

TRANSFORMATION OF DOG KIDNEY CELLS INFECTED WITH SV 40 AND SV 40-ADENO 7 HYBRID VIRUS

V. VONKA, L. KUTINOVÁ, H. ZÁVADOVÁ

Research Institute of Immunology, Virus Department, Prague, Czechoslovakia

Received July 4, 1968

Summary. — Dog kidney cells were infected with either SV 40 or SV 40-Adeno 7 hybrid virus. The cultivation of the infected cells resulted in cell transformation; this occurred earlier in cells infected by the hybrid virus than in SV 40-infected cells. The cells transformed by either virus were morphologically similar; the cultures consisted of small tightly packed epitheloid cells which formed multilayered mold-like colonies after prolonged cultivation. Cells transformed by the hybrid virus contained the T antigen of SV 40; this antigen was not detected in cells transformed after infection with SV 40.

Introduction

The papovavirus SV 40 has been shown to be capable of transforming *in vitro* cells of hamster (Ashkenazi and Melnick, 1963; Rabson and Kirchstein, 1962; Black and Rowe, 1963*a*), simian (Fernandez and Moorhead, 1965), human (Shein *et al.*, 1962; Ashkenazi and Melnick, 1963; Koprowski *et al.*, 1962, etc.), bovine (Diderholm *et al.*, 1965), porcine, murine, rabbit (Black and Rowe, 1963*b*), guinea pig and rat (Diderholm *et al.*, 1966) origin. In the respective experiments changes in cell morphology, growth rate, metabolism and karyotype were employed as indices of transformation. It has also been shown that the transformed cells possess new cellular antigens. One of them, called the tumour or T antigen, is localized in the nucleus and has been detected by the complement-fixation (CF) reaction (Black *et al.*, 1963; Koch and Sabin, 1963) or by immunofluorescence (Pope and Rowe, 1963; Rapp *et al.*, 1964). Another antigen, termed surface or S antigen, has been revealed by the transplantation rejection test (Defendi, 1963; Habel and Eddy, 1963; Khera *et al.*, 1963; Koch and Sabin, 1963), immunofluorescence (Tevethia *et al.*, 1965) and cytotoxic test (Tevethia and Rapp, 1965); it is probable that all these methods identify the same substance. Transformed hamster cells were capable of inducing tumours after transplantation to homologous hosts (Khera *et al.*, 1963; Habel and Eddy, 1963; Koch and Sabin, 1963).

Similarly, the SV 40-Adeno hybrid viruses the population of which consists of true adenovirions and defective particles, called PARA, carrying a portion of SV 40 genome encased in adeno capsid (for references see the recent review by Rapp and Melnick, 1966) were reported to transform *in vitro* cells of hamster and human origin. The transformed cells carried the SV 40 T anti-

gen (Huebner *et al.*, 1964) and SV 40 transplantation antigen (Rapp *et al.*, 1966). Strong evidence was obtained that the adenovirus genome was also active in the transformation process. This was revealed by the presence of cells remembering both SV 40 and adeno transformed cells in the tumours induced by the SV 40-Adeno hybrids (Rapp *et al.*, 1967; Igel and Black, 1967) and, moreover, by the presence of adenovirus antigen in the transformed cells (Black and White, 1967; Wells *et al.*, 1966; Vonka *et al.*, 1968).

As far as we are aware no report has been published on the transformation of cells of dog origin by either SV 40 or SV 40 adeno hybrid virus or any other DNA viruses. The present paper describes transformation of dog kidney cells infected with SV 40 virus and SV 40-Adeno 7 hybrid virus.

Materials and Methods

Viruses. The SV 40 and SV 40 — Adeno 7 hybrid virus, denoted SP-2, were obtained through the courtesy of Dr. F. Rapp. The prototype adeno 7 virus was kindly supplied by Dr. Brůčková. They were passaged as described previously (Vonka *et al.*, 1967). The SV 40 virus was titrated in green monkey kidney cells (GMKC) using five tube cultures per each virus dilution. The test was read after 3 weeks. The SP-2 virus was titrated for the content of adeno 7 virus in the human diploid cell strain LEP-14, and for the content of defective PARA in GMKC infected simultaneously with prototype adeno 7 (Gomen) virus at the approximate multiplicity of 1 LEP TCD₅₀ per cell (Kutinová and Vonka, 1968).

Cells. Horák's inbred dog (Horák, 1964) puppies aged three months were used. The dog kidneys were trypsinized and cultivated in 1200 ml Roux bottles as described by Mareš and Dřevo (1965). Confluent cultures formed after 6-day cultivation at 36° C.

Cell infection and cultivation of infected cells. Confluent dog kidney cell cultures grown in 1200 ml Roux bottles were infected with either SV 40 virus or SP-2 virus. The input multiplicity of infection with the SV 40 virus was about 6 TCD₅₀ per cell, with the SP-2 virus it was about 0.2 LEP TCD₅₀ of adeno 7 and about 2 TCD₅₀ of PARA per cell. The cells were incubated with the virus for 2 hours at 37° C. After rinsing out the unadsorbed virus, 100 ml of Earle-lactalbumin hydrolysate (0.3%) medium supplemented with 3% of heated calf serum was added. Four days later the infected and uninfected cultures were trypsinized and from each culture two bottles were seeded in medium 199 supplemented with 10% heated calf serum. This medium was used throughout.

Immune sera. The immune sera reacting with the T antigen of SV 40 in the immunofluorescence and CF tests were obtained from hamsters bearing large tumours induced by SV 40-transformed cells. The cell line employed, TU-B, originated from the H-50 cells derived by Ashkenazi and Melnick (1963) shown to be virus free (Melnick *et al.*, 1964). The serum pool used contained a high level of T antibody and did not react in the CF test with the viral (V) antigen of SV 40, or with antigens from untransformed hamster cells. The SV 40 immune sera were prepared in monkeys as described previously (Vonka *et al.*, 1967); a monkey serum not reacting with SV 40 T antigen was employed. Adeno 7 immune serum was likewise prepared in monkeys, using Gomen virus; this serum contained a high level of antibody reacting with homotypic adeno CF antigens.

CF reaction. The microdrop technique described previously (Závadová *et al.*, 1967; Vonka *et al.*, 1967) was employed. Cell antigens were prepared as follows. Ten per cent cell suspension in phosphate buffered saline (PBS; pH 7.3) was frozen and thawed, and clarified by low speed centrifugation. The supernate was used as antigen. The following reagents were used as controls or references: a) antigen from primary dog kidney cells; b) antigen from untransformed hamster cells; c) antigen from cells transformed by SV 40; d) serum pool from healthy untreated hamsters; e) serum pool from hamsters bearing tumours induced by SV 40-transformed cells.

Immunofluorescence. The indirect technique used has been described (Vonka *et al.*, 1967). The sera of hamsters bearing tumours induced by SV 40-transformed cells were used for demonstrating T antigen of SV 40. As controls, the following reagents and cells were employed: a) negative hamster serum; b) SV 40-transformed hamster cells; c) hamster embryo fibroblasts; and d) primary dog kidney cells.

Transplantation test. Cultures of transformed cells were trypsinized and resuspended in PBS (pH 7.3). Ten ml of cell suspension were injected subcutaneously into the interscapular region of four months old Horák's dogs, which had been daily injected for three days with 25 mg of hydrocortisone (Hydro-Adreson, N. V. Organon OSS) per kg body-weight. They were checked for the presence of tumour once a week.

Results

During the first days after infection no marked differences were observed on examination of the infected and control cells, with the exception of a lower pH in cultures infected with the SV 40 virus. From the passage No. 1 of each culture (see Materials and Methods), several subcultures were initiated; the cultures were passed shortly after confluent cells sheets had formed (lines A), or after keeping the cultures without passaging for periods of three weeks to more than two months (lines B).

The earliest marked indication of a change was observed in SP-2 infected cells; among the fibroepithelial cells of the dog kidney culture, foci of small epitheloid cells piling up on each other appeared. They were first observed in the third passage of subline A, on the 27th day after infection. Upon the transformed cell colonies multiple round cells appeared; such cells were only rarely observed upon the layer of the larger fibroepithelial cells. Islets of small epithelial cells of similar shape were also observed in the second passage of subline B of the SP-2 infected cells, on the 44th day after infection. In this subline, which was less frequently passed, the new cell type formed macroscopically recognizable colonies of mold-like character. In subsequent passages of both sublimes the small epithelial cells quickly became the dominant cell type and a marked increase in the growth rate occurred. Prolonged cultivation without passage resulted in the formation of large colonies and syncytial forms of bizarre shape.

A control culture, a focus of small epithelial cells and a culture of transformed cells are shown in Figs 1—3.

The transformation of SV 40 infected cells proceeded much slower. From the first passage several sublimes were derived which were subjected to further

Table 1. Immunofluorescence tests with DK SP-2 and DK-SV 40 cells

Cells	Line	Cells from passage No.	Day after infection	Immunofluorescence*
DK SP-2	A	2	11	1
		3**	39	80
		4**	60	100
DK SV 40	A	2	11	0
	A	3	39	0
	A	4	60	0
	B	5**	129	0
	B	10**	168	0

* The figures represent approximate percentage of cells containing T antigen.

** Transformed culture.

passages. The cells grew at a rate comparable to that of uninfected control cultures, and finally spontaneously degenerated or exhibited no evidence of transformation during a period exceeding 5 months. Only in one first passage bottle culture kept without passaging with occasional change of medium, two colonies recognizable by the naked eye were detected on days 67 and 80 after infection. Microscopic investigation revealed multilayered colonies of tightly packed small epithelial cells comparable to those observed in the SP-2 infected cells. The two cell colonies were scraped off the glass, resuspended in the growth medium and seeded into a new bottle. A cell line was established from this seed. In the original first passage culture, 67 macroscopically visible colonies developed within 14 days after scraping off the two colonies; it is probable that all or most of them were secondary colonies originating from the cells freed from the primary colonies during the scraping procedure. None of the uninfected control cultures was transformed.

The cells originating from the cultures infected with the SV 40 virus and SP-2 virus were examined for the presence of SV 40 T antigen in immunofluorescence tests. The results of several experiments in which cells at different passage levels were examined are summarized in Table 1. Groups of cells exhibiting intranuclear fluorescence were observed already in the second passage of SP-2 infected cells; in the next passage, nearly 80% of cells were positive. It was evident that the small epitheloid cells were positive, while the bigger elements were without fluorescence. From the fourth passage on, specific intranuclear fluorescence was detected in all cells (Fig. 4). On the other hand, the cells infected with SV 40 virus were T antigen negative, both before and after morphological transformation had occurred.

The presence of T antigen in SP-2 transformed dog kidney cells (DK SP-2) was also revealed by the CFR test. Results of titration of antigen from the SP-2 transformed cells are in Table 2. It can be seen that undiluted preparation of the transformed cells contained 4 units of T antigen. The undiluted antigen from DK SP-2 cells did not react with either SV 40 or adeno 7 immune monkey sera.

Table 2. Results of CF tests with DK SP-2 cells and different sera

Antigen	Antigen dilution	Hamster SV 40 tumour serum	Hamster control serum	SV 40 monkey serum	Adeno 7 monkey serum
DK SP-2	Undil.	64	<4	<4	<4
	1 : 2	64			
	1 : 4	32			
	1 : 8	<4			
	1 : 16	<4			
DK (control)	Undil.	<4	<4	<4	<4

The figures are reciprocals of the highest serum dilution which prevented the lysis of more than 50% sensitized red blood cells in the presence of appropriate antigen dilution and 2 units of complement.

The SP-2 transformed cells and the SV 40 transformed cells were injected each into one Horák's puppy which had been treated with cortisone (see Materials and Methods). The dose of DK SV 40 cells was 3×10^7 and the dose of DK SP-2 cells was 1.2×10^8 . There was no evidence of tumour development in the course of 7 months.

Discussion

The infection of in vitro cultivated dog kidney cells with SV 40 and SV 40-Adeno 7 (SP-2) hybrid virus resulted in cell transformation. In both instances the transformed cell type was represented by tightly packed small epitheloid cells, which formed multilayered colonies recognizable with the naked eye after prolonged cultivation. There was, however, a marked difference in the properties of the two cell lines derived from the SV 40 and SP-2 infected cells: the SP-2 transformed cells contained the SV 40 T antigen, which was demonstrable by immunofluorescence in all the cells, but this antigen was not detected in cells transformed in the SV 40-infected culture. Two explanations may be considered: 1) The SV 40 virus was not responsible for the transformation, in which case the transformation should be attributed to other factors. Although the transformation was not observed in the control cultures, this possibility cannot be ruled out because the number of controls cannot be considered sufficient to rule out a rare transformation event in uninfected cell cultures. 2) The cells were transformed by the SV 40 virus, but the SV 40 T antigen was not formed because the proper viral gene was not integrated or because its expression was repressed. A similar observation was reported by Diamandopoulos and Enders (1965). Using the immunofluorescent technique, they failed to detect the SV 40 T antigen in cells of hamster origin which had been transformed in vitro by SV 40 virus and which had acquired an oncogenic potential (Diamandopoulos and Enders, 1965). Sabin (1966) also reported that the Stoker-Sanders cloned line of H-1 cells transformed by polyoma virus, another member of the papova group, did not contain detectable amounts of polyoma T antigen; however, following injection into hamsters the tumour bearing animals possessed antibody reacting with the polyoma T antigen. This phenomenon might have been associated with the quantity or form in which the T antigen was present in the transformed cells, or it might have been conditioned by a derepression of the T antigen synthesis during cell growth in vivo. Unfortunately, our cells failed to induce tumours in homologous host; thus the possible similarity between the H-1 cells and DK SV 40 cells could not be examined.

References

- Ashkenazi, A., and Melnick, J. L. (1963): Tumorigenicity of simian papovavirus SV 40 and of virus-transformed cells. *J. nat. Cancer Inst.* **30**, 1227—1265.
- Black, P. H., and Rowe, W. P. (1963a): Transformation in hamster kidney monolayers by vacuolating virus SV 40. *Virology* **19**, 107—109.
- Black, P. H., and Rowe, W. P. (1963b): SV 40 induced proliferation of tissue culture cells of rabbit, mouse, and porcine origin. *Proc. Soc. exp. Biol. (N. Y.)* **114**, 721—727.

- Black, P. H., Rowe, W. P., Turner, H. C., and Huebner, R. J. (1963): A specific complement-fixing antigen present in SV 40 tumor and transformed cells. *Proc. nat. Acad. Sci. (Wash.)* **50**, 1148—1156.
- Black, P. H., and White, B. J. (1967): In vitro transformation by the adenovirus hybrid viruses. II. Characteristics of the transformation of hamster cells by the Adeno 2-, Adeno 3- and Adeno 12- SV 40 viruses. *J. exp. Med.* **125**, 629—646.
- Defendi, V. (1963): Effect of SV 40 virus immunization on growth of transplantable SV 40 and polyoma virus tumors in hamsters. *Proc. Soc. exp. Biol. (N. Y.)* **113**, 12—16.
- Diamandopoulos, G. T., and Enders, J. F. (1965): Studies on transformation of syrian hamster cells by simian virus 40 (SV 40): Acquisition of oncogenicity by virus exposed cells apparently unassociated with the viral genome. *Proc. nat. Acad. Sci. (Wash.)* **54**, 1092—1099.
- Diderholm, H., Berg, R., and Wesslén, T. (1966): Transformation of rat and guinea pig cells in vitro by SV 40 and the transplantability of the transformed cells. *Int. J. Cancer* **1**, 139—148.
- Diderholm, H., Stenkvist, B., Pontén, J., and Wesslén, T. (1965): Transformation of bovine cells in vitro after inoculation of simian virus 40 or its nucleic acid. *Exp. Cell Res.* **37**, 452—459.
- Fernandez, M. V., and Moorhead, P. S. (1965): Transformation of African green monkey kidney cultures infected with simian vacuolating virus (SV 40). *Tex. Rep. Biol. Med.* **23**, 242—258.
- Habel, K., and Eddy, B. E. (1963): Specificity of resistance to tumor challenge of polyoma and SV 40 virus-immune hamsters. *Proc. Soc. exp. Biol. (N. Y.)* **113**, 1—4.
- Horák, F. (1964): Specially breed dogs for general labs use are termed unique. *Med. Trib. and News* **5**, 86.
- Huebner, R. J., Channock, R. M., Rubin, B. A., and Casey, M. J. (1964): Induction by adenovirus type 7 of tumors in hamsters having the antigenic characteristic of SV 40 virus. *Proc. nat. Acad. Sci. (Wash.)* **52**, 1333—1340.
- Igel, H. J., and Black, P. H. (1967): In vitro transformation by the Adenovirus — SV 40 hybrid viruses. III. Morphology of tumors induced with transformed cells. *J. exp. Med.* **125**, 647—656.
- Khera, K. S., Ashkenazi, A., Rapp, F., and Melnick, J. L. (1963): Immunity in hamsters to cells transformed in vitro and in vivo by SV 40. Tests for antigenic relationship among papovaviruses. *J. Immunol.* **91**, 604—613.
- Koch, M. A., and Sabin, A. B. (1963): Specificity of virus induced resistance to transplantation of polyoma and SV 40 tumors in adult hamsters. *Proc. Soc. exp. Biol. (N. Y.)* **113**, 4—12.
- Koprowski, H., Pontén, J. A., Jensen, F., Raudin, R. G., Moorhead, P., and Saksela, E. (1962): Transformation of cultures of human tissue infected with simian virus SV 40. *J. cell. comp. Physiol.* **59**, 281—292.
- Kutinová, L., and Vonka, V. (1968): Growth of SV 40 — Adeno 7 hybrid virus in human diploid cells. *Int. J. Cancer*, **3**, 344—350.
- Mareš, I., and Dřevo, M. (1965): Cultivation of measles virus in dog kidney cell cultures. *Acta virol.* **9**, 152—159.
- Melnick, J. L., Khera, K. S., and Rapp, F. (1964): Papovavirus SV 40: failure to isolate infectious virus from hamster cells synthesizing SV 40 induced antigens. *Virology* **23**, 430—432.
- Pope, J. H., and Rowe, W. P. (1964): Detection of specific antigen in SV 40 transformed cells by immunofluorescence. *J. exp. Med.* **120**, 121—128.
- Rabson, A. S., and Kirchstein, R. L. (1962): Induction of malignancy in vitro in newborn hamster kidney tissue culture infected with simian vacuolating virus (SV 40). *Proc. Soc. exp. Biol. (N. Y.)* **111**, 323—328.
- Rapp, F., Butel, J., and Melnick, J. L. (1964): Virus-induced intranuclear antigen in cells transformed by papovavirus SV 40. *Proc. Soc. exp. Biol. (N. Y.)* **116**, 1131—1135.
- Rapp, F., and Melnick, J. L. (1966): Papovavirus SV 40, adenoviruses and their hybrids: transformation, complementation and transcapsidation. *Progr. med. Virol.* **8**, 349—399.
- Rapp, F., Melnick, J. L., and Levy, B. (1967): Correlation of immunology and histopathology of tumors induced by defective SV 40 — Adenovirus hybrids. *Amer. J. Path.* **50**, 849—858.
- Rapp, F., Tevethia, S. S., and Melnick, J. L. (1966): Papovavirus SV 40 transplantation immunity conferred by an adenovirus SV 40 hybrid. *J. nat. Cancer Inst.* **36**, 703—708.
- Sabin, A. B. (1966): Summary of Symposium on Malignant Transformation by Viruses, pp. 164 to 177. In F. H. Kirsten (ed.): *Malignant transformation by viruses*, Springer Verlag, New York 1966.
- Shein, H. M., Enders, J. F., and Levinthal, J. D. (1962): Transformation induced by simian virus 40 in human renal cell cultures. II. Cell — virus relationships. *Proc. nat. Acad. Sci. (Wash.)* **48**, 1350—1357.

- Tevethia, S. S., Katz, M., and Rapp, F. (1965): New surface antigen in cells transformed by simian papovavirus SV 40. *Proc. Soc. exp. Biol. (N. Y.)* **119**, 896—901.
- Tevethia, S. S., and Rapp, F. (1965): Demonstration of new surface antigens in cells transformed by papovavirus SV 40 by cytotoxic tests. *Proc. Soc. exp. Biol. (N. Y.)* **120**, 455—458.
- Vonka, V., Kutinová, L., and Závadová, H. (1968): Studies with SV 40 — Adeno 7 hybrid virus. Proc. 10th Congr. permanent Sect. microbiol. Standard., Prague 1967 (Karger, Basel/New York), in press.
- Vonka, V., Závadová, H., Kutinová, L., and Řezáčová, D. (1967): Development of antibodies against viral and tumor antigens of papovavirus SV 40 in monkeys. *Proc. Soc. exp. Biol. (N. Y.)* **125**, 790—794.
- Wells, S. A., Jr., Rabson, A. S., Malmgren, R. A., and Ketcham, A. S. (1966): In vitro neoplastic transformation of newborn hamster salivary-gland tissue by oncogenic DNA viruses. *Cancer* **19**, 1411—1415.
- Závadová, H., Kutinová, L., and Vonka, V. (1967): Preparation of antisera against the S antigen of influenza A virus by immunization of guinea pigs with internal S antigen. *Arch. ges. Virusforsch.* **20**, 421—429.

Explanation of Photomicrographs:

Fig. 1. Uninfected secondary dog kidney cells, unstained, $\times 150$.

Fig. 2. Dog kidney cells infected with SV 40 — Adeno 7 hybrid virus, forty days after infection. Focus of small epitheloid cells. Unstained, $\times 150$.

Fig. 3. Dog kidney cells transformed by SV 40 — Adeno 7 hybrid virus; unstained, $\times 150$.

Fig. 4. Immunofluorescence of DK SP-2 cells. Treated with serum from hamster bearing SV 40 induced tumour and stained with fluorescein isothiocyanate-labelled pig antihamster globulin. $\times 750$.