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PRODUCTION OF "TUMOR-SPECIFIC" ANTIGENS BY ONCOGENIC VIRUSES DURING ACUTE CYTOLYTIC INFECTIONS

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Virus specific complement-fixing (CF) antigens in adenovirus type 12 tumor extracts which react with adenovirus type 12 tumor-bearing hamster sera have been described by Huebner *et al.*[1] Similar specific antigens have been found in SV40-transformed cells and SV40-induced hamster tumors by Black *et al.*[2]

Much evidence indicates that these antigens are under viral genetic control.[1–4] However, since they have not been found in ordinary acute virus preparations harvested after 4+ cytopathogenic effect (CPE), it has been suggested[2] that they may be produced only in virus-induced tumors or viral-transformed cells.

We present data herein that show that these or similar CF antigens are produced during early phases of the normal virus reproductive cycle in acutely infected cells. Further evidence is presented that these antigens are soluble, but closely associated with cell or cell debris and are less stable than the antigens associated with the intact virus particle.

Materials and Methods.—SV40 virus: Two strains of SV40 virus were used. Strain 776[5] (pool #3-457) had an infectivity titer of $10^{7.4}$ $TCID_{50}$ per 0.1 ml in Cercopithecus kidney [African green monkey (AGMK)] cell cultures. Strain 777 (pool #01370) was originally obtained from Dr. Paul Gerber and has been previously described.[6] Since receipt in this laboratory, it has been passed five times in AGMK and twice in BSC-1 cells (a continuous line of AGMK).[7] Pool #01370 had an infectivity titer of $10^{6.4}$ $TCID_{50}$ per 0.1 ml in AGMK and BSC-1 cells.

Adenovirus type 12: One pool (#3-453) of prototype Ad. 12 virus (Huie strain[8]) was used in all experiments reported here. It had an infectivity titer in human embryonic kidney cultures (HEK) of 10^7 $TCID_{50}$ per 0.1 ml. (SV40 pool #3-457 and Ad. 12 virus pool #3-453 were prepared on contract #PHS-43-62-200 by Microbiological Associates, Inc., Bethesda, Maryland.)

Cell cultures: Growth media were as follows. KB and HEK cells: Eagle's basal medium (BME) and 10% calf serum; AGMK cells: BME and 10% calf serum with 0.2% anti-SV5 rabbit serum; BSC-1 and hamster kidney (HK) cells: BME and 5% calf serum. Except for infectivity titrations, cells were grown in 32-oz prescription bottles; all cultures were maintained in BME and 2% heated agammaglobulinic calf serum. (Agamma calf serum was obtained from Hyland Laboratories, Los Angeles, California.) All media contained penicillin and streptomycin.

Infectivity titrations: SV40 titrations were done in AGMK or BSC-1 cells, and Ad. 12 infectivity was determined in HEK cells. At least three tubes per tenfold dilution were used, and the

tubes were observed every 2 days for at least 21 days. Fluids were changed twice each week. $TCID_{50}$ per 0.1 ml were determined by the method of Reed and Muench.[9]

Complement-fixation tests: The various preparations under examination were tested for complement-fixing antigens as previously described.[1, 2] The CF antigens have been previously classified as "viral" or "tumor" antigens.[1, 2] The "viral" antigens have been considered to be those antigens produced by replicating virus in acutely infected cell cultures and which are found in the tissue culture fluid containing cell debris after 4+ CPE. In this communication such preparations will be referred to as "standard virus harvests" and the antigens again classed as "viral antigens."

Antigens found in saline extracts of tumors and which are distinct from previously recognized viral antigens will be referred to as "T antigens." It should be pointed out that even though standard virus antigens generally cannot be detected in such extracts, animals which carry tumors for extended periods may develop antivirion antibodies, with both primary and transplanted tumors in the case of Ad. 12 tumors,[1] and predominantly with primary tumors in the case of SV40.[2]

A third type of antigen preparation, concentrated cell packs of acutely infected cells, contains antigens which react with antiviral sera and antigens which react with antitumor sera. These harvests will be called "cell pack antigen preparations"; their manner of preparation is described below.

Two types of sera were used in the SV40 system: (1) hamster sera from SV40-induced tumor-bearing animals which reacted to higher titer (160 or >) against SV40 tumor extracts or SV40-transformed cell extracts, but not at all against standard virus harvests; (2) SV40 virion-reactive sera consisting of convalescent sera from monkeys or baboons injected with SV40 virus. Some of these primate sera reacted only with virus antigen at low or high serum dilution, while other sera reacted against both tumor extracts and virus preparations at low dilutions. However, at the serum dilution used in tests for virion antigen (8 antibody units), there was no reaction with tumor extract.

Three types of sera were used as reagents for defining Ad. 12 CF activity: (1) "narrow reacting" sera from hamsters with Ad. 12-induced tumors; these sera react with Ad. 12 tumor extracts, but not with standard Ad. 12 virus pools; (2) "broad reacting" sera from tumor-bearing hamsters which react both against tumor and virus antigen preparations; and (3) a pool of human convalescent sera (#33917) which in the CF test reacts against the group antigen(s) of all adenoviruses, but does not react against Ad. 12 type-specific antigens or Ad. 12 tumor extracts. This human serum pool shall be called "group reactive serum." Checkerboard titrations were done on each serum pool and they were then used at dilutions which were eight times more concentrated than a dilution giving a 3+ reaction with the optimal dilution of antigen.

Experimental design: Replicate cell cultures in 32-oz prescription bottles were inoculated with multiplicities of virus ranging from 0.3–120 $TCID_{50}$ per cell. The virus was allowed to adsorb for 2–4 hr at 37°C. Each culture was then washed four times with 25 ml of BME and then renewed with 24 ml of maintenance medium. After various intervals of incubation at 37°C, infected and control bottles were harvested as follows: cells were scraped into the supernatant fluid, which was then centrifuged at 1500 rpm for 10 min. Samples of supernate were removed and the cell packs were suspended in 5–10 ml of PBS. Cell counts were then made utilizing Trypan Blue. The cells and debris were recentrifuged and resuspended in PBS at a concentration of $5–50 \times 10^6$ cells per ml. The preparations were then stored at −60°C until tested. Unless specified, they were tested without further disruption or centrifugation. All kinetic data have been adjusted to represent the antigen units per cell.[15]

Results.—Sequential appearance of SV40 antigens in acutely infected AGMK (primary and BSC-1 cells): Figure 1 shows a typical time experiment using BSC-1 cells infected with SV40 strain 776, at a multiplicity of 35 $TCID_{50}$ per cell. Beginning at 24 hr, antigens reactive with tumor-bearing hamster serum appeared in the cell pack, reaching a peak at 2 days and declining thereafter. Virion antigen was first detected in the cell pack at 2 days, and in the supernatant fluid at 3 days. At no time was tumor serum-reactive antigen detected in the supernatant fluid.

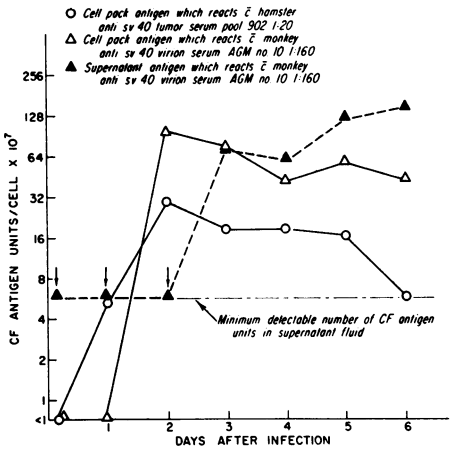

FIG. 1.—CF antigen formation by SV40 virus strain 777 in BSC-1 cells.

Similar experiments carried out using AGMK cells gave similar results. Also, SV40 strain 777 gave comparable results in both BSC-1 and AGMK cell cultures. In all instances the tumor antigen appeared prior to the viral antigen.

Failure to demonstrate SV40 CF antigens in hamster kidney cells during short-term experiments: Primary HK cultures were inoculated with multiplicities of 10 and 40 $TCID_{50}$ of SV40 virus per cell. The cells and supernatant fluid were harvested from replicate cultures in the usual manner (one bottle each day for 12 days). Neither virion nor tumor CF antigens were found at any time. Since the parameter of CF antigen formation may be relatively insensitive, this result would not be too surprising if only a small proportion of cells were involved.[10]

Comparison of the SV40 CF antigens from standard virus harvests, tumors, and cell pack antigens: Black *et al.*[2] showed that the antigens found in SV40-induced tumor or transformed cells differed from SV40 viral antigen(s) in sedimentability and heat lability, as well as in serologic reactivity. Table 1 compares the heat stability of the three types of preparation; it is evident that the acutely infected cell pack preparations contain both heat-labile tumor serum-reactive antigens and heat-stable SV40 virion-reactive serum antigens.

TABLE 1

EFFECT OF HEAT (50°C 30 MIN) ON SV40 CF ANTIGENS

Antigen preparation	Treatment	Hamster SV40 Tumor Serum		AG Monkey Anti-SV40 Virion Serum	
		a	*b*	*c*	*d*
Standard virus harvest #3-457	Unheated	0	0	4	16
	Heated	0	0	4	32
Tumor extract (primary SV40) 02151	Unheated	16–32	...	0	
	Heated	0		0	
Tumor extract (Melnick line) H50-1[e]	Unheated	...	8	..	0
	Heated	...	0	..	0
SV40-transformed C_3H mouse kidney cell 01472	Unheated	...	4	..	0
	Heated	...	0	..	0
SV40-infected BSC-1 cell pack 01372 (1:2)	Unheated	16	16	4	64
	Heated	0	0	8	64
SV40-infected AGMK cell pack 01034	Unheated	...	16	..	256
	Heated	...	0	..	256
Standard virus harvest Ad. 12 #3-453	Unheated	...	0	0	...
Ad. 12 tumor extract #12	Unheated	0	...	0	...

[a] Hamster serum pool 00190, 1:20.
[b] Hamster serum pool 902, 1:20.
[c] AG monkey serum MS37-2, 1:160. (No antitumor antibody at 1:20.)
[d] AG monkey serum #13, 1:160. Kindly supplied by Dr. Joseph Melnick.
[e] Transformed hamster embryo cell line received from Dr. Joseph Melnick and transplanted 4× in weanling hamsters in our laboratory.

Effect of sonication on the sedimentability characteristics of SV40 cell pack antigens: Black *et al.*[2] have shown that only a small fraction of the CF activity of the tumor antigen is sedimentable at 30,000 rpm (59,310 *g* av) for 2 hr while virtually all of the CF activity of standard virus harvests is sedimentable under the same conditions. Table 2 shows the effects of low (350 × *g* for 10 min at 4°C) or high speed (74,000 *g* for 1 hr in a 39SW Spinco rotor) centrifugation both before and after sonication of cell pack antigen preparations. The antigens had been frozen and thawed once prior to centrifugation.

It is obvious that even low-speed centrifugation removed some CF-reactive antigens from the cell pack preparation before it was sonicated. High-speed centrifugation of otherwise untreated cell pack antigens removed virtually all of the reactivity against antivirion serum but little of the reactivity against the antitumor serum.

After sonication very little of the CF antigen activity against either antitumor or anti-SV40 virion serum was sedimented by slow-speed centrifugation. However, after high-speed centrifugation virtually all of the remaining CF activity against tumor serum was in the supernate, while all CF activity against virion-reactive serum was in the pellet. In both high-speed centrifugations some antigen activity was lost, particularly of viral antigen, which could not be recovered even after dissolving the high-speed pellets overnight at 0–4°C. The reason for this is not completely clear, but we have felt that insoluble aggregates of the virion may form during ultracentrifugation. It should also be pointed out that sonication caused a one-log increase in the infectivity titer of the cell pack preparations.

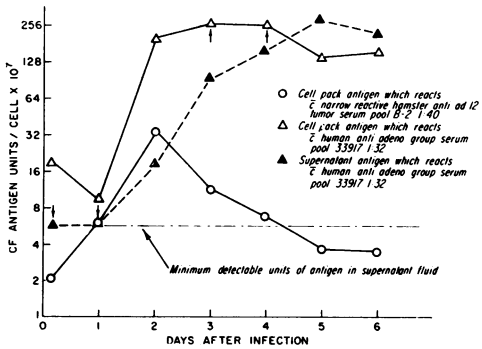

FIG. 2.—CF antigen formation by adenovirus type 12 in stationary KB monolayer cultures.

Thus, the "tumor-reactive" antigen in the cell pack preparation not only shared the serological reactivity of that in tumor extracts, but resembled it in its heat lability and failure to sediment.

Sequential appearance of Ad. 12 CF antigens in acutely infected KB cells: As expected, Ad. 12 CF antigens which reacted against the group-reactive human serum were easily demonstrated in cell pack preparations. A representative experiment carried out in stationary KB cultures is shown in Figure 2; a multiplicity of 48 $TCID_{50}$ per cell was used. Adenovirus group-reactive antigen (that demonstrated by the human pool #33917) began increasing in the cells between 24 and 48 hr, and continued to increase until it reached an apparent peak between 72 and 96 hr. It decreased by 6 days at which time the experiment was terminated. This same antigen was first detectable in the supernatant fluid at 48 hr, and reached rather high levels by 5 days.

Some antigen reactivity with narrow-reacting hamster serum was noted at 4 hr in the cell pack preparations. It increased by 24 hr, reached its maximum level by 48 hr, and then decreased progressively until the sixth day. At no time were more than trace amounts of this reactivity in the supernatant fluid.

TABLE 2

EFFECT OF SONICATION AND CENTRIFUGATION ON SV40 AND ADENOVIRUS 12 CELL PACK PREPARATION

Treatment	SV40-Infected AGMK Cell Pack #01372*			Ad. 12-Infected KB Cell Pack #01474*		
	CF titer with hamster anti-SV40 tumor serum pool 902, 1:20	CF titer AGM anti-SV40 virion serum #13, 1:160	Infectivity titer $TCID_{50}$ per 0.1 ml log_{10}	CF titer hamster anti-Ad. 12 tumor serum narrow-reactive pool 9, 1:20	CF titer human antiadeno group-reactive serum 1:16	Infectivity titer $TCID_{50}$ per 0.1 ml log_{10}
None	32	128	6.3	32	512	7.5
Low-speed centrifugation 350 × *g*—10 min						
Supernate	16	32	5.5	16	256	7.3
2 × pellet	32	128	6.5	32	512	6.7
High-speed centrifugation 74,000 × *g*—1 hr (39SW Spinco rotor)						
Top half supernate	16	0	4.7	16	256	4.7
Bottom half supernate	16	0	5.4	16	256	4.5
2 × pellet	16	32	6.7	16	256	5.7
Sonication in Bronson sonicator for 1 min at 2.5 amp	16	128	7.5	64	512	8.7
Low-speed centrifugation of sonicate 350 × *g*—10 min						
Supernate	16	128	7.0	64	512	8.0
2 × pellet	2	16	6.7	8	128	7.0
High-speed centrifugation of sonicate 74,000 × *g*—1 hr (39SW Spinco)						
Top half supernate	8	0	3.7	32–64	256	5.7
Bottom half supernate	8	0	5.5	64	256	3.0
2 × pellet	0	16	6.5	16	256	8.7

* The antigen had been frozen and thawed once before centrifugation.

The results of similar experiments carried in KB spinner cultures will be reported in detail elsewhere.[11]

Formation of antigens in Ad. 12 infected hamster cell cultures: Hamster kidney (HK) cultures were infected with Ad. 12 virus at a multiplicity of 25 $TCID_{50}$ per cell and cell pack preparations made at intervals. The results of CF antigen and infectivity assays are summarized in Figure 3 and Table 3. The table also shows comparable data obtained with KB cell cultures in the experiment shown in Figure 2. The hamster cells produced antigens which reacted with tumor-reactive hamster sera, the titers reaching maximal levels within 1–3 days and remaining at comparable levels through at least the ninth day. In contrast, no group-reactive antigen was formed, and there was no indication of formation of infectious virus. This pattern of antigen development resembled that seen in Ad. 12-induced hamster tumors, in which there is abundant T antigen but no group (A) antigen or infectious virus. The cytopathic changes which occurred in Ad. 12 inoculated HK cultures during the 9-day period studied were negligible, and 85 per cent of the cells were viable at 9 days. Attempts to pass Ad. 12 virus serially (either measured by infectivity or production of CF antigen) in HK failed.

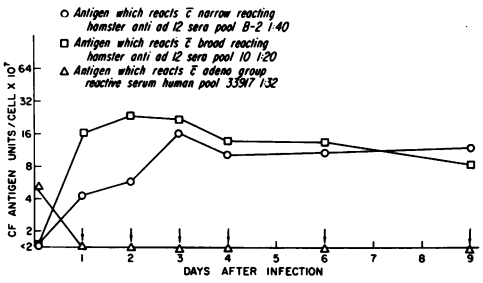

FIG. 3.—CF antigens found in adenovirus type 12 infected hamster kidney cell pack preparations.

In marked contrast, when similar experiments were done with Ad. 2 virus, there was marked CPE with the production of infectious virus and group-reactive antigen, but no antigens reactive with either narrow or broad-reactive Ad. 12 tumor serum. The infectivity titer of the cell pack after seven HK passages was over 10^8 $TCID_{50}$ per 10^7 cells, and the group-reactive CF antigen titer was over 1:64. Ad. 7 virus (Gomen strain) gave similar results on passage in HK cells, but the titers were lower (infectivity 10^5 $TCID_{50}$ and 8–16 CF antigen units per 10^7 cells after seven passages).

Comparison of some biological and physical properties of antigens in virus, tumor, and cell pack preparations: Like the SV40 tumor antigen, the antigens in Ad. 12

TABLE 3

FORMATION OF ADENOVIRUS CF ANTIGENS AND INFECTIOUS VIRUS IN ADENOVIRUS 12 INFECTED HAMSTER KIDNEY AND KB CELL CULTURES

	Ad. 12 Infected HK Cell Pack			Ad. 12 Infected KB Cell Pack		
Time	CF antigen units per 10^7 cells with narrow tumor-reactive sera pool B2 1:40	CF antigen units per 10^7 cells with group-reactive sera pool 33917 1:32	$TCID_{50}$ per 10^7 cells $\log_{10}$	CF antigen units per 10^7 cells with narrow tumor-reactive sera pool B2 1:40	CF antigen units per 10^7 cells with group-reactive sera pool 33917 1:32	$TCID_{50}$ per 10^7 cells $\log_{10}$
2–4 hr	<2	5.5	6.2	2.5	21.2	6.4
1 day	4.2	<2	5.8	6.4	10.6	7.5
2 days	6.1	<2	5.8	25.5	206	9.1
3 "	16.1	<2	5.8	12.5	>256	9.1
4 "	11	<2	4.5	7.5	>256	9.2
5 "	...	...	...	3.9	144	9.2
6 "	8	<2	4.5	3.5	165	8.0
9 "	13.6	<2	2.7	...	...	...

tumors demonstrate greater thermal lability than the previously described viral antigens, and are not sedimented by centrifugal forces which sediment the virus particles.[1] To determine if these properties are also characteristic of the tumor-reactive antigens in cell pack preparations, experiments were carried out similar to those described above for SV40 antigens (Tables 2 and 4).

TABLE 4

COMPARISON OF HEAT STABILITY OF VARIOUS ADENOVIRUS 12 CF ANTIGENS

Antigen preparation	Heating	CF antigen titer with narrow tumor-reactive sera pool B2 1:40	CF antigen titer with broad reactive sera pool 10 1:20	CF antigen titer with group reactive sera pool 33917 1:32
Standard virus harvest #3-453	—	0*	16	128–256
	56° 30′	0	8	128–256
Standard Ad. 12 tumor extract #6	—	32	128	0
	56° 30′	8	8–16	0
Acute Ad. 12 infected KB cell pack #1474	—	16	128	256
	56° 30′	4	8	512
Normal KB cell pack	—	0	0	0

* 0, negative at 1:2 dilution.

Tests for heat (56°C 30 min) stability of standard virus, tumor, and cell pack preparations (Table 4) confirmed the stability of the group-reactive antigen in viral and cell-pack preparations and the partial inactivation of the antigens in tumor extracts. The tumor-reactive antigen in the cell pack was also largely inactivated by the heating.

The sedimentation and sonication studies (Table 2) of the tumor antigen gave results essentially identical to those with SV40. The tumor-reactive antigen in the frozen-thawed preparation was chiefly associated with large cellular particles, but after sonication the bulk of the antigen was not sedimentable. It is noteworthy that the group-reactive antigen showed the same pattern; this suggests that the apparent difference between tumor-reactive and group antigen with respect to appearance in culture fluid may be merely a function of the lower titer and instability of the former. Sonication of the Ad. 12 cell-pack preparation also resulted in an increase of one log or greater in the infectivity titer.

Discussion.—The studies reported here indicate the identity between the antigens previously found only in tumors and those in the cell-packs of tissue culture preparations. Further evidence of this identity in the Ad. 12 system was obtained in immunodiffusion tests by Berman and Rowe,[12] cell-pack and tumor extract antigens giving a continuous precipitation line when reacted with tumored hamster serum, this line being distinct from that obtained with standard viral harvests. Also, the immunofluorescent studies of Pope and Rowe[13, 14] showed Ad. 12 and SV40 antigens in acutely infected cells when stained with tumor-specific hamster antisera. The failure of these antigens to be recognized in previous studies of antigenic composition of SV40 and adenoviruses is due to a number of causes, including their failure to appear in culture fluids, relative instability, and low antigenicity, as well as the previous lack of appropriate immune sera.

In both the immunofluorescent and CF antigen studies there are indications that the tumor-reactive antigens appear earlier than the virion antigens. These antigens

may well represent virus-induced precursor enzymes, persistence of which may be important in determining the abnormal functioning of the tumor cells.

Although cell-pack preparations have been used by a number of workers to obtain high titers of infectious virus, the present study indicates that they may also be of much use for preparing high-titered CF antigens and for defining antigenic composition of viruses.

Note added in proof: Since the submission of this paper, "tumorlike" antigens in SV40-infected cells have been reported by A. B. Sabin and M. A. Koch [these PROCEEDINGS, **52**, 1131 (1964)], and by F. Rapp, T. Kitahara, J. S. Butel, and J. L. Melnick [these PROCEEDINGS, **52**, 1138 (1964)].

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[14] *Ibid.*, 577 (1964).

[15] Calculation of CF antigen units: In order to be able to standardize all bottles in a given experiment and to be able to equate the results from experiment to experiment, the data from all kinetic experiments were calculated on the basis of CF antigen units per cell (*CFU*). The three parameters which must be considered are (1) complement-fixation titer (*CFT*) which equals the reciprocal of the dilution of antigen which gives a 3-plus reaction with 8 units of antibody, (2) the total volume (V) of a preparation (usually 0.5–1.0 ml of a cell pack antigen and 25 ml of supernatant fluid), and (3) the total number (N) of cells in the preparation at the time of harvest.

$$CFU = \frac{CFT \times V}{N}, \text{ e.g.,}$$

	Cell pack	Supernate
CFT	16	2
V (ml)	1	25
N	8×10^6	8×10^6
CFU	$\frac{16 \times 1}{8 \times 10^6} = 20 \times 10^{-7}$/cell	$\frac{2 \times 25}{8 \times 10^6} = 62.5 \times 10^{-7}$/cell