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Transformation of Hamster Embryo Cells and Tumor Induction in Newborn Hamsters by Simian Adenovirus SV11

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Simian adenovirus, SV11, readily transformed hamster embryo cell cultures in vitro and produced tumors in vivo when inoculated into newborn hamsters. Foci consisting of small, loosely attached, rounded cells could be seen as early as 7 days postinoculation. Many of these cells contained several nuclei or the nucleus was multilobed. The cells grew without extensive cell to cell contact or formed small chains or clusters when passaged in vitro. This pattern of cell morphology and growth has not been reported with other simian or human adenovirus-transformed cells. Linearity of foci formation with virus dilution was observed when the virus multiplicity was less than 3 plaque-forming units (PFU)/cell. The PFU to focus-forming units ratio for SV11 was found to be 2×10^4 to 4×10^4, which is approximately 5- to 10-fold and 50- to 100-fold lower than those reported for simian adenovirus, SA7, and human adenovirus type 12, respectively. Cells transformed by SV11: (i) produced tumors when inoculated into young hamsters, (ii) contained tumor antigen which reacts with serum obtained from hamsters bearing SV11 passaged tumors, and (iii) could be propagated in vitro through an indefinite number of generations.

The report by Trentin et al. (15) of the oncogenicity of human adenovirus type 12 (Ad12) for newborn hamsters has now been extended to include adenoviruses from other animal species (2, 5, 7, 11, 12). Thus far, 11 simian adenoviruses have been reported to be oncogenic in newborn hamsters. These include SV1 (6), SA7, SV20, SV33, SV34, SV37, and SV38 (7), SV11, SV23, and SV25 (5), and SV30 (14). With the exception of SA7, SV25, and SV37, the oncogenic simian advenoviruses comprise hemagglutination group III as determined by Rapoza (9).

Gilden et al. (5) grouped the tumor and T antigens of the oncogenic and several nononcogenic simian adenoviruses on the basis of their cross-reactivity in the complement-fixation test. Tumor and T antigens induced by simian adenoviruses SV1, SV11, SV25, SV33, SV34, and SV38 have been tentatively placed in subgroup I. SV20 and SV23 are classified in subgroup II, SA7 in subgroup III, and SV37 was listed as ungrouped. SV30 was classified as nononcogenic, although Slifkin et al. (14) previously had shown this virus to produce tumors in newborn hamsters.

Over the past few years, we have been conducting investigations with various simian adenoviruses in an effort to classify the oncogenic potential of these viruses, based on their relative transforming efficiency, in vitro, for hamster embryo cells. Of the viruses studied thus far, SV11 has presented an unusual and distinctive type of transformation which distinguishes it from the reported transformation induced by other adenoviruses. The SV11 transformation of hamster embryo cells can be characterized as follows: (i) a rapid appearance of identifiable transformed cell foci within 7 days after virus inoculation; (ii) a type of transformed cell which morphologically and culturally is quite distinct from other adenovirus-transformed cells; and (iii) a relatively low ratio of plaque-forming units (PFU) to focus-forming units (FFU).

This report presents these features of the SV11 transformation and, in addition, confirms the finding by Gilden et al. (5) of the oncogenicity for newborn hamsters.

MATERIALS AND METHODS

Virus. SV11 was obtained from R. N. Hull, Eli Lilly Laboratories. The identity of the virus was verified by R. L. Heberling (*personal communication*)

and, in this laboratory, with sera obtained from N. P. Rapoza. The virus stocks used in these experiments were derived from inocula which had been doubly plaque-purified in LLC-MK2 cells.

The titer of one such stock, used in most of the experiments presented herein, was 10^8 PFU/ml when titrated in BSC-1 cells.

Cell cultures and transformation assays. LLC-MK2 cells used for the plaque purification and preparation of stocks of SV11 were cultured in Eagle's minimal essential medium (MEM) with 5 or 2% heat-inactivated (56 C for 30 min) fetal calf serum (FCS).

BSC-1 cells, used for plaque assay of the virus stocks, were cultivated in Eagle's basal medium enriched with four times the usual concentration of amino acids and vitamins and supplemented with 10% heat-inactivated FCS. Methods for the preparation of cells for plaque assay and details of the virus assay procedure were previously reported (1).

Hamster embryo cultures (HEC) were prepared from decapitated and eviscerated embryos taken after approximately 14 days of gestation. Details of the cell culture methods and methods used for transformation assays have been presented (1).

RESULTS

Tumor induction in hamsters by SV11. Two litters of hamsters, 1- and 3-days-old, were injected subcutaneously on two occasions with 5×10^6 PFU of SV11. Animals were observed weekly for tumors for as long as 4 months after virus inoculation. Seven of 10 hamsters, inoculated within 1 day after birth, developed tumors within 91 days. Tumors were demonstrable as early as 50 days postinoculation in three animals. In the second litter, inoculated 3 days after birth, 3 of 12 hamsters were positive for tumors by 82 days. All animals were sacrificed at 122 days, by which time two additional animals in the latter group had become positive. Of the 12 animals developing tumors, 6 developed tumors in the axillary area in the absence of tumor formation at the site of inoculation, whereas the remaining developed tumors either at the site of inoculation or at both sites. One animal had developed multiple tumors predominantly in the dorsal cervical region. Although most tumors were confined to the subcutaneous space, one tumor had invaded through the dorsal musculature and into the thoracic cavity. Microscopic observation of sectioned tumors showed them to more closely resemble poorly differentiated lymphosarcomas (Fig. 1). Selected tumors were minced, washed twice, resuspended to 30% (v/v), and passaged into eight 10-day-old animals by injecting 0.25 ml of the mince subcutaneously. Tumors from this passage were harvested after 19 days and subsequently passaged intradermally into sixteen 4-week-old hamsters, from which sera were obtained to be used for detecting tumor antigen in cells transformed in vitro.

Appearance of SV11-transformed cell foci. Small areas of SV11-transformed cells were observed within 7 days after inoculation of virus. Unlike the foci induced by SA7 or Ad12 (1), foci resulting from SV11 inoculation consisted primarily of small, rounded, loosely attached cells (Fig. 2). Only rarely were tightly adhering colonies, similar to SA7 or Ad12, observed. Upon subsequent transfer in vitro, the SV11-transformed cells rarely established discrete colonies, but remained separate from each other or formed small clusters or chains of cells. Microscopic observation of stained cells revealed many in which the nuclei were lobulated and possibly binucleate. Some multinucleated cells were observed in which the nuclei were arranged in a horseshoe-shaped or, in many instances, a doughnut-shaped manner around the periphery of the cell with only a small area of cytoplasm visible in the center (Fig. 2). After four passages in vitro, 2×10^6 SV11-transformed cells were inoculated subcutaneously into 1-week-old hamsters. By 3 weeks, 8 of 8 animals had developed tumors, which ranged in weight from 1.4 to 15.5 g. These tumors also resembled lymphosarcomas (Fig. 1), and cells from these tumors, re-established in culture, again grew as rounded, separate cells with polymorphic nuclei.

Correlation between SV11 dose and numbers of transformed cell foci. Four 60-mm dishes of HEC were inoculated with 0.2 ml of one of a series of twofold dilutions of SV11. Inoculated cells were transferred into new plates as described previously (1), and were overlaid after 5 to 10 days with 0.5% agar in Eagle's medium with 0.1 mM $CaCl_2$ (3) and 10% FCS. Final focus counts were made 3 weeks after virus inoculation.

The numbers of transformed cell foci were linear when there was less than 2.8 PFU per cell (Fig. 3). At this latter multiplicity (M), the number of expected foci in two of three experiments decreased by 40 to 60%. If the virus inoculum was doubled ($M = 5.6$), most of the inoculated cells failed to attach and no foci of transformed cells appeared.

Relationship between PFU and FFU. Results from six separate transformation experiments with SV11 are shown in Table 1. The data include several experiments in which serial twofold dilutions of SV11 were inoculated onto hamster embryo plates. Within any one of these experiments, there was good agreement between virus dilution and number of foci when the PFU to FFU ratios were calculated. However, in all experiments, there was approximately a four-fold difference between the highest and lowest PFU to FFU ratios calculated from individual experiments. Such variation between results was observed previously (1), and presumably most

of the variation is due to the inherent differences in susceptibility between different preparations of hamster embryo cultures. However, with the data from all experiments, the average PFU to FFU ratio was 3.8×10^4, as compared to 1.8×10^6 and 2.5×10^5 reported earlier for Ad12 and SA7, respectively.

Detection of tumor antigen in SV11-transformed cells. Sera from tumor-bearing hamsters (transformed cell-induced and tertiary passaged virus-induced tumors) were titrated by the complement-fixation test (13) against antigens prepared from cultures of SV11-transformed hamster embryo, primary hamster embryo, and

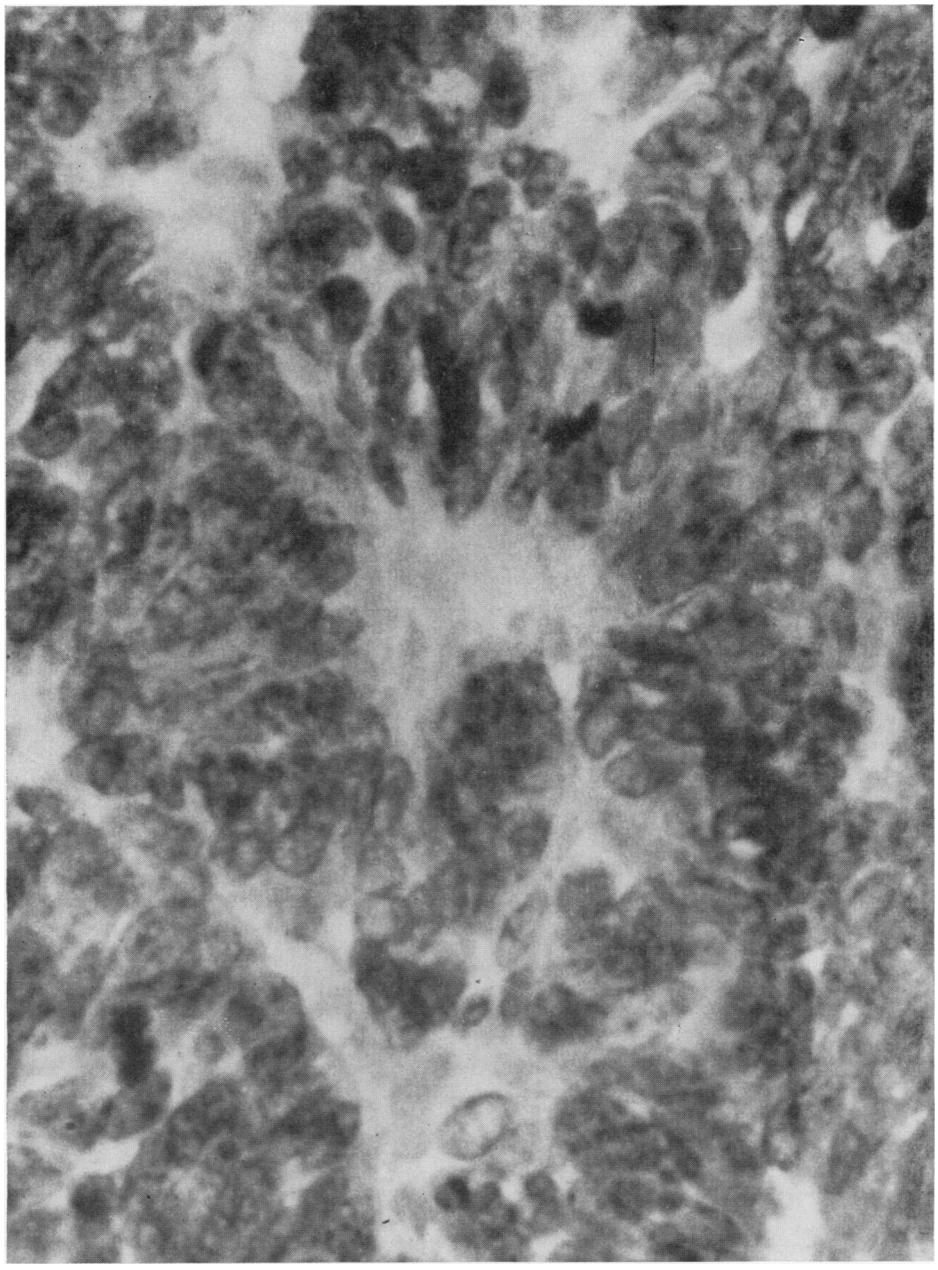

FIG. 1. *Section of a tumor produced in hamsters by the injection of SV11-transformed cells. Tumors resulting from inoculation of virus were similar in appearance. Hematoxylin and eosin stain.* × *2,400.*

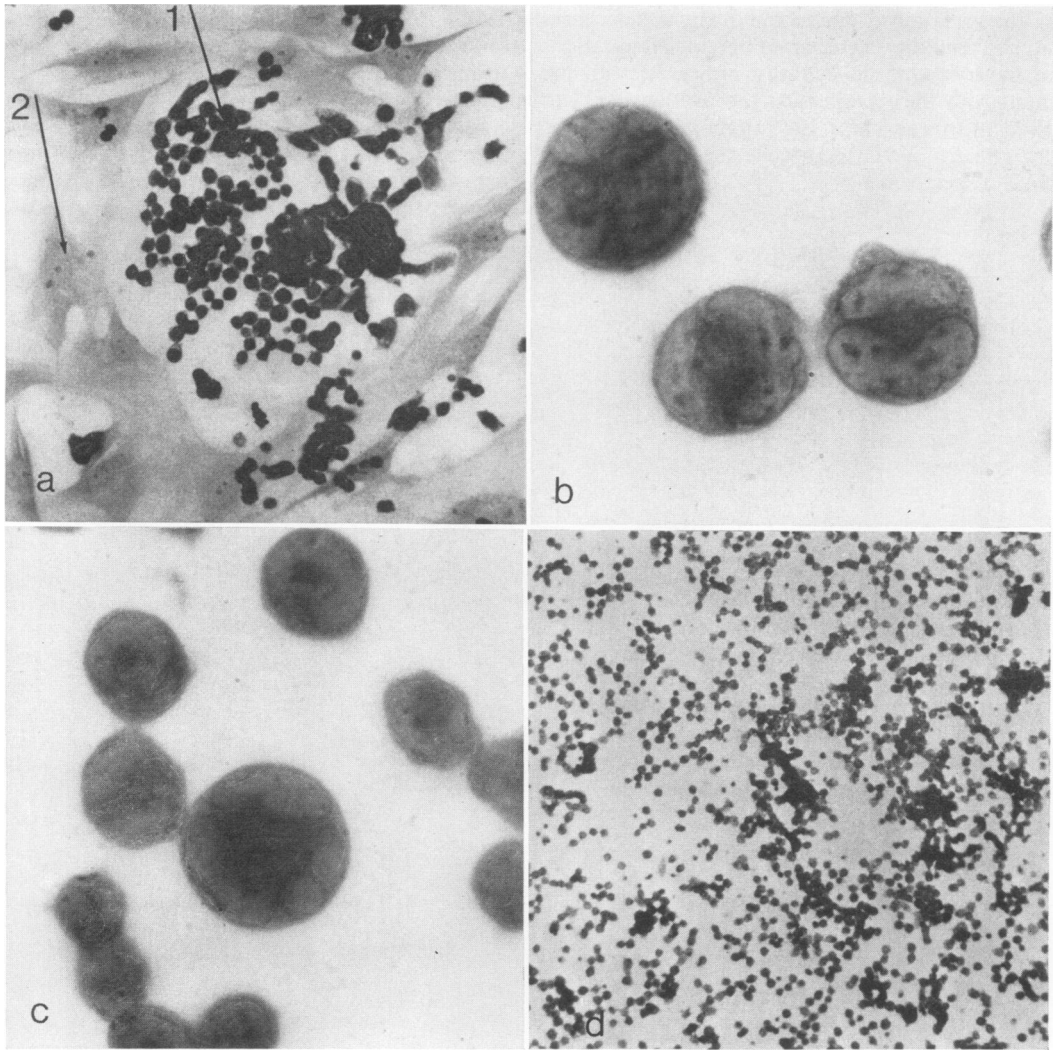

FIG. 2. *Appearance of SV11-transformed hamster embryo cells in culture. May-Grunwald stain. (a) Transformed cells (arrow 1) as contrasted to normal hamster cells (arrow 2). × 400. (b) Apparent binucleated cells. × 4,000. (c) Multinucleated giant cell. × 4,000. (d) Typical growth pattern with cells in chains or small clusters. × 160.*

FSa3B cells. Antigens were prepared by harvesting cells from twenty 100-mm plates. The cells were washed two times in Veronal buffer and were resuspended to 20% (v/v) in buffer. The cell suspension was frozen-thawed four times, and either the crude suspension or a clarified extract thereof was used in the test. Table 2 shows the homologous reactivity between SV11 tumor antigens and several SV11 antisera and the lack of reactivity against antigens prepared from primary hamster embryo and FSa3B cell cultures (10).

Representative antisera from each hamster group, bearing either cell-induced tumors or passaged virus-induced tumors, were employed in testing several clones of SV11-transformed cells for tumor antigen. In addition, antigens from SA7-and SV20-transformed cells were used to test the specificity of these sera, since Gilden et al. (5) had shown lack of cross-reactivity between the SV11 (subgroup I), the SV20 (subgroup II), and the SA7 (subgroup III) antigens. Table 3 shows the complement-fixing titers of five SV11 tumor antigens. Homologous reactivity of these antigens ranged from 1:16 to 1:64 with

antisera from hamsters bearing cell-induced tumors and from 1:32 to 1:128 with antisera from hamsters with passaged virus-induced tumors. A low level of reactivity was observed with SA7 (1:4) and SV20 (<1:4 to 1:8) antigens (with homologous antigen titers of 1:32).

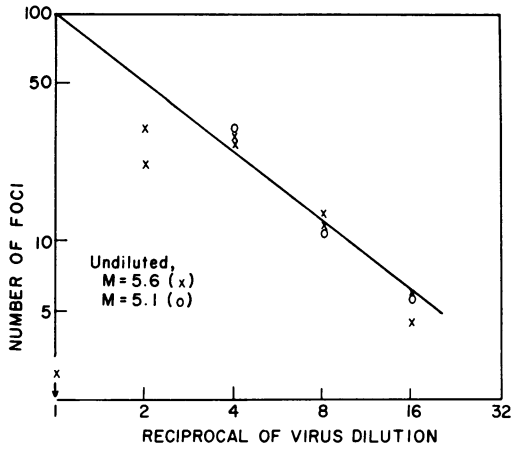

FIG. 3. *Linear relationship between number of transformed cell foci and concentration of SV11 (normalized values). Each point represents a separate experiment and was determined from the average number of foci arising from four inoculated plates. Total number of foci from four plates at the 1:4 dilution of SV11 ranged from 250 to 400. M = multiplicity of infection (in PFU) with undiluted virus.*

TABLE 1. *Relationship between PFU and FFU in the SV11-hamster embryo transformation system*

Expt no.	Virus dose (PFU)[a]	Virus multiplicity	No. of transformed foci (FFU)[b]	PFU/FFU
1	2.0×10^7	3.50	Toxic	
	5.0×10^6	0.90	276	1.8×10^4
	1.3×10^6	0.25	70	1.7×10^4
2	8.3×10^5	0.25	67	1.2×10^4
	4.1×10^5	0.13	23	1.7×10^4
3	5.0×10^6	1.33	92	5.4×10^4
	2.5×10^6	0.66	32	7.8×10^4
	1.3×10^6	0.33	17	7.6×10^4
4	2.3×10^6	0.71	23	10^5
5	10^7	2.80	372	2.7×10^4
	5.0×10^6	1.40	334	1.5×10^4
	2.5×10^6	0.70	150	1.7×10^4
	1.3×10^6	0.35	51	2.5×10^4
6	5.0×10^6	1.00	148	3.4×10^4
Avg				3.8×10^4

[a] Each of three or four plates was inoculated with 0.2 ml of virus suspension.

[b] Total number of foci arising from a single inoculated plate. Each figure represents the mean of three or four plates.

TABLE 2. *Complement-fixing antibody, to SV11 tumor antigens, in sera from hamsters bearing SV11-transformed cell-induced tumors or tertiary passaged SV11 virus-induced tumors*

Antigens	Cell-induced tumors						Virus-induced passaged tumors					
	158[a]	159	161	163	164	165	177	179	180	181	175	190
SV11-transformed hamster embryo cell culture	8[b]	ND[c]	32	16	>32	>32	32 64	16 32	16 16	ND	32	128
Normal hamster embryo cell culture[d]	ND	ND	<4	<4	<4	ND	<4	<4	<4	<4	ND	
FSa3B[d]	ND	ND	<4	<4	<4	ND	<4	<4	<4	<4	ND	
SV11-induced hamster tumor (50% homogenate)	16	64	16	8	ND	ND	32	16	4	4	ND	ND

[a] Each number represents an individual animal.

[b] Reciprocal of the antibody titer with 2 units of complement and 4 units of SV11 tumor antigens as determined in the homologous reaction.

[c] Not done.

[d] Hamster embryo cell (20% suspension) and FSa3B (20% suspension) antigens were diluted 1:8.

DISCUSSION

Inoculation of SV11 onto primary hamster embryo cultures resulted in a morphological alteration of these cells which is unlike that observed with any of the other adenoviruses thus far reported. Small foci of transformed cells were observed as early as 7 days after inoculation of SV11 and transfer of primary hamster embryo cells. Foci consisted of a loose aggregation of rounded cells which exhibited little affinity either for the substrate of normal cells or for each other. Discrete foci could be maintained only by covering the cell sheet with a soft agar overlay, 7 to 10 days after inoculation and transfer. Six of six clones, isolated by picking colonies from under the agar, grew as isolated, rounded cells or in small clusters or chains of cells. When stained, a large proportion of the cells appeared to be binucleate or contained a highly lobulated nucleus. Multinucleated cells containing six to eight nuclei were frequently observed in which the nuclei were arranged around the periphery of the cells.

TABLE 3. *Titration of complement-fixing antigens from clones of SV11-transformed hamster embryo cells*

Antigens[a]	Antisera	
	Cell-induced tumor	Passaged virus-induced tumor
SV11-HE-P7	16[b]	32
SV11-923-2	32	64
SV11-923-6	64	128
SV11-923-7	64	128
SV11-923-8	64	128
SA7-O-1	4	4
SV20-924-6	<4	8

[a] Antigens were prepared from the fourth or fifth passage of transformed cells derived from an isolated cell clone, with the exception of SV11-HE-P7, which was prepared from the seventh passage of transformed cells.

[b] Reciprocal of the complement-fixing antigen titer in the presence of 4 units of antibody and 2 units of complement.

Freeman et al. (3) did not observe differences either in cell or colonial morphology in rat embryo cells transformed by human adenovirus types 2, 3, or 12. However, there are easily recognizable morphological differences in hamster embryo cells transformed by simian adenoviruses SA7, SV11, or SV20 or human adenovirus type 12 (Fig. 4). Examination of a large number of transformed cell clones derived from hamster embryo cultures inoculated with the latter four viruses showed that each virus gives rise to a characteristic transformed cell type.

Inoculation of 2×10^6 SV11-transformed

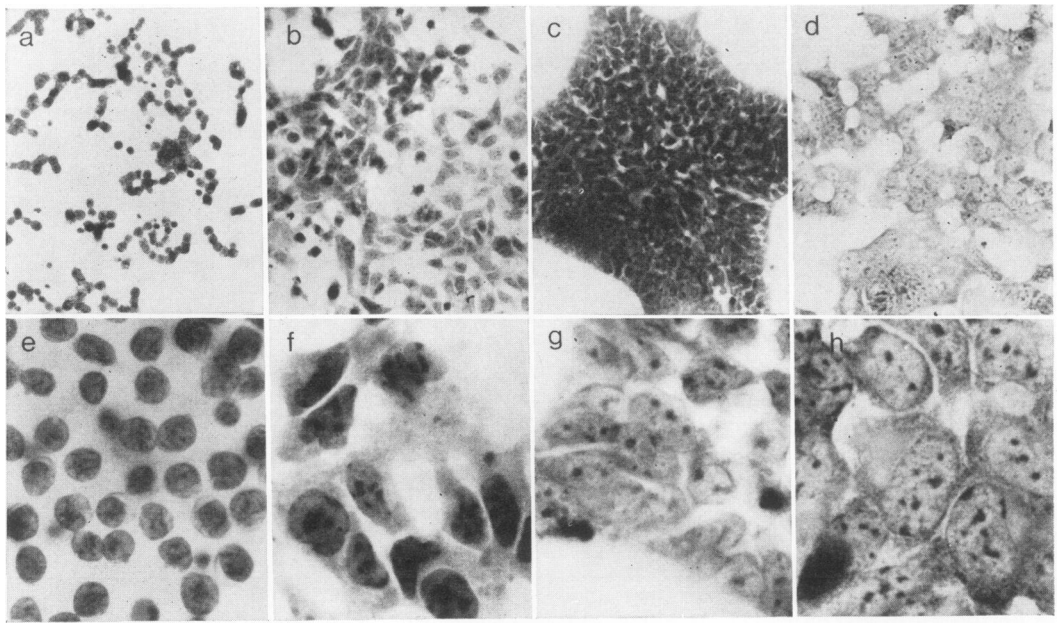

FIG. 4. *Morphology of SV11-transformed hamster cells in culture (a, e), as contrasted to cells transformed by SA7 (b, f), Ad12 (c, g), and SV20 (d, h). Top: × 260. Bottom: × 1,600. May-Grunwald stain.*

cells into suckling hamsters resulted in tumor formation in 100% of the animals within 3 weeks, and sera from all animals tested developed complement-fixing antibodies to antigens derived from SV11-transformed cells or from virus-induced passaged tumors. The same sera failed to react with antigens prepared from primary hamster embryo cultures or from cultures of FSa3B cells. The latter cells have been shown to possess an antigen which reacts in the complement-fixation test with sera from hamsters bearing SV40, polyoma, or nonviral-induced tumors (10).

Sera from 6 of 10 hamsters with third-passaged, virus-induced tumors contained complement-fixing antibody to antigens from SV11-transformed cells, with titers ranging from 1:4 to 1:128. A low level of cross-reactivity (1:4) was observed between high-titered SV11 tumor antisera and antigens from SA7- or SV20-transformed cells. SA7 tumor antisera with homologous titers of 1:64 to 1:128 also demonstrated this low level of reactivity in the complement-fixation test with 4 or 8 units of SV11 tumor antigen.

Serial twofold dilutions of SV11, when inoculated onto HEC, resulted in a proportionate decrease in the number of transformed cell foci. Data combined from several experiments showed that the PFU to FFU ratio in the SV11-HEC system is approximately 3.8×10^4. However, in the majority of the experiments, the ratio was found to be about 2×10^4. In either case, the values found for SV11 are still 50- to 100-fold and 5- to 10-fold lower than those found for Ad12 and SA7, respectively (1, 8).

The amount of SV11 which can be added to HEC before a demonstrable decrease in the expected number of foci occurs is also less than that which can be used with Ad12 or SA7. When 5 PFU/cell of SV11 was inoculated onto HEC, no foci of transformed cells appeared, and linearity with dilution occurred more regularly when 2 or less PFU/cell was used. Earlier, with Ad12 (1), fewer than the expected number of foci were found when more than 100 PFU per cell was used for inoculation. Other dose-response studies with SA7 (*unpublished data*) showed that the number of expected foci deviated from linearity when the input multiplicity was 40 to 80 PFU/cell, but was linear when approximately 20 or less PFU was used.

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