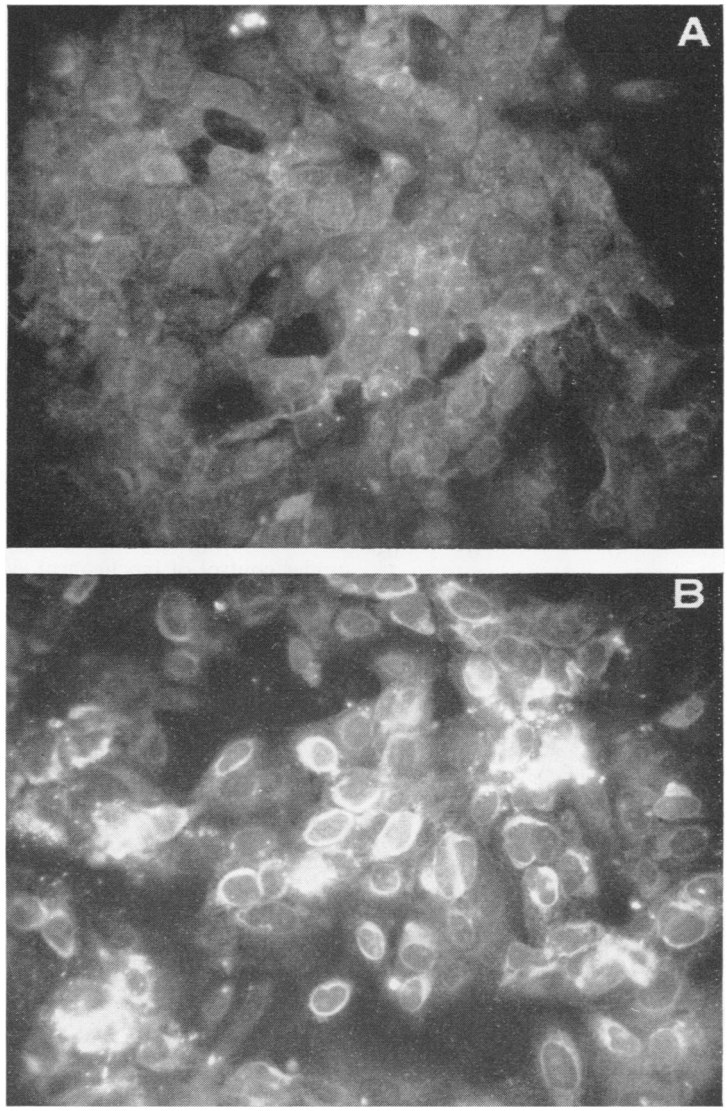

FIG. 1. *FA reactions of sera from tumor-bearing hamsters with Ad2$^+$ND$_1$-infected cells. (A) Ad2$^+$ND$_1$-infected HEK cells stained with T$^+$U$^-$ hamster serum. (B) Ad2$^+$ND$_1$-infected HEK cells stained with T$^+$U$^+$ hamster serum.*

intensity as indicating that the Ad2$^+$ND$_1$-immune monkey sera do not react with SV40 T antigen by FA. As all five lines contained heat-stable SV40 antigen by CF, we conclude that the staining of these lines by Ad2$^+$ND$_1$ monkey serum represents SV40 U antigen.

Effects of inhibitors of DNA synthesis on SV40 U antigen formation as detected by CF. By using the reagents for SV40 T and U antigens, we next compared the effects of inhibitors of DNA synthesis on the production of heat-stable and heat-labile SV40 CF antigens produced in cells infected with Ad2$^+$ND$_1$ and SV40. Cell packs were prepared by infecting AGMK cells in the presence or absence of 10 μg of cytosine arabinoside (CA) per ml; the drug was continually present in the medium from 1 hr prior to infection until the time of harvest. Each cell pack was titrated both unheated and after heating at 50 C for 30 min against 4 to 8 units of SV40 U antibody in the T$^+$U$^+$ hamster serum pool and of SV40 T antibody in the T$^+$U$^-$ pool (Table 5). From these data, it is apparent that the heat-stable SV40 U antigen in Ad2$^+$ND$_1$-infected cells was reduced fourfold by

TABLE 3. *Reaction of Ad2+ND1 hyperimmune monkey sera with Ad2 and SV40 antigens*

Animal no.	Immunizing antigen	Antibody titer in serum after 7th inoculation			
		Fluorescent-antibody[a]		Neutralizing[b]	
		SV40	Ad2	SV40	Ad2
1 C269	Ad2+ND1 AGMK cell pack	40	>160	0[c]	320
2 C270	Ad2+ND1 AGMK cell pack	20	>160	0	160
3	Ad2 AGMK cell pack	0	160	0	80
4	Ad2 AGMK cell pack	0	40	0	160
5	Uninoculated cell pack	0		0	0
6	Uninoculated cell pack	0		0	0

[a] Sera were titrated against AGMK infected with SV40 strain 777 and HEK cells infected with Ad2 prototype strain.

[b] Sera were tested for SV40 neutralizing antibody by plaque neutralization and for Ad2 antibody with a tube assay.

[c] 0 = <1 to 5.

the CA, whereas the heat-stable SV40 antigen induced by SV40 was not affected by CA. The Ad2+ND1 cell packs did not react with SV40 T or V antibody but did contain high titers of Ad2 group-specific antigen. SV40-infected monkey cells contained heat-labile, CA-resistant SV40 T antigen that reacted with both T+U+ and T+U− hamster sera. The SV40 V antigen in these cells was completely heat stable and its synthesis was reduced 32-fold by CA. Similar results have been obtained in other tests by using 10^{-5} M 5-fluorodeoxyuridine as an inhibitor.

Sequential FA studies on the development of T, U, and V antigens in cells infected with Ad2+ND1 and SV40. The preceding studies indicated that the synthesis of the heat-stable SV40 antigen detected in Ad2+ND1-infected cells was partially inhibited by treating the cells with CA, whereas the heat-stable antigen induced by SV40 was unaffected by CA. This suggests that the synthesis of SV40 U antigen by Ad2+ND1 virus is probably under control of biochemical events surrounding the synthesis of viral DNA (i.e., a "late" Ad function) although the synthesis of SV40 U antigen by SV40 is an early event. Sequential studies by FA on the development of T, U, and V antigen in cells infected with these viruses were undertaken to confirm these findings.

Figure 2 compares the development of FA-stainable SV40 U antigen and Ad2 T and V anti-

TABLE 4. *Heat-labile and heat-stable SV40 antigens in SV40-induced tumors or transformed cells*

Antigen	Antigen titer by complement fixation test				Reaction by fluorescent-antibody test	
	T+U+ hamster serum		T+U− hamster serum		Ad2+ND1 immune monkey serum	T+U− hamster serum
	Unheated	Heated[a]	Unheated	Heated[a]		
Hamster tumor[b] induced by						
THK-1	64	16	32	0[c]		
THK-3	16	4	16	0		
SV40 virus	64	16	64	0		
RSV[d]	0		0			
Ad12[d]	0		0			
Polyoma	0		0			
Transformed cell[e]						
Hamster, THK-1	128	64	128	0	90 (3+)[f]	95 (3+)
Hamster, THK-3	64	16	32	0	10 (±)	95 (3+)
Hamster, T+S+	64	32	64	0	75 (1+)	95 (3+)
3T3, clone 479	16	8	16	0	15 (±)	95 (3+)
Human, W18 VA2	64	32	64	0	95 (3+)	95 (4+)

[a] Antigen heated at 50 C for 30 min.

[b] 20% Tumor extract.

[c] 0 = <2.

[d] Induced by Ahlstrom strain of Rous sarcoma virus (RSV) and Huie strain of Ad12.

[e] Cell pack.

[f] Per cent positive cells. (Staining intensity ± very dull, 4+ brilliant).

TABLE 5. *Effects of cytosine arabinoside and heat on SV40 U antigen*

Antigen	Heat treatment (50 C, 30 min)	Antigen titer by complement fixation			
		SV40 hamster sera		SV40 V serum	Ad2 V serum
		T^+U^+	T^+U^-		
$Ad2^+ND_1$-AGMK	−	32	0[a]	0	128
$Ad2^+ND_1$-AGMK	+	32	0	0	
$Ad2^+ND_1$-AGMK + CA	−	8	0	0	0
SV40-AGMK	−	32	32	64	
SV40-AGMK	+	8	0	64	
SV40-AGMK + CA	−	64	32	2	
SV40-AGMK + CA	+	16	0	2	

[a] 0 = <2.

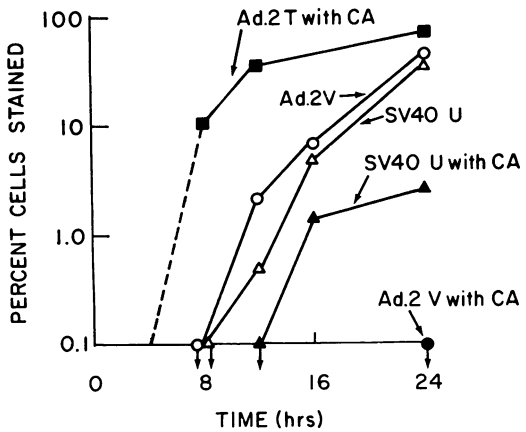

FIG. 2. *Sequential studies on the development of SV40 U and Ad2 T and V antigens in HEK cells infected with $Ad2^+ND_1$ virus at a multiplicity of 100 to 200 PFU/cell. SV40 U antigen was stained with T^+U^+ hamster serum, Ad2 T antigen was stained with serum from a hamster bearing an Ad12 tumor (17), and Ad2 V antigen was stained with Ad2-immune rabbit antiserum.*

gens in HEK cells infected with $Ad2^+ND_1$. From these curves, it is apparent that SV40 U antigen developed in parallel with Ad2 V antigen 4 to 6 hr later than Ad2 T antigen. Although Ad2 V antigen was completely inhibited by CA, the appearance of SV40 U antigen in $Ad2^+ND_1$-infected cells was delayed by 4 hr, and the number of cells containing SV40 U antigen was reduced 20-fold.

Figure 3 compares the development of SV40 T, U, and V antigens in monkey cells infected with SV40. SV40 U antigen as induced by SV40 virions

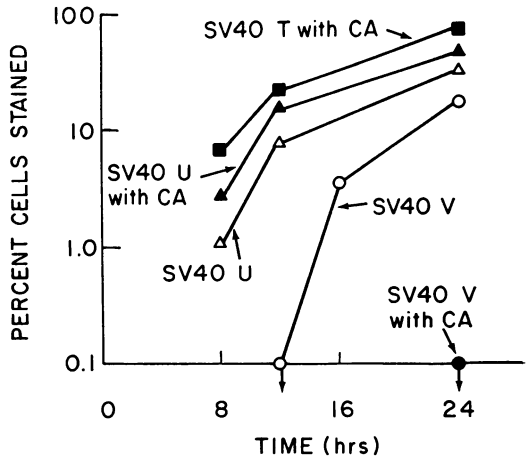

FIG. 3. *Sequential studies on the development of SV40 T, U, and V antigens in AGMK cells infected with SV40 strain 777 at a multiplicity of 100 to 200 PFU/cell. SV40 T antigen was stained with T^+U^+ hamster serum, SV40 U antigen was stained with $Ad2^+ND_1$-immune monkey serum, and SV40 V antigen was stained with serum from a rabbit immunized with ultraviolet-inactivated SV40 virus.*

developed early, in parallel with SV40 T antigen, and was unaffected by the presence of CA, which eliminated SV40 V-antigen formation. The same patterns were seen in three different experiments with each virus.

Both the CF and FA studies indicated that U antigen is an early product of SV40 infection, having the same time course and independence of DNA synthesis as T antigen. However, it is not clear whether U antigen is an early or late product in $Ad2^+ND_1$ infection. Although it seems to be synthesized at the same time as Ad V antigen, its synthesis shows a much greater degree of resistance to inhibitors of DNA synthesis.

DISCUSSION

A brief survey of the results of the foregoing experiments shows that sera from hamsters bearing SV40 tumors can be segregated into two groups—those that react by FA and CF with the SV40 antigen in $Ad2^+ND_1$-infected cells (T^+U^+ sera) and those that react with SV40 T antigen only (T^+U^- sera). Sera from monkeys immunized with $Ad2^+ND_1$ cell pack antigen do not react by FA with SV40 T antigen but do react with an antigen in the nucleus of SV40-transformed cells and with an early CA-resistant antigen present in the nucleus of SV40-infected cells. The SV40 antigen present in $Ad2^+ND_1$ cell packs is heat-stable, and its synthesis is partially sensitive to inhibitors of DNA synthesis. A heat-stable SV40 antigen de-

tectable by CF with T^+U^+ hamster sera is present in extracts of SV40-induced hamster tumors and in cell packs of SV40-infected or -transformed cells. These data lead us to conclude that we have succeeded in defining at least one of the uncharacterized SV40 antigens other investigators have detected in cells infected with or transformed by SV40 (6, 18).

By our definition, U antigen is a heat-stable SV40 antigen which reacts with a T^+U^+ but V-negative hamster serum in CF or any SV40 antigen detected by FA with $Ad2^+ND_1$-immune monkey serum. This antigen closely resembles T antigen in its kinetics of synthesis, morphological appearance in SV40-infected and -transformed cells, and its presence in all tumors and transformed cell lines examined. It is clearly different from T antigen in its serological reactivity and heat stability. It cannot be ruled out that U antigen is a variant or subunit of T antigen. Since T^+U^+ hamster sera fail to react with either purified or disrupted SV40 virions, U antigen does not appear to be a structural SV40 protein.

A major obstacle in these studies has been the presence of antibodies other than SV40 U antibody in the T^+U^+ hamster serum and the $Ad2^+ND_1$-immune monkey serum, i.e., SV40 T and multiple Ad2 antibodies, respectively. The high concentrations of these antibodies have made absorption impractical. Standardization of these reagents by FA against $Ad2^+ND_1$-infected cells grown in the presence of DNA inhibitors to block Ad2 V antigen was considered unreliable since evaluation of $Ad2^+ND_1$-immune monkey serum would require differentiating between reactions with SV40 U and early Ad2 antigens in the same cell. Consequently, it has been necessary to define the SV40 U antigen with reagents that were standardized against different antigens. Although the selection and preparation of each of these reagents depended upon the presence of SV40 U antigen in $Ad2^+ND_1$-infected cells, we must consider the possibility that more than one antigen is being detected.

The discrepancies noted in the properties of U antigen induced by different viruses, i.e., perinuclear versus homogeneous nuclear staining and partial sensitivity versus resistance to inhibitors of DNA synthesis, could be explained by the presence of antibodies in the T^+U^+ serum to additional uncharacterized SV40 antigens in infected cells. The $Ad2^+ND_1$-immune monkey sera should be quite specific for U antigen as biophysical studies indicate that the $Ad2^+ND_1$ genome contains only one-tenth of the SV40 genome (Crumpacker et al. and Levin et al., *in preparation*). Thus, it is unlikely that this virus is inducing more than one SV40 protein.

The morphological difference between the perinuclear accumulation of U antigen in $Ad2^+ND_1$-infected cells and the diffuse nuclear staining in SV40-infected cells is not inconsistent with our conclusions, as the intracellular localization or morphological appearance of an antigen by FA cannot be accepted as an inherent antigenic property. The perinuclear accumulation of U antigen during $Ad2^+ND_1$ infection could be due to the absence of the "virus-mediated transport mechanism" proposed by Butel et al. (5), which transfers early SV40 antigens from cytoplasm to nucleus.

In evaluating the differences in the effects of CA on the synthesis of SV40 U antigen in cells infected with SV40 and $Ad2^+ND_1$, the location of the small piece of SV40 DNA in the $Ad2^+ND_1$ genome must be considered. It is possible that a piece of SV40 DNA containing an early function could be linked to Ad2 DNA in such a position that it would be transcribed as a late function during the replicative cycle of the hybrid virus. An equally plausible alternative is that U antigen is produced in undetectable quantities early in the replicative cycle of $Ad2^+ND_1$ virus but is amplified to detectable levels later in the cycle during the replication of viral DNA.

Having confirmed the presence of multiple early SV40 antigens in SV40-induced tumors and transformed cells and having demonstrated the presence of high-titer antibody in the sera of tumor-bearing hamsters to at least two such SV40 antigens, we are obliged to point out the need for a careful appraisal of studies characterizing SV40 T antigen. Future work will require a thorough evaluation of T-reactive hamster sera for the presence of SV40 U antibody as well.

Preliminary studies on other Ad-SV40 hybrids indicate that the Ad7-SV40 hybrid population ($E46^+$) induces heat-labile SV40 antigen reactive with T^+U^- hamster sera and smaller quantities of heat-stable SV40 antigen reactive only with T^+U^+ hamster sera. Thus, this hybrid also induces SV40 U as well as SV40 T antigen.

Since SV40 U antigen is present in both tumors and transformed cells, it could be involved in SV40-mediated transformation. However, as the $Ad2^+ND_1$ virus does not seem to transform hamster cells in vitro (11) or produce tumors when inoculated into newborn hamsters (Lewis and Rowe, *unpublished data*), it seems unlikely that this antigen is the SV40 gene product responsible for oncogenesis. However, if transformation by oncogenic DNA viruses is a multiple-step process requiring some gene products for integration and others for transformation, the $Ad2^+ND_1$ virus could be missing one or more of these SV40 genes, and U antigen could still be involved in malignant transformation.

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