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Transformation of Hamster Kidney Cells by Simian Papovavirus SA12[1] (40421)

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Simian Agent 12 (SA12 virus) was originally isolated from an uninoculated kidney culture of the vervet monkey *Cercopithecus pygerythrus* (1). It was recently identified as a new immunologically distinct member of the SV40-polyoma subgroup of papovaviruses and it was suggested that the South African chacma baboon (*Papio ursinus*) may be its principal natural host (2). We report here that SA12 virus readily transformed hamster kidney cells in culture and that the inoculation of transformed cells into syngeneic hosts produced both undifferentiated sarcomas and well-differentiated adenocarcinomas.

Materials and methods. Virus and cell cultures. Two preparations of this virus were used (2): (i) SA12 (RMK) which had been passaged three times in primary and early passage kidney cultures derived from juvenile SV40-antibody free rhesus monkeys; and (ii) SA12 (MA104) derived by plaque purification and propagation of SA12 (RMK) on continuous rhesus embryonic kidney cell line MA-104. Both preparations had infectivity titers of $10^{7.0}$ $TCID_{50}$ per ml in their respective cell types. Hamster kidney (HK) cells were prepared by trypsinization of a pool of normal kidneys from 10 day old outbred Syrian hamsters (Charles River, Lakeview, Wilmington, MA). They were grown on Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS) for outgrowth and with 5% FBS for maintenance.

Production of transformed cells. First or second passage subconfluent HK cells were inoculated with approximately 10 infectious units/cell of SA12 (RMK) virus. Virus was adsorbed onto cells for one hour. The inoculated cultures were passaged 10 days after virus inoculation and subsequently at 7-10-day intervals. Frequency of transformation was estimated by the method described by Todaro and Green (3). Flasks with subconfluent monolayers of HK cells were exposed to SA12 (MA104) virus for 3 hr at an estimated multiplicity of infection of 10 infectious units per cell. Twenty-four hours later, the monolayers were trypsinized and planted in duplicate 60 mm petri dishes at concentrations of 500, 1000 and 10,000 cells per petri dish. Twelve days later, the cells were fixed for 10 min in 100% methanol, stained for 10 min with 10% Giemsa. Transformed colonies were recognized by their morphology and were counted under low power of an inverted light microscope.

Characterization of transformed cells. A cell line of transformed HK cells obtained after inoculation of SA12 (RMK) virus was designated SAHK-1 line. These and control HK cells were grown on coverslips, fixed in Bouin's and stained with hematoxylin and eosin for cytological examination. Cells at alternate passage levels were also examined for SA12 viral and T antigens by indirect immunofluorescence (IF) tests (4). The SA12 anti-viral antibodies were obtained from immunized rabbits (4) and anti-T antibodies from hamsters bearing SA12 tumors (2). SAHK-1 cells at passage 22 and HK cells at passage 1 were tested for their dependence on serum growth factors. Replicate 25 cm^2 falcon flasks seeded with 10^5 cells/ml were grown with FBS concentrations of 5%, 2% and 0.5% and cell yields at different time intervals were determined. The ability of the cells to form colonies in soft agar was tested by the method of MacPherson and Montagnier (5) as modified by Kakunaga and Kamahara (6). SAHK-1 cells, passage 25, and normal HK cells, passage 1, were planted at concentrations of 10^2–10^6 in duplicate 60 mm plastic petri dishes and colonies were counted on day 20. Clonal cultures were initiated from seven soft agar colonies which were removed individually using finely drawn capillary pasteur pipettes.

Attempts to demonstrate infectious virus in

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transformed cells. Culture supernatants and extracts of $10^{6.0}$ or more frozen and thawed SAHK-1 cells were periodically examined for the presence of SA12 viral hemagglutinin by tests with human 0 cells (7) and for infectious SA12 virus by inoculation into permissive MA104 cells. Attempts were also made to rescue SA12 virus by fusion of 5×10^6 SAHK-1 cells, passage 22 with an equal number of MA104 cells using betapropiolactone (BPL)-inactivated Sendai virus (8). The treated cells were diluted to a concentration of 500,000 cells per ml and planted in falcon flasks and in tissue culture plates with coverslips. These cultures were passed twice, on days 10 and 22 postfusion, and were monitored for infectious virus by immunofluorescence tests, hemagglutination tests and by cytological examination for viral cytopathic effects.

Tumorigenicity of transformed cells. Four- to five-week-old female outbred Golden Syrian hamsters (Charles River, Lakeview, MA) were inoculated subcutaneously with SAHK-1 cells at different passage levels and the inoculated animals were observed for at least 8 weeks for tumor occurence at the site of inoculation. Selected tumors were surgically removed and used for initiation of tissue culture, passage back into hamsters and for histopathological examination. For morphological studies tumor tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. The sera of tumor-bearing hamsters were tested for the presence of SA12 T antibodies by indirect IF tests with late passage SAHK-1 cells.

Results. Transformation of HK cells with SA12 virus. Foci of cells of altered morphology appeared approximately 10 days after inoculation of HK cells with SA12 (RMK) virus. Three morphologically different cell types were seen: large, bizarre shaped, multinucleated cells and colonies of small, densely packed cells which were either epithelioid or fibroblast-like. The epithelioid cells appeared to grow faster and on passage they became the predominant cell type. The cells tended to pile up even before the cultures reached confluency. The cultures contained a large number of cells in mitosis. The cells in control HK cultures were heterogenous with a predominance of large fibroblast-like cells. These cells had one or two uniformly dense nuclei, prominent nucleoli and abundant cytoplasm. In contrast, the predominant cells in the SA12-transformed culture (designated SAHK-1) were cuboidal to slightly spindle shaped, epithelioid, and smaller than normal cells. They had greatly reduced cytoplasm, granular nuclei and non-prominent nucleoli. The SAHK-1 cells have continued to grow vigorously for over 35 passages whereas normal HK cells could not be maintained beyond five passages. As determined by cell yields at the time of passages, the SAHK-1 cells reached saturation densities which were 8–12 times that of normal HK cells. HK cells were exposed to SA12 (RMK) virus in two additional experiments. In each instance, foci of transformed cells appeared 10–12 days after inoculation of virus but no attempt was made to establish permanent cell lines from these cells.

In the first and second passages of SAHK-1 cells, the intranuclear SA12 T antigen was detected by IF tests in small clusters of cells having many bizarre nuclear shapes. In cultures of the third and all subsequent passages, 95–100% of the cells were T antigen-positive.

The frequency of transformation was determined using SA12 (MA104) virus on subconfluent HK cells. Transformed colonies were recognized as densely packed groups of small, polygonal, granular cells with scanty cytoplasm. As shown in Table I, the fraction of cells planted which gave rise to transformed colonies was estimated to be 0.25% for cell concentration of 10,000 cells.

SAHK-1 cells were much less dependent on serum growth factors than normal HK cells. At the serum concentration of 5%, SAHK-1 cells multiplied rapidly to achieve a 50-fold increase in number in 10 days as compared to a two fold increase in number of normal HK cells. There was a nine-fold and 12-fold increase, respectively, in the

TABLE I. FREQUENCY OF TRANSFORMATION OF HAMSTER KIDNEY CELLS BY SA12 VIRUS.

Number of cells/plate	Number of transformed colonies		Transformation frequency
	Plate 1	Plate 2	
500	1	0	.10%
1000	1	2	.15%
10,000	27	23	.25%

number of SAHK-1 cells at FBS concentration of 0.5% and 2%, whereas normal HK cells could not even be maintained at these serum concentrations. SAHK-1 cells also had the ability to form colonies in soft agar. At the cell concentration of $10^{5.0}$, approximately 0.05% of SAHK-1 cells formed colonies in soft agar; in contrast, normal cells did not develop colonies at $10^{5.0}$ and $10^{6.0}$ cell concentrations. Colonies were first visible on day 10 by light microscope scanning as three-dimensional clusters of cells and they were later visible macroscopically as small white foci suspended in soft agar. Seven colonies were successfully grown out. Cells from each of these seven clones exhibited the intranuclear SA12 T antigen in IF tests.

Attempts to demonstrate infectious virus in SAHK-1 cells. In IF tests, viral structural antigens were not detected at any time in SAHK-1 cells. Extracts from several passages of SAHK-1 cells failed to produce viral CPE when inoculated into MA104 cells. Attempts to rescue infectious SA12 virus by Sendai virus-induced fusion of transformed cells with permissive cells were also unsuccessful. Examination of stained cells on day 4 after fusion showed that 30–50% of the cells were mutlinucleated and contained 2–20 nuclei per cell and that a large proportion of the multinucleated cells contained, as judged by morphology, nuclei from both transformed and permissive cells. Neither virus nor viral structural antigen was demonstrated in the fused cultures by any of the following: (a) observation of fused cultures for viral CPE for 52 days; (b) tests of supernates or frozen and thawed cell extracts, obtained on days 15 and 52 postfusion, for SA12 hemagglutinin; (c) IF tests for viral structural antigen on day 12 postfusion; and (d) virus isolation attempts by inoculation of cell extracts, prepared on day 22 postfusion, into MA104 cells.

Tumorigenicity of transformed cells. Inoculation of early and late passage SAHK-1 cells as well as of clones derived from soft agar colonies of transformed cells consistently produced tumors in weanling hamsters and the tumors were readily maintained by passage in hamsters (Table II). In the first attempt to produce tumors, SAHK-1, passage 8 cells were titrated in hamsters. Tumors occurred after inoculation of 2.6×10^5 or greater number of cells. In the first two hamster passages, 10 of 17 hamsters inoculated with $10^{6.0}$ or more cells developed tumors after a latent period of 45–92 days. In the third and fourth tumor passage, 11 of 11 and 6 of 6 inoculated hamsters developed tumors after a latent period of 17–36 days. The cells appeared to become more tumorigenic after successive hamster passage.

Several histological types of tumors were seen: pure adenocarcinomas, undifferentiated sarcomas, and mixed tumors containing both carcinomatous and sarcomatous elements (Fig. 1). The adenocarcinomas were distinguished by the presence of cuboidal to columnar epithelial cells arranged in tubular or glandular formations. They were either cystic or papillary and the stroma of most was infiltrated by lymphocytes. The sarcomas were composed of elongate spindle shaped and polyhedral cells arranged in poorly defined bundles or whorls. In both tumor types, there were high nuclear-cytoplasmic ratio, abundant mitotic activity, and numerous foci of necrosis. Three of five tumors which were

TABLE II. TUMORIGENICITY OF SA12-TRANSFORMED CELLS FOR JUVENILE HAMSTERS

Inoculum			Tumor		
SAHK-1 passage	Hamster passage	Number of cells	Proportion with tumor	Time of appearance (days)	Histological diagnosis[a]
8	1	2.6×10^5	1/5	45	Mixed (1)
		2.6×10^6	2/4	45	Carcinoma (1)
	1	4.5×10^6	2/5	62–92	Carcinoma (1)
	2	2.5×10^6	6/8	9–46	Undiagnosed
	3	1.7×10^6	11/11	17–36	Carcinoma (1)
	4	Not known	6/6	17–28	Carcinoma (6)
25, clone 1	1	2.5×10^6	4/4	13–20	Sarcoma (4)
25, clone 2	1	2.5×10^6	3/4	27–50	Sarcoma (3)

[a] Carcinoma - cystic or papillary adenocarcinoma; sarcoma = undifferentiated sarcoma. Number of tumors examined is indicated in parentheses.

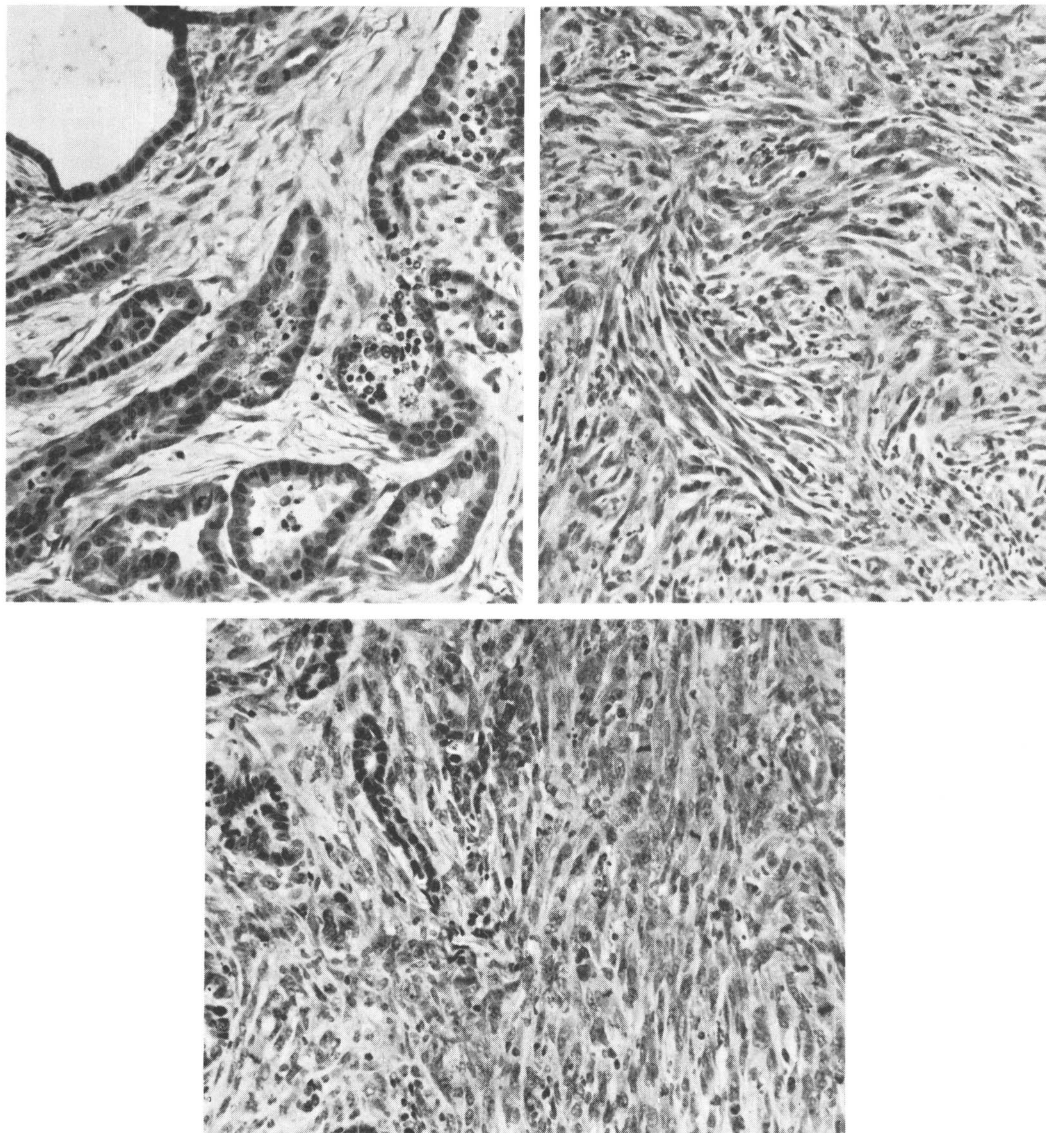

FIG. 1. Hamster tumors induced by SA12-transformed cells. *Upper left:* Section of adenocarcinoma showing glandular orientation of tumor cells. Foci of necrosis are also present. ×250. Hematoxylin and eosin (H&E). *Upper right:* Section of sarcomatous tumor showing whorls of spindle-shaped cells. ×250. (H&E). *Bottom:* Carcinosarcoma containing both neoplastic connective tissue and epithelial elements. Both cell types have histologic features of neoplasia including high mitotic activity. ×250. (H&E).

produced by the inoculation of SAHK-1, passage 8, were examined histologically. Two were pure adenocarcinomas and the third was a compound lesion composed of an undifferentiated sarcoma with an adjoining well differentiated cystic adenocarcinoma. The tumor used for passage to the other hamsters was a pure adenocarcinoma. Of the 23 tumors which were produced in subsequent hamster passages, 7 were examined histologically. All were adenocarcinomas. In contrast, the seven tumors produced by inoculation of two soft agar clones of SAHK-1, passage 25, were all undifferentiated sarcomas. The tumors of all histological types were fast growing and noninvasive.

In tissue culture, cells from the tumors tended to pile up even when the cultures were not confluent and they reached high saturation densities. Cytologically, the cells derived

from undifferentiated sarcomas could not be distinguished with certainty from those derived from adenocarcinomas. SA12 T antigen was detected in cells of each of 11 tumor-derived cultures tested, six from adenocarcinomas, four from undifferentiated sarcomas, and 1 from an undiagnosed tumor. SA12 T antibodies were detected in each of 16 sera from individual tumor-bearing animals (eight bearing adenocarcinomas, six bearing sarcomas and two bearing mixed tumors) as well as in two sera representing pooled bleedings of 17 hamsters.

Discussion. Simian papovavirus SA12 resembled SV40 in the ease with which it transformed hamster kidney cells. The transformed cells had altered morphology, loss of contact inhibition, an indefinite life span, increased saturation density, reduced serum requirement for growth and an ability to form colonies in soft agar. These cells also have abnormal numbers of chromosomes with numerous structural anomalies (Bempong, M. A.: Personal communication). The virus-specific intranuclear T antigen was detected in transformed cells maintained by tissue culture passage as well as in cells derived from soft agar colonies and from hamster tumors. Virus was not rescued from transformed cells.

Inoculation of transformed cells into hamsters produced undifferentiated sarcomas, well-differentiated adenocarcinomas and tumors with both carcinomatous and sarcomatous elements. Tumors of all histologic types were virus-specific. T antigen was present in cells derived from all types and T antibodies were demonstrable in sera of animals bearing all histologic types of tumor. The primary cultures of hamster kidney cells are heterogeneous and contain epithelial cells of tubular origin as well as mesenchymal cells. It is possible that SA12 transformed both types of cells which continued to grow simultaneously in the culture and that adenocarcinomas resulted from transformed epithelial cells, undifferentiated sarcomas from transformed mesenchymal cells, and mixed tumors by adjacent growth of both types of cells. The following observations would appear to support this explanation. Both epithelioid and fibroblastic foci of transformed cells were seen in the original transformed culture. Inoculation of this culture produced mixed tumors and pure adenocarcinomas. Tumors raised in successive hamster passages of an adenocarcinoma were all adenocarcinomas; however, tumors derived by inoculation of soft agar clones were all undifferentiated sarcomas. The absence of mixed tumors in these two groups suggests that the epithelial and mesenchymal elements in the culture may have "separated out", respectively, in the course of hamster passage and soft agar cloning. On the other hand, clones derived from single cells of an SV40 transformed epithelial hamster line have produced mixed tumors, suggesting that undifferentiated embryonic metanephric cells, when transformed by a virus, may retain their capacity to differentiate into either mesenchymal or epithelial elements (9).

Summary. SA12, a newly recognized SV40-related primate papovavirus, transformed early passage hamster kidney cells. These cells, designated SAHK-1, were characterized by increased saturation density, altered morphology, loss of contact inhibition, increased growth rate, an indefinite life span, reduced serum requirements for growth, an ability to grow in soft agar, and the presence of SA12-specific T-antigen. Infectious virus was not rescued by Sendai virus-induced fusion of SAHK-1 cells with permissive cells. Inoculation of transformed hamster cells into syngeneic hosts produced adenocarcinomas, undifferentiated sarcomas, and mixed tumors containing both elements. SA12 T antibodies were demonstrated in all the tested sera from tumor bearing animals.

1. Malherbe, H., Harwin, R., and Ulrich, M., S. Afr. Med. J. **37**, 407 (1963).
2. Valis, J. D., Newell, N., Reissig, M., Malherbe, H., Kaschula, V. R., and Shah, K. V., Infect. Immun. **18**, 247 (1977).
3. Todaro, G. J., and Greene, H., Virology **23**, 117 (1964).
4. Shah, K. V., Ozer, H. L., Ghazey, H. N., and Kelly, T. J., Jr., J. Virol. **21**, 179 (1977).
5. MacPherson, I., and Montagnier, L., Virology **23**, 291 (1964).
6. Kakunaga, T., and Kamahara, J., Biken J. **11**, 313 (1968).
7. Shah, K. V., Valis, J. D., and Daniel, R. W., J. Clin. Microbiol. **7**, 396 (1978).
8. Neff, J. M., and Enders, J. F., Proc. Soc. Exp. Biol. Med. **27**, 260 (1968).
9. Black, P. H., and Rowe, W. P., Proc. Nat. Acad. Sci. U. S. A. **50**, 606 (1963).

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