PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 180-186 (1976)

# Experimental Infection of Human Foreskin Cultures with BK Virus, A Human Papovavirus[1] (39506)

KEERTI V. SHAH, CLARE HUDSON, JANET VALIS, AND JOHN D. STRANDBERG

*Department of Pathobiology, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore, Maryland 21205*

*Introduction.* BK virus (BKV), an SV40-related papovavirus, was first isolated by Gardner *et al.* (1) from the urine of a renal transplant recipient. The virus is a common infection of childhood (2, 3), but is not yet related to any illness. The virus probably persists in the individual after primary infection and is reactivated in times of immunological impairment, e.g., in transplant recipients and cancerous patients on immunosuppressive therapy (4-6) and in individuals with immune deficiency diseases (7). The virus transforms hamster cells in tissue culture (8) and produces tumors in neonatally inoculated hamsters (9).

BKV produces a lytic infection and grows to high titers in cells of human origin. Cells derived from human embryonic kidney (HEK) or brain and cells of the diploid fibroblast line WI38 are highly susceptible to lytic infection by the virus (10). We report here the results of BKV infection of early passage cell cultures derived from foreskins of newborn infants. In order to test the capacity of BKV to transform human cells, the lytic activity of the virus was decreased by irradiation of the virus and, in some instances, by the incorporation of BKV antiserum in the medium.

*Materials and methods. Foreskin cultures.* Foreskins from circumcisions were obtained from the Johns Hopkins Hospital, B-2 nursery. The donors were black or white infants, approximately 3 days of age. The tissue was minced with scissors and trypsinized to initiate cell cultures. Cells were grown in Falcon plastic 25-cm$^2$ tissue culture flasks in Eagle's minimum essential medium (Earle's salt base) supplemented with nonessential amino acids, antibiotics, and 20% inactivated fetal calf serum and were maintained on the same medium but with 5 or 10% fetal calf serum. The cells were serially passed in culture until sufficient numbers were obtained for experiments.

*Virus.* The prototype BKV strain isolated by Gardner *et al.* (1) was used at two passage levels (Table I). Virus was concentrated 10-fold by ultrafiltration at 4° in an Amicon Stirred Cell using a PM-30 membrane. For irradiation, petri dishes containing virus were placed 15 cm away from a uv germicidal lamp (Sylvania G15T8) so that 40 W/cm (400 erg/sec/cm$^2$) were delivered at the surface. The time of exposure to the uv irradiation was varied.

*Infection of cultures.* Two experiments were done. In the first, third to fifth passage cultures derived from 19 foreskins were exposed to unirradiated virus or to virus irradiated for 8 min. In addition, cultures from 12 and 10 of these foreskins were exposed to virus irradiated, respectively, for 10 and 15 min. In the second experiment, seventh passage cultures from four of the foreskins were subjected to virus irradiated for 30 min, and one group of virus-exposed cultures was subpassaged serially and maintained with medium containing BKV antiserum. The passage levels and titers of the virus and the treatment of cultures in the two experiments are indicated in Table I. Two-tenths milliliter (0.2 ml) of concentrated virus, with or without irradiation, was adsorbed on about three-fourths confluent cultures grown in 25-cm$^2$ flasks. The multiplicity of infection for the unirradiated virus was about 100 $TCID_{50}$. As shown in Table I, irradiation for 8 min resulted in a 50-fold, and for 30 min in a greater than 10,000-fold, reduction in infectivity titers. The hemagglutinin titer (1) of the unirradiated virus in both prepara-

[1] Supported by Public Health Service Grant CA-13478 and research contract NO1-CP-43266 from the National Cancer Institute.

TABLE I. A SUMMARY OF THE EXPERIMENTAL PROTOCOL: VIRUS TITERS AND TREATMENT GROUPS.

| | | BK virus | | | |
|---|---|---|---|---|---|
| | Cultures | Passage level | Titer of unirradiated virus* | Titer of irradiated virus[a] | Treatment of cultures |
| Expt. 1 | 19 cultures at 3–5 passage levels | 10 in vero, 1 in HEK | $10^{8.9}$ | $10^{7.2}$ (8 min of irradiation) | 1 A, Inoculated with unirradiated virus<br>1 B, inoculated with irradiated virus<br>1 C, uninoculated |
| Expt. 2 | 4 of above cultures at passage 7 | 9 in vero, 2 in WI38 | $10^{9.2}$ | $10^{4.4}$ (30 min of irradiation) | 2 A, inoculated with irradiated virus<br>2 B, inoculated with irradiated virus and maintained and passed with antiserum<br>2 C, uninoculated, maintained and passed with antiserum |

[a] $TCID_{50}$/ml in WI38 cell culture tubes, observed for 30 days.

tions was 1:640–1:1280 and was unchanged after irradiation.

*Passage of 2B cultures with BKV antiserum.* Except for cultures in treatment group 2B, all others exposed to irradiated or unirradiated virus were observed for BKV CPE without subpassage and without addition of BKV antiserum to the medium. The four cultures in group 2B were subpassaged serially once a week, at a 1:3 split ratio, for 9 weeks. Two flasks were prepared at each passage level, of which one was used for further passage and the other held for observation. BKV hyperimmune rabbit serum with a hemagglutination-inhibition (HI) titer of 1:1280 was incorporated in the medium at a concentration of 0.1% for 2B cultures beginning immediately following exposure to irradiated virus and then for a period of 1 week after each passage. Culture flasks at various passage levels held for observation were maintained without antiserum. Uninoculated cultures, group 2C, were treated in the same way as group 2B; i.e., they were subpassaged and maintained on medium containing BKV antiserum.

*Observation of cultures.* Infected and uninfected cultures were observed for BKV CPE and for cell morphology. Representative cultures were fixed in absolute methanol and stained with Jenner Giemsa stain. Supernatant fluids were assayed for BKV hemagglutinin each week in Experiment 1 and periodically in Experiment 2. Immunofluorescence (IF) tests for BKV viral and T antigens were done, as described (3), on cells grown on coverslips or LabTek slides (Miles Laboratories, Napierville, Virginia). The reference sera for viral antibodies were a BKV-immune rabbit serum and a human serum pool with high titers of BKV HI antibodies. Both of these sera were nonreactive in IF tests with BKV and SV40 T antigens. The reference T antibody serum was derived from hamsters bearing SV40 tumors. This serum reacted equally well with SV40 and BKV T antigens. For some of the cultures, concentrated supernatant fluids were examined, after negative staining, for virus particles by electron microscopy (EM) (6).

*Results. Response of foreskin cultures to unirradiated and irradiated BKV.* All inoculated cultures, whether they were infected with irradiated or unirradiated virus, eventually developed BKV CPE. The effect of increased time of irradiation was to progressively delay the onset of BKV CPE. The course of BKV infection in cultures of Experiment 1 is summarized in Table II. On Day 5 postinfection, in 14 foreskin cultures infected with unirradiated virus, an average of 24% of the cells exhibited BKV viral antigen by IF tests. The proportion of fluorescing cells was lower, averaging between 9.9 and 1.4% in the three groups of cultures which were infected with irradiated virus.

TABLE II. RESPONSE OF FORESKIN CULTURES TO UNIRRADIATED AND IRRADIATED BKV.

| | Treatment of cultures | | | |
|---|---|---|---|---|
| | Unirradiated virus | Virus irradiated 8 min | Virus irradiated 10 min | Virus irradiated 15 min |
| 1. Number of cultures examined | 14-19 | 15-19 | 9-12 | 5-10 |
| 2. Percentage of immunofluorescent cells, 5 days postinoculation[a] | 24 ± 3.4 | 9.9 ± 2.2 | 4.3 ± 0.8 | 1.4 ± 0.7 |
| 3. Time of onset of CPE, range (weeks) | 1-2 | 2-6 | 4-6 | 5-9 |
| 4. Time of appearance of HA in supernate, range (weeks) | 2 | 3-6 | 5-8 | 5-8 |
| 5. Proportion of cultures showing regrowth | 13/19 | 7/19 | 4/12 | 1/10 |
| 6. Time of beginning of regrowth, range (weeks) | 3-7 | 3-13 | 6-9 | 9 |

[a] Mean percentage of immunofluorescent cells for all cultures in the group ± standard error of the mean.

BKV hemagglutinins in titers ranging from 1:16 to 1:4096 were detected in almost all cultures which exhibited BKV CPE, but were not detected in infected cultures prior to the development of CPE. All of the uninfected control cultures remained normal and were negative for viral hemagglutinins and IF.

All of the foreskin cultures were fibroblastic in appearance at the time of inoculation with the virus. BKV CPE was first manifest by the appearance of scattered large rounded cells which, with time, increased in numbers and led to a complete destruction of the cell sheet, leaving behind only matted debris attached to the culture flasks. The time between the first appearance of CPE and complete cell destruction varied from between 2 to 7 weeks for the different cultures. The supernatant fluids contained large amounts of BKV hemagglutinins during this period.

A significant proportion of the cultures which had previously undergone extensive BKV CPE showed regrowth of cells and remained persistently infected with BKV (Table II). The regrown cells were fibroblastic in some cultures, epithelioid in others, and mixed in still others. A culture flask often had some areas of fibroblastic and others of epithelioid cells. The cells grew very slowly, often taking weeks before there were enough for passage. Rounded cells and cells with vacuolated or granular cytoplasm were common. The supernatant fluids from these cultures were mostly negative for BKV hemagglutinins but had low levels of infectious BKV, as indicated by successful isolation of the virus in inoculated WI38 cells. In IF tests, between 1 and 5% of the cells from regrown cultures stained for BKV viral and T antigens. Virions of papovavirus morphology were detected by EM examination, after negative staining, of concentrated supernatant fluids from some of these cultures. In observation periods ranging from 12 to 24 weeks after the initial infection, none of the regrown cultures became virus free or grew rapidly enough to permit frequent passage.

*Regrowth and passage of two cultures (JV-1 and JV-2) following BKV CPE.* In the second experiment, an attempt was made to further decrease the lytic activity of BKV. The time of virus irradiation was increased to 30 min and one group of cultures (2B) was maintained on antiserum and weekly serial passage.

BKV CPE developed in each of the four cultures of group 2A, which were maintained without antiserum and without passage. CPE was first seen 4-11 weeks postinoculation and was extensive within 1 to 2 weeks of onset. As in the first experiment, there was regrowth of cells with development of persistent infection in two of the four cultures.

In cultures of the treatment group 2B, BKV CPE was not seen as long as the cultures were maintained with BKV antiserum. At each successive passage level, all cultures which were held without further passage and without antiserum developed BKV CPE within 1-3 weeks of discontinuation of the antiserum. A total of 32 culture flasks representing cells from four foreskins at pas-

sage levels between 7 and 16 were observed. Following extensive BKV CPE in all 32 flasks regrowth occurred in 18. The regrowing cultures showed the characteristics described earlier of persistently infected cultures, viz. slow growth, cells with varying morphology, some cells showing BKV CPE and low levels of infectious BKV. Cultures in two of the 18 flasks, representing 16th and 10th passage levels of one foreskin (74-7) grew rapidly after an initial period of slow growth and exhibited some of the properties of transformed cells. These cultures were named JV-1 and JV-2, respectively, and were characterized with respect to presence of BKV-specific antigens, morphology, and susceptibility to BKV infection. All passages of the uninoculated cultures (2C) remained normal.

*Characteristics of JV-1 and JV-2 cell cultures.* The history of the JV-1 culture is given in Table III. Following inoculation of a seventh passage culture with irradiated BKV, CPE was first seen after nine further subpassages, at 9 weeks postinoculation. The progression of CPE to 4+ and regrowth of the culture to a stage where it could be passed took 7 weeks. In IF tests, <0.01% of the cells in the first two passages postinoculation showed viral antigen, but this proportion increased to 25% by 9 weeks. During this period, a comparable proportion of cells stained for T antigen. In contrast, in the regrown cultures, viral antigen-positive cells were absent or infrequent, while the proportion of T antigen-positive cells rapidly increased to >90% (Fig. 1) by the 19th passage and remained subsequently at that level. BKV hemagglutinins were absent in periodic examinations of the supernatant fluids. The history of JV-2 was essentially similar to that of JV-1 except that viral CPE and regrowth occurred in the 10th passage of foreskin culture 74-7, instead of in the 16th passage as in JV-1.

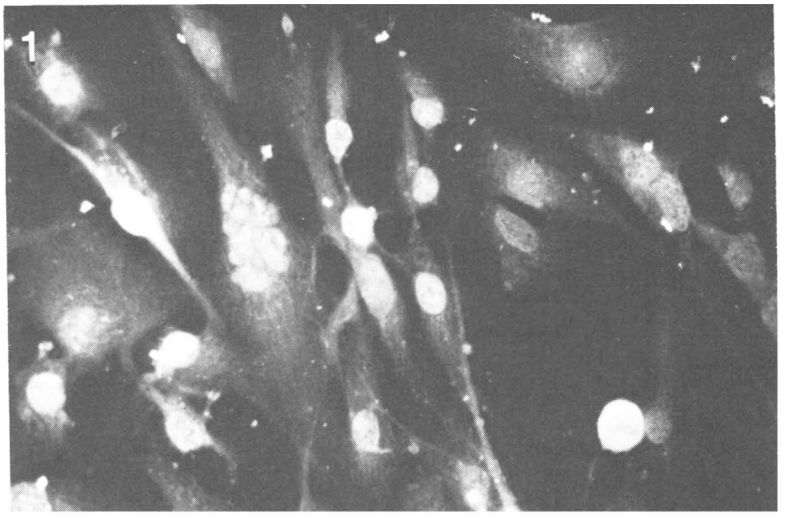
JV-1 and JV-2 cultures were resistant to BKV infection (Table IV). Following inoculation of BKV at a multiplicity of greater than 100, these cultures did not develop viral CPE, were negative for BKV hemagglutinins in the supernatant fluids, and had only 0.01 to 2% of cells showing viral antigen. In contrast, control cells derived from the same parent foreskin were susceptible to BKV infection.

Cells of JV-1 and JV-2 cultures looked alike and were readily distinguished from the control cells (Figs. 2 and 3). The control cultures were fibroblastic with long, spindle-shaped cells, essentially uniform in size, arranged in whorls or bands. Multinucleated cells were uncommon and the cytoplasm was free of granules. The cells of JV-1 and JV-2 cultures were granular and pleomorphic. Stellate cells rather than spindle cells were predominant and multinucleated cells were commonly found. Some of the nuclei had an irregular outline. Cytoplasmic vacuoles were frequently seen. Rounding cells which appeared to detach from the glass were always present. The cytologic characteristics of these cells were similar to those described for SV40-transformed cells in "crisis" (11).

JV-1 and JV-2 cells were contact inhibited and did not reach high saturation densities. Their growth rate was not greater than that of the control cells as judged by

TABLE III. BKV CYTOPATHIC EFFECT AND REGROWTH (JV-1) IN CULTURES OF FORESKIN 74-7.

| | Weeks of observation after exposure to irradiated BKV | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22–29 |
| Passage number | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | | | | | | | | 17 | 18 | 19 | 20 | 21 | 22–27 |
| Antiserum in medium | + | + | + | + | + | + | + | + | 0 | | | | | | | | + | + | + | 0 | 0 | 0 |
| Cytopathic effect | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1+ progression to 4+ and regrowth | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 |
| Percentage of cells, BKV viral antigen | <0.01 | <0.01 | | | | | 1 | 2 | 25 | | | | | | | | <0.01 | <0.01 | 0 | 0 | 0 | 0–≤0.01 |
| Percentage of cells, BKV T antigen | <0.01 | <0.01 | | | | | | 5 | 25 | | | | | | | | 25 | 50 | 90 | 80 | 90+ | 90+ |



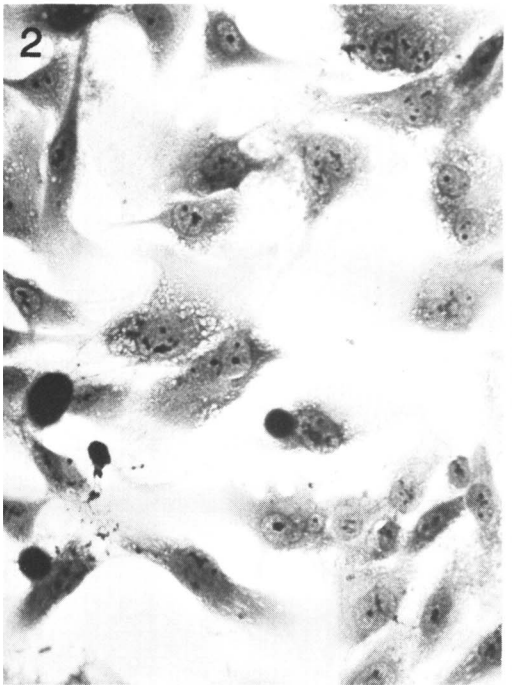





FIG. 1. Immunofluorescence test of JV-1 cells, passage 21, with SV40 T antibody showing the intranuclear T antigens. These cells were negative for BKV viral antigen. ×267.

FIG. 2. JV-1 cells in a coverslip preparation showing lack of confluent growth and presence of numerous giant cells. The cells are stellate and nucleoli are prominent. Jenner–Giemsa stain. ×267.

FIG. 3. Control human foreskin culture with a monolayer of uniform spindle cells containing regular oval nuclei. ×267.

the time required to become confluent and by the number of cells recovered at various times after initiation of the cultures. JV-1 and JV-2 cells could not be maintained beyond the 30th passage since the primary culture.

*Discussion.* All of the 19 cultures derived from foreskins of newborn infants devel-

TABLE IV. RESPONSE OF JV-1, JV-2, AND NORMAL CELLS, ALL DERIVED FROM FORESKIN 74.7, TO BKV INFECTION.

| Culture | Response | Days postinfection | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 3 | 8 | 12 | 16 | 20 | 24 | 28 |
| JV-1(p26) regrown after CPE | Percentage with viral antigen | 1 | 2 | | | | | |
| | cytopathic effect | | 0 | 0 | 0 | 0 | 0 | 0 |
| | hemagglutinin in supernate[a] | | | Neg[b] | Neg | Neg | | |
| JV-2(p14) regrown after CPE | Percentage with viral antigen | 0.01 | 0.01 | | | | | |
| | cytopathic effect | | 0 | 0 | 0 | 0 | 0 | 0 |
| | hemagglutinin in supernate[a] | | | Neg | Neg | Neg | Neg | |
| 74-7(p16) control | Percentage with viral antigen | 20 | 50 | | | | | |
| | cytopathic effect | | 1+ | 2+ | 3-4+ | 4+ | 4+ | 4+ |
| | hemagglutinin in supernate[a] | | | 32 | 256 | 1024 | | 512 |

[a] reciprocal of titer.
[b] Neg: hemagglutinin titer of <1:10.

oped lytic infection after inoculation of BKV. Several investigators have successfully prevented the lytic viral activity in permissive cells by exposure of the virus to irradiation; for instance, in the transformation of African green monkey kidney cells by SV40 (12) and of hamster fibroblasts with herpes simplex virus (13). In this study, irradiation of BKV and maintenance of infected cells with medium containing BKV antiserum delayed but did not prevent viral cytopathic effect. Although regrowth occurred in about one-half of the cultures following extensive BKV CPE, these cultures were persistently infected and, as a rule, grew very slowly. In only two instances, regrowing cultures could be passed regularly. Cells of these two cultures met some of the criteria of viral transformation. Their morphology was altered, and they had acquired resistance to infection with BKV. Almost all cells possessed the intranuclear T antigen, and the viral antigen was either absent or detectable in a very small proportion of cells. The cell cultures, however, did not exhibit the accelerated growth rate and high saturation densities that are characteristic of some transformed cell lines, and they could not be maintained beyond the 30th passage. Human cells transformed by SV40 characteristically also have a limited life span (14, 15).

*Summary.* Cell cultures derived from foreskins of 19 newborn infants were exposed to irradiated or unirradiated BK virus (BKV), an SV40-related papovavirus infection of man, and were observed for viral cytopathic effect (CPE) and for possible transformation. Four of the cultures were passed serially and maintained for a period of 9 weeks on medium containing BKV antiserum. All infected cultures developed viral CPE. Irradiation of virus delayed but did not prevent viral CPE. In nearly one-half of the cultures, some regrowth occurred following extensive virus-induced destruction of the cell sheets but the regrown cells, as a rule, remained infected with BKV and could not be passed. In two instances, regrown cells (JV-1 and JV-2) could be passed, and these had some of the characteristics of transformed cells. They were essentially free of BKV viral antigen but contained, in over 90% of the cells, the intranuclear T antigen. They were resistant to superinfection with BKV. In contrast to the uninfected control cells which were uniformly fibroblastic in appearance, cells in JV-1 and JV-2 cultures were pleomorphic with a predominance of stellate cells and multinucleated giant cells. However, JV-1 and JV-2 cultures were contact inhibited, did not reach high saturation densities, and could not be maintained beyond the 30th passage.

1. Gardner, S. D., Field, A. M., Coleman, D. V., and Hulme, B., Lancet **1**, 1253 (1971).
2. Gardner, S., Brit. Med. J. **1**, 77 (1973).
3. Shah, K. V., Daniel, R. W., and Warszawski, R. M., J. Inf. Dis. **128**, 784 (1973).
4. Coleman, D. V., Gardner, S. D., and Field, A. M., Brit. Med. J. **3**, 371 (1973).
5. Dougherty, R. M., and DiStefano, H. S., Proc. Soc. Exp. Biol. Med. **146**, 481 (1974).
6. Reese, J. M., Reissig, M., Daniel, R. W., and

Shah, K. V., Infect. Immunol. **11,** 1375 (1974).
7. Takemoto, K. K., Rabson, A. S., Mullarkey, M. F., Blaese, R. M., Garon, C. F., and Nelson, D., J. Natl. Cancer Inst. **53,** 1205 (1974).
8. Portolani, M., Barbanti-Brodano, G., and La-Placa, M., J. Virol. **15,** 420 (1974).
9. Shah, K. V., Daniel, R. V., and Strandberg, J. D., J. Natl. Cancer Inst. **54,** 945 (1975).
10. Takemoto, K. K., and Mullarkey, M. F., J. Virol. **12,** 625 (1973).
11. Girardi, A. J., Jensen, F. C., and Koprowski, H., J. Cell. Comp. Physiol. **65,** 69 (1965).
12. Shiroki, K., and Shimojo, H., Virology **45,** 163 (1971).
13. Rapp, F., and Duff, R., J. Virol. **8,** 469 (1971).
14. Shein, H. M., Enders, J. F., Palmer, L., and Grogan, E., Proc. Soc. Exp. Biol. Med. **115,** 618 (1964).
15. Moyer, A. W., Wallace, R., and Cox, H. R., J. Natl. Cancer Inst. **33,** 227 (1964).

Received May 6, 1976. P.S.E.B.M. 1976, Vol. 153.