PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 158, 437-441 (1978)

Effects of BK Virus Infection on Primary Cultures of Rodent and Primate Cells[1] (40220)

JOHN E. GREENLEE[2], OPENDRA NARAYAN, AND RICHARD T. JOHNSON

Departments of Neurology, Comparative Medicine and Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

BK virus, a human papovavirus originally isolated from urine (1), is capable of inducing cell transformation *in vivo* and *in vitro*. In hamsters, the virus resembles simian virus 40 (SV40) in producing sarcomas following subcutaneous (sc) inoculation (2–4) and choroid plexus papillomas following intracerebral inoculation (5, 6), although with a single exception (6), the frequency of tumor induction with BK virus has been less than that with SV40 (7, 10). In individual reports, BK virus has been found to transform hamster (11–13) and rat (4) cells *in vitro*. Hamster cells transformed *in vivo* (5) or *in vitro* (13) by BK virus appear less oncogenic in hamsters than SV40 transformed cells. Human cells have been reported to develop persistent infection without definite transformation (14). The present study compares the effect of inoculations of rat, hamster, monkey and human cells with BK virus at high multiplicity and describes the biologic properties of resultant lines of transformed rodent cells.

Materials and methods. Virus. BK virus, originally isolated by Gardner (1) was obtained from Dr. Keerti Shah. Virus was propagated in cultures of human fetal skin and muscle (HFSM) and supernatant fluids were concentrated 100 fold by centrifugation at 100,000*g*. Stock virus had a titer of 100,000 hemagglutinating units (HAU).

Preparation of cell cultures. Newborn hamster kidneys (NHK), fetal rat lungs (FRL), and rhesus monkey choroid plexus were washed with Hanks' basic salt solution, minced and transferred to Falcon flasks in 3 ml of Eagle's Minimal Essential Media (MEM) with 20% fetal calf serum and 0.5% gentamicin. In addition, the fetal rats (FR) at late gestation were decapitated and minced. The minced fetal tissue was filtered through sterile gauze, agitated for 30 min at 37° in 50 ml of 0.05% trypsin solution, centrifuged for 10 min at 2000 rpm, and resuspended in 3 ml of MEM with 20% fetal calf serum and 0.5% gentamicin. Human colloid cyst cells were derived from a cerebral tumor explanted into tissue culture. HFSM and human embryonic kidney (HEK) cells were obtained commercially. All cell cultures beyond the first passage were grown in MEM with 10% fetal calf serum and 0.5% gentamicin and maintained in MEM with 0.2% fetal calf serum and 0.5% gentamicin.

Infection of cell culture flasks. Subconfluent flasks of each cell culture were inoculated with 50,000 HAU concentrated BK virus stock, incubated for 2 hr at 37°, overlaid with growth medium, grown to confluence, and subcultured at ratios of 1 to 6. Cells were seeded overnight in growth medium, changed to maintenance media, and observed daily thereafter for islands of transformed cells.

Histologic and ultrastructural studies. Cell cultures for electron microscopy were pelleted, fixed in 3.5% glutaraldehyde and embedded in epon. Sections were stained with uranyl acetate and lead citrate. Sections of transplanted tumors were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Fluorescent antibody studies. Antiserum to tumor (T) antigen was obtained from hamsters bearing transplanted SV40-induced sc tumors. Antiserum to BK virus viral (V) antigen was prepared in rabbits given 250 HAU of virus iv at weekly intervals for 6 weeks and bled 10 days after the final injection. Coverslip cultures were fixed in cold acetone, in-

[1] Supported by Public Health Service Grant Nos. NC1-43266 and NS 07000. Oster-Granite, M. L. unpublished data. Histologic sections of tumors derived from transplanted BK virus transformed cells were reviewed by Dr. John Strandberg, Department of Pathology, The Johns Hopkins University School of Medicine. The technical assistance of Ernestine Barber and secretarial help of Linda Kelly is greatly appreciated.

[2] Present address: Department of Neurology, University of Virginia Medical Center, Charlottesville, Virginia 22901.

0037-9727/78/1583-0437$01.00/0

cubated with appropriate sera and stained with fluorescein-conjugated goat anti-hamster or anti-rabbit globulin. SV40-transformed hamster cells and BK infected human embryonic kidney cells were used as positive controls for T and V antigen respectively. Normal human embryonic kidney or fetal hamster cells were used as negative controls. Normal rabbit and hamster sera served as serum controls.

Hemagglutination (HA). Cell cultures were prepared and tested for HA activity after treatment with receptor destroying enzyme (RDE) as described below. Doubling dilutions of each sample were prepared in phosphate-buffered saline. An equal volume of 0.5% human type O red blood cells was mixed with each dilution and samples were incubated at 4° for 4 hr.

Characterization of transformed cells. Islands of cells were trypsinized and cells transferred to Linbro plates. Cells were grown to confluence and passed to Falcon flasks. Cells were tested for the presence of T and V antigens, for their ability to grow after multiple subcultivations, to grow in serum-free medium, and to form colonies in soft agar (15). Ability of cells to form clones from single cells was evaluated by diluting cell suspensions to a concentration of 2 cells per 0.2 ml and seeding 0.2 ml into individual wells of a microtiter plate (16). Microtiter plates were examined at 2 and 12 hr. Wells containing single cells were observed until confluent monolayers had formed, and then subcultivated into larger flasks or onto coverslips.

Virus rescue. NHK cells at passages 5 and 15 and FR and FRL cells at passages 12 and 15 were fused to permissive human epithelial cells (colloid cyst and HEK) using inactivated Sendai virus (17). Cultures were maintained for 3 weeks, frozen, thawed, and sonicated for 2 min in an ice bath. RDE was added at a concentration of 0.5 ml per 2 ml of suspension and the samples were incubated overnight at 37°. RDE was inactivated at 56° for 20 min. Five blind passages were made onto both colloid cyst and human embryonic kidney cells. RDE-treated cell suspensions were evaluated for HA activity at each passage level.

Results. Rodent cells. Four islands of cells with altered growth patterns appeared in 6 flasks of NHK cells approximately 6 weeks after inoculation with BK virus. One island of surviving cells was found in 6 flasks of FR cells and 2 in 6 flasks of FRL cells at 6 weeks. Cell lines were established from two colonies of NHK cells, (NHK-1 and NHK-2), one of FR cells, and one of FRL cells. Fluorescent antibody staining of coverslip preparations of NHK cells, FR cells and FRL cell lines showed T antigen in 90–100% of the cells at multiple passage levels. V antigen was not detected (Table I).

All 4 cell lines grew rapidly to form dense cell layers characterized by cellular pleomorphism and numerous multinucleated giant cells. Cells could be subcultivated at least 30 times without evident changes in growth or morphology. At least 6 clones were derived from each cell line and prepared as coverslip cultures. All clones showed T antigen in 90–100% of the cells and no V antigen. Cultured cells grew well in 0.2% fetal calf serum but did not survive in serum-free media. No growth occurred in soft agar with NHK-2 cells, but colonies formed from 12.5% of NHK-1 cells, 15% of FR cells, and 51% of FRL cells. Electron microscopic examination failed to show viral particles in any of the cell lines.

After fusion with colloid cyst cells or HEK cells, NHK and FRL cells failed to yield virus detectable by hemagglutination. Virus was recovered after fusion of FR cells to HEK and colloid cyst cells as determined by hemagglutination and electron microscopy showing papovavirus virions.

Oncogenicity of transformed rodent cells for hamsters. Cell suspensions from two NHK cell lines at passage level 18 and FR and FRL cells at passage level 16 were inoculated into hamsters. Newborn and weanling hamsters received 2×10^6 NHK cells sc and adult hamsters 10^7 NHK, FR or FRL cells into the left cheek pouch. One-half of the newborn hamsters in each group also received serial injections of rabbit anti-hamster thymocyte serum (ATS) as described previously (5). Animals were observed for 6 months.

Only BK virus-transformed NHK cells induced tumors (Table II). Furthermore, mature hamsters developed tumors only after cheek pouch inoculation. Newborn hamsters

TABLE I. CHARACTERISTICS OF RODENT CELL LINES TRANSFORMED *in vitro* BY BK VIRUS.

Cell type	Transformed cell lines	Antigens in cell lines		Growth characteristic of cells				
		T	V	Number of subcultivations	Minimal serum required	Growth in soft agar[a]	Cells oncogenic for hamsters	Virus rescue
Hamster Kidney	NHK I	90–100%	0%	30	0.2%	12.5%	Yes	No
	NHK II	90–100%	0%	30	0.2%	0%	Yes	No
Rat Fibroblast	FRL	90–100%	0%	30	0.2%	51%	No	No
Rat Epithelial	FR	90–100%	0%	30	0.2%	15%	No	Yes

[a] Figure indicates per cent of cells seeded forming colonies.

TABLE II. ONCOGENICITY OF BK VIRUS, TRANSFORMED CELL LINES FOR HAMSTERS.

Cell line	Age of animals	Route of inoculation	No. of cells	Anti-thymocyte serum treatment	Hamster with tumors/Total no. of hamsters	First appearance of tumor (days after inoculation)	Histologic description of tumors
NHK Line 1	Weanling	sc	2×10^6	None	0/5	—	—
	Adult	Cheek pouch	10^7	None	4/9	94	Spindle cell sarcoma
	Newborn	sc	2×10^6	None	3/9	68	Spindle cell sarcoma
	Newborn	sc	2×10^6	Days 1, 2, 5, 10	15/15	27	Spindle cell sarcoma
NHK Line 2	Weanling	sc	2×10^6	None	0/5	—	—
	Adult	Cheek pouch	10^7	None	3/9	174	Spindle cell sarcoma
	Newborn	sc	2×10^6	None	3/10	108	Spindle cell sarcoma
	Newborn	sc	2×10^6	Days 1, 2, 5, 10	7/9	46	Spindle cell sarcoma
FR	Adult	Cheek pouch	10^7	None	0/5	—	
FRL	Adult	Cheek pouch	10^7	None	0/7	—	

had higher tumor incidence and shorter latency when treated with ATS. All tumors were composed of spindle-shaped cells with oval, often vesicular nuclei, prominent nucleoli, and poorly demarcated cytoplasm. Frequent mitotic figures were present in many, but not all sections, as were occasional multinucleated giant cells. A small amount of collagen was present between tumor cells. Virtually all sections contained areas of coagulative necrosis and/or hemorrhage, and a few sections contained foci of calcification. Local invasion of skeletal muscle and fat by tumor cells was observed, but there was no evidence of distant metastasis. Cultures derived from transplanted tumors showed T antigen in 90–100% of cells and no V antigen. Serum of hamsters bearing tumors produced intranuclear fluorescence when reacted with homologous tumor cells or with cultured cells transformed by BK, SV40 of JC viruses,[3] but not with control cells.

Monkey cells. Primary rhesus monkey choroid plexus cells could be subcultivated only with difficulty and did not survive more than three subcultivations. Inoculation of these cultures with BK virus caused no cytopathic effect (CPE) and did not alter growth characteristics. No T or V antigen could be demonstrated in inoculated cells, and no virus

[3] Hamster cells transformed by JC virus were kindly provided by Dr. Billie Padgett, Department of Microbiology, University of Wisconsin, Madison.

could be identified by hemagglutination.

Human cells. Cultures of both HEK and colloid cyst cells inoculated with BK virus developed focal CPE consisting of cytoplasmic vacuolation and lysis; CPE appeared at day 10–14, with loss of all cells by day 21–24. When cells were subcultivated prior to the appearance of CPE and were serially passaged to maintain low cell density, CPE and cell lysis were retarded but involved all cells by 5–8 weeks. Cultures of HFSM cells inoculated with BK virus slowly developed CPE and virus was detected by HA through the third subcultivation, but not thereafter. Coverslip cultures of HFSM cells at passage levels 5–8 after inoculation showed T antigen in 15–20% of the cells but did not have V antigen. Electron microscopic examination of HFSM cells at passage level 5 failed to show virus particles in any cell. HFSM cells at passage levels 5–8 grew slowly in 10% fetal calf serum, would not grow in 0.2% fetal calf serum or in soft agar, and could not be cloned from single cells. Although three foci with piling up of cells developed at passage level 6, cells from these foci could not be subcultivated, and no flask of HFSM cells could be maintained beyond the eight passage level after inoculation.

Discussion. In the present study BK virus produced multiple transforming events in rodent cells, without evidence of replication of infectious virus. Primary rhesus monkey choroid plexus cells appeared insusceptible to BK virus infection. Of the three human cell types studied, the two epithelial cell types, colloid cyst cells and HEK, underwent lytic infection without evidence of cell transformation. The fibroblastic HFSM cells also developed CPE, but a small percentage (15–20%) of surviving cells showed T antigen and contained no viral particles as judged by hemagglutination or electron microscopy. The presence of T antigen suggests the presence of at least a portion of the viral genome in these cells. However, the inability of these cells to survive subcultivation to passage levels greater than control cells, to grow in low serum concentrations, or to survive in soft agar indicate that the cells were not truly transformed.

The *in vitro* transformation of rodent cells observed here is in agreement with previous reports (4, 11–13). The effect of BK virus on simian cells appears to be more variable. BK virus has been shown to cause lytic infection in cultures of fetal rhesus and cynomolgus monkey glial cells*, but did not lyse or transform primary African green monkey kidney cells or monkey choroid plexus cultures in this study. Furthermore, although BK virus was initially cultivated in a continuous line of green monkey kidney cells (Vero) (1), its growth in these cells is less efficient than in most human cells.

In human cells, BK virus usually produces lytic infection without evidence of transformation. Our results with HFSM cells inoculated with BK are similar to the findings reported by Shah *et al.* (14) that human foreskin fibroblasts inoculated with UV-irradiated BK virus expressed T antigen but failed to fulfill other criteria for transformation.

In our study, virus could be rescued from only one of four transformed rodent cell lines, and our results are similar to previous reports in which virus could not be rescued (3) or could be rescued from only occasional lines of tumor cells (4, 7). The infrequency with which BK virus has been recovered from transformed cells might suggest that the viral genome is not readily excised from the host genome during cell fusion. However, virus can be readily rescued from cells transformed by BK viral DNA (18), suggesting that the failure to rescue virus from cells transformed by virions may indicate instead the lack of complete viral genomes in these cells. In both our study and a previous report (4), BK virus could be recovered only from rat cells and not hamster cells. This finding is of interest in that rat embryo cells are among the few cell types from which recovery of infectious virus has been reported after transformation by polyoma virus (19).

Although BK virus readily transformed rodent cells *in vitro*, transformed cells were poorly oncogenic for hamsters. Transformed cells produced tumors in newborn hamsters uniformly only with immunosuppression, and would grow in adult hamsters only if inoculated into the cheek pouch, an immunologically privileged site (20, 21). These data complement our results from a BK-induced choroid plexus papilloma (5) in supporting the hypothesis that the poor oncogenicity of

BK virus *in vivo* may represent, at least in part, an immunological recognition and rejection of cells transformed by BK virus.

Summary. The effect of BK virus at high multiplicity was studied on cultures of hamster, rat, monkey, and human cells. BK virus induced multiple transforming events in cells of both rodent species. Rhesus monkey choroid plexus cells were insusceptible to the virus. Epithelial and fibroblastic human cell strains developed lytic infection. Fifteen to 20% of the fibroblastic cells (skin and muscle) contained T antigen at later passage levels when no evidence of productive infection could be detected; these cells did not, however, meet other criteria for transformation. Transformed rodent cells exhibited T antigen in 90–100% of cells. BK viral particles could be rescued from only one line of transformed rat cells. BK virus-transformed rat cells could not be transplanted, and the transformed hamster cells were poorly oncogenic for newborn and adult hamsters unless cheek pouch inoculation or immunosuppression was used, suggesting that the poor oncogenicity reported for BK virus *in vivo* may reflect immune recognition and rejection of transformed cells by the host.

1. Gardner, S. D., Field, A. M., Coleman, D. V., and Hulme, B., Lancet **1,** 1253 (1971).
2. Shah, K. V., Daniel, R. W., and Strandberg, J. D., J. Nat. Cancer Inst. **54,** 945 (1975).
3. Nase, L. M., Karkkainen, M., and Mantyjarvi, R. A., Acta Pathol. Microbiol. Scand. Section B **83,** 347 (1975).
4. van der Noordaa, J., J. Gen. Virol. **30,** 31 (1976).
5. Greenlee, J. E., Narayan, O., Johnson, R. T., and Herndon, R. M., Lab. Invest. **36,** 636 (1977).
6. Uchida, S., Watanabe, S., Aizawa, T., Kato, K., Furuno, A., and Muto, T., Gann **67,** 857 (1976).
7. Diamandopoulos, G. T., J. Nat. Cancer Inst. **50,** 1347 (1973).
8. Duffell, D., Hinz, R., and Nelson, E., Amer. J. Pathol. **45,** 59 (1964).
9. Eddy, B. E., Borman, G. S., Berkeley, W. H., and Young, R. D., Proc. Soc. Exp. Biol. Med. **107,** 191 (1961).
10. Girardi, A. J., Sweet, B. H., Slotnick, V. B., and Hilleman, M. R., Proc. Soc. Exp. Biol. Med. **109,** 649 (1962).
11. Major, E. O., and DiMayorca, G., Proc. Nat. Acad. Sci. U.S.A. **70,** 3210 (1973).
12. Portolani, M., Barbanti-Brodano, G., and LaPlaca, M., J. Virol. **15,** 420 (1975).
13. Seehafer, J., Salmi, A., and Colter, J. S., Virology **77,** 356 (1977).
14. Shah, K. V., Hudson, C., Valis, J., Strandberg, J. D., Proc. Soc. Exp. Biol. Med. **153,** 180 (1976).
15. MacPherson, L., and Montagnier, L., Virology **23,** 291 (1964).
16. Rosenbaum, M., Sullivan, E. J., and Edwards, E. A. *in* "Animal Tissue Culture" (G. O. Wasley, ed.), p. 49. The Williams and Wilkins Co., Baltimore (1972).
17. Watkins, J. F., *in* "Methods in Virology " (K. Maramorosch and H. Koprowski, eds.) Vol. 2, p. 1. Academic Press, New York and London (1971).
18. Takemoto, K. K., and Martin, M. A., J. Virol. **17,** 247 (1976).
19. Fogel, M., and Sachs, L., Virology **37,** 327 (1969).
20. Greenblatt, M., Progr. Exp. Tumor Res. **16,** 380 (1972).
21. Handler, A. H., Progr. Exp. Tumor Res. **16,** 368 (1972).

Received September 29, 1977. P.S.E.B.M. 1978, Vol. 158.