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Mutagenic activity of BKV and JCV in human and other mammalian cells

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Summary. We present data suggesting that human polyomaviruses BKV and JCV, widely distributed throughout human populations, are able to induce gene mutations in cultured cells. In this study, using different infecting agents, cell lines to be infected, mutation expression periods, and selection systems, we observed mutagenic effects of varying extent with values of spontaneous mutant frequencies being increased after BKV infection up to 100-fold in BHK cells (6-thioguanine resistance) and nearly 35-fold in virus-transformed human Lesch-Nyhan cells (ouabain resistance). In experiments with BKV the viral mutagenic potential was found to be raised both in moderately uv-irradiated cells, or when wild-type virus was replaced by the variant BKV-IR isolated from a human tumor [27]. Since BKV-IR is defective in the expression of small-t antigen, the viral mutagenicity does not require this protein to be active. BKV was shown to mutate, besides different established cell lines, human peripheral blood lymphocytes. Moreover, as demonstrated by comparing mutagenicities of DNAs from BKV, JCV, and the related polyomavirus SV40, the mutagenic effects of the three viruses do not appear to be essentially different. Implications of these findings are discussed.

Introduction

The genetic stability of the human genome has been proposed to be influenced, among other factors, by its interaction with the genetic material of different viruses present in the human environment (e.g., [10]). This idea was substantiated by the finding that some viruses of the papova, adeno, and herpes groups as well as retroviruses are able to mutate cells cultured in vitro [5, 17, 28, 34, for further review see 11]. The oncogenic polyomavirus SV40 was shown to

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induce resistance mutants affecting different cellular genes, a process which depends on the expression of the early region of the viral genome as well as, most likely, on a function involved in specific binding of large T antigen to viral DNA; the virus acts synergistically in combination with different chemical and physical mutagens; and, though the mutagenic activity is essentially detectable at early stages of viral infection, clones of SV40-induced specific-locus mutants were found to show increased spontaneous mutability of other markers [11, 39, 40]. In view of these findings it should be borne in mind that genotoxic effects might be caused by two related polyomaviruses, BKV and JCV, whose natural host in man.

These viruses [for review see 35, 45, 48] infect 70 to 80% of the world's population with primary infection occurring during childhood and showing no obvious deleterious effects; however, antibody levels are maintained throughout life suggesting a persistent, latent infection. Both viruses appear to be reactivated in either naturally (e.g., pregnancy) or therapeutically immunosuppressed individuals as well as under conditions of diabetes and other chronic diseases. They are highly oncogenic in rodents, particularly hamsters; JCV also induces brain tumors in lower primates and is the etiological agent of the progressive multifocal leukoencephalopathy, a rare disease found predominantly in patients with impaired cell-mediated immunity. While the capacity of BKV to transform neoplastically nonpermissive or semipermissive cultured cells has been well demonstrated, transformation by JCV, having an extremely restricted host range, is limited to a few cell types at low frequency. There is no definitive evidence that polyomaviruses are involved in human cancer. Recent findings have been discussed, however, in relation to a possible cooperation between BKV and other factors in human neoplasia [8, 9].

Mutagenic activity of human polyomaviruses would signalize them as possible genetic risk factors present, especially, under conditions connected with increased virus multiplication in the human body and, in addition, might have to be accounted for when applying polyomavirus derived replicating vectors.

In this report, an increase in mutant frequencies found in different rodent and human cell cultures after human polyomavirus infection will be described and discussed.

Materials and methods

Cells, virus, and viral DNA

The cell lines used were Chinese hamster cells V79-4, baby hamster kidney cells BHK21-C13, the human larynx carcinoma cell line HEp-2, LNSV-R31, a clone derived from human Lesch-Nyhan fibroblasts being transformed by SV40 and reverted from HPRT deficiency [21, 36], XP25RO-SV, a derivative from the human xeroderma pigmentosum complementation group A fibroblast line XP25RO [18] which was immortalized in our laboratory following SV40 infection, normal human embryonic lung fibroblasts HEL, isolated in our laboratory, and Vero monkey kidney cells. Cells were grown in Eagle MEM with supplements described elsewhere [38] and 10% fetal bovine serum (human cells) or 8% newborn calf serum plus 2% fetal bovine serum (rodent and Vero cells). Preparation and culture of

human lymphocytes: Heparinized 10-ml-blood samples from 5–6 healthy donors were pooled and the mononuclear fraction was immediately separated on Ficoll-Visotrast 370 using the Visotrast modification [33] of the method of Boyum [4] and washed twice in serum-free RPMI-1640. The cells were counted, tested for viability by dye exclusion (trypan blue) and resuspended in lymphocyte growth medium (LM) consisting of RPMI-1640 with 10% heat-inactivated fetal bovine serum, glutamin (2 mM), Hepes buffer (24 mM), PHA (Wellcome; 22.5 µg/ml), penicillin (100 IU/ml), and streptomycin (100 µg/ml).

Wild-type BK virus (Gardner strain) and the virus variant BKV-IR [27] were grown in Vero cells and purified by CsCl density gradient centrifugation based on the method described for SV40 [16]. Virus was titrated by measurement of both its hemagglutinating activity for human type 0 erythrocytes [29] and its infectivity in HEL cells according to Reed and Muench [24], read after 22 days and expressed in terms of $TCID_{50}$/0.5 ml. The purified virus preparations had titers of $10^{4.6}$ (wild-type) and $10^{4.9}$ (IR) hemagglutinating units (HAU)/ml. One HAU of BKV was found to correspond to 15.244 $TCID_{50}$ units.

The plasmids pBKV and pJCV (obtained from Dr. P. Howly, NIH, Bethesda, Md.) as well as pSV40 consist of the complete BKV (prototype)-, JCV (Mad-1)-, or SV40-genome cloned each into the BamHI site of pBR322. They were amplified in *E. coli* and purified by centrifugation to equilibrium in CsCl-ethidium bromide gradients [22] and used as closed circular DNAs for infection.

Infection and selection of mutants

For infection with virus subconfluent cell monolayers (10^6 cells) were inoculated with 1 ml, duplicate samples of 2×10^6 freshly isolated lymphocytes deprived of culture medium with 2 ml virus suitably diluted in corresponding serum free medium to give the concentration indicated in the tables. After 2 h the cells were washed and further incubated for the expression time indicated. Infection with DNA by the DEAE-dextran technique (final concentration 2.0 µg viral DNA per ml with correspondingly higher values of plasmid DNAs) as well as mutant selection with the adherent cell lines were performed as described elsewhere [38]. Optimum conditions for selection of human cell mutants had been determined by preceding chemical mutagenesis experiments. The media for selection contained 6-thioguanine (TG; 0.25×10^{-5} M for HEp-2 cells; 0.5×10^{-5} M for other cell lines), ouabain (Oua; 1×10^{-3} M for V79 cells; 0.5×10^{-6} M for LNSV-R31 cells) or aminopterin (AP; 5.5×10^{-8} M). Mutant frequencies were calculated in terms of mean number of mutant colonies per plate/number of inoculated cells per plate × plating efficiency. Each calculation was based on six to eight 70 mm plates inoculated with 1×10^5 cells. The stability of a number of mutants of the different cell lines and resistant phenotypes was checked after prolonged growth in the absence of selective agent. Most or all of the mutant clones tested so far in each case proved to be stable regarding their resistant phenotype.

TG resistant lymphocytes were assayed autoradiographically according to the method described by Albertini and Sylvester [1] with some modifications. Samples of both 10^6 infected and 10^6 noninfected cells suspended in 0.9 ml LM plus either 0.1 ml TG solution (dissolved in LM to give a final concentration of 2×10^{-4} M) or 0.1 ml pH-adjusted LM were cultured in triplicate sets for 64 h in the presence of PHA and, during the last 16 h, ^{3}H-thymidine (10 µCi/ml; 1 Ci/mmol) using glass tubes in a humidified atmosphere containing 5% CO_2. Following termination by adding 4 ml of 0.1 M citric acid and centrifugation pellets were washed once in fixative (methanol-acetic acid, 5 : 1), recentrifuged and resuspended in 0.2 ml fixative. After at least 1 h, free nuclei were counted and added in measured volumes to coverslips afixed to slides which were then dried, covered with nuclear emulsion (ORWO K6) and autoradiographed. Variant frequencies were calculated as the ratio of labeling indexes of TG- and no TG-containing cultures by counting each of the numbers of labeled nuclei per test culture (TG) and per 500 nuclei of the control culture (no TG) respectively.

All data shown in the tables and figures are representative, in each case, of at least two independently performed experiments.

Immunofluorescence staining

Virus T antigen was detected in cells infected with BKV by indirect immunofluorescence using hamster anti-SV40-T-serum prepared from hamsters with tumors that were induced by SV40-transformed hamster cells, taking advantage of the strong immunological cross reactivity of SV40 and BKV T antigens [48], and fluorescein-conjugated antiglobulin according to the method described elsewhere [38].

Results

Mutagenic activity of BKV and JCV

Hamster cells being highly sensitive in vivo and in vitro to the oncogenic potential of human polyomavirus may also be suitable indicators for assaying mutation induction. On the other hand, specific locus mutation systems are well established for cell lines such as V79 or BHK (e.g., [6]). Results obtained with BK virus in these cells are shown in Table 1.

When infected with 6×10^{-4} HAU/cell (corresponding to 9.1 $TCID_{50}$/cell) V79 cells responded with an increase in the frequency of both TG-resistant (TG^r) and Oua-resistant (Oua^r) mutants by a factor of about 6, as compared to the mock infection values, whereas infection with 1×10^{-4} HAU/cell produced lower or nonessential effects. The use of substantially higher multiplicities of infection strongly reduced the fraction of V79 cells surviving infection. Even for an infection with 6×10^{-4} HAU/cell the colony forming efficiency of single cells seeded a few hours after virus adsorption reached only 25%, whereas mock infected cells plated with an efficiency of more than 70%. However, the infected mass culture when incubated for several days tended to recover from infection (see Table 1, plating efficiency of cells seeded 4 and 7 days after virus adsorption) and could be kept for a very long time. In contrast to V79, BHK cells showed tolerable surviral from infection with higher multiplicity. This difference between the Chinese hamster and Syrian hamster cell lines obviously reflects the finding of different rodent cells being either nonpermissive or semipermissive for BKV to a varying degree [30, 41]. After infection with 1×10^{-2} HAU/cell the frequency of TG^r mutants in these cells increased by about 10- to 100-fold depending on the length of time following infection allowed for mutation expression. The mutagenic potential of BKV was further demonstrated when the cells were pretreated, prior to infection, with a moderate dose of uv irradiation (253.7 nm). The combined action of both mutagens resulted in a mutant production in V79 cells much exceeding the sum of mutants produced by single treatments (Table 1). Therefore, in BKV-infected mammalian cells virus-induced mutations are detectable in dependence on the viral dose, the operative mutation expression time and possible synergistic effects elicited by combined action of the virus and another mutagen – findings that seem to be analogous to effects described earlier for the simian papovavirus SV40 [11].

Table 1. Mutations of rodent cells induced by infection with BK virus

Cell mutation system[a]	Treatment (HAU/cell)[b]	Expression time (days)	Plating efficiency (%)	Mutant frequency ($\times 10^{-5}$)	Increase in mutant frequency[c]
V79-TG^r	mock infection	7	73.3	0.4	–
V79-TG^r	BKV (1×10^{-4})	7	72.0	0.8	2.0
V79-TG^r	BKV (6×10^{-4})	7	63.2	2.4	6.0
V79-TG^r	uv[d]	7	53.7	3.2	8.0
V79-TG^r	uv[d] plus BKV (6×10^{-4})	7	54.8	11.7	29.25
V79-TG^r	BKV-IR (6×10^{-4})	7	60.0	6.7	16.75
V79-Oua^r	mock infection	4	75.1	0.2	–
V79-Oua^r	BKV (1×10^{-4})	4	74.6	0.3	1.5
V79-Oua^r	BKV (6×10^{-4})	4	64.6	1.1	5.5
BHK-TG^r	mock infection	3	69.0	0.3	–
BHK-TG^r	BKV (1×10^{-2})	3	59.4	3.4	11.3
BHK-TG^r	mock infection	7	72.2	0.2	–
BHK-TG^r	BKV (1×10^{-2})	7	60.1	8.3	41.5
BHK-TG^r	mock infection	13	65.0	0.2	–
BHK-TG^r	BKV (1×10^{-2})	13	60.0	20.5	102.5

[a] TG^r, resistance to 6-thioguanine (5×10^{-6} M); Oua^r, resistance to ouabain (1×10^{-3} M)

[b] Multiplicities of viral infection, expressed as haemagglutinating units per cell, are given in parentheses

[c] Increase in mut. freq. is defined as mut. freq. after treatment (infection and/or uv irradiation)/mut. freq. after mock infection

[d] Cells as monolayers overlayed with PBS were irradiated by a constant dose using a low-pressure uv lamp

Since, especially in the case of BKV, tumorigenic and transforming properties of the virus have been correlated with rearrangements in the viral regulatory region [7, 47] it seemed to be intersting to compare the mutagenic effects of wild-type virus and BKV-IR, a virus variant isolated from a human tumor and bearing both an insertion and a deletion in the early region of the genome [27]. In consequence of the insertion enhancer elements are rearranged while the deletion abolishes the expression of small-t antigen [27]. As is shown in Table 1, under conditions of infection permitting a 6-fold increase in the mutant frequency by wild-type infection, an infection with the variant resulted in a 16.7-fold increase relative to the mock infection value. It may be concluded (*i*) that the rearrangement which happened in BKV-IR has raised the viral capacity to mutate rodent cells and (*ii*) that small-t protein activity is not necessarily required for polyomaviruses to be mutagenic.

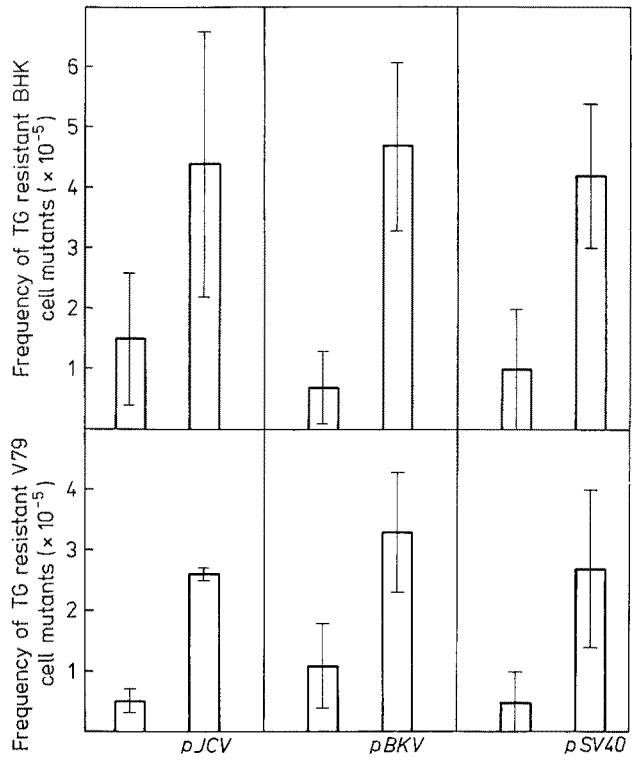

Fig. 1. Mutagenicity of DNAs from different polyomaviruses in rodent cells. BHK cells (top) and V79 cells (bottom) were infected with DNAs from JCV, BKV, or SV40 (cloned in pBR322, final concentration 2.0 µg viral DNA per ml) and plated on the 6th day for selection of mutants resistant to 6-thioguanine (5×10^{-5} M) after intermediate passage. The left columns in each case show corresponding mutant frequencies obtained by infection with pBR322 alone. The plating efficiencies in non-selective medium ranged from 0.65 to 0.85 for differently infected cells. Bars represent 95% confidence intervals based on Student's t-distribution. For further details see Materials and methods

For mutagenicity testing of JC virus, owing to complications connected with preparing highly titered JCV [45] cells were infected with viral DNA as an alternative [40]. As shown in Fig. 1, pJCV-infection increased the mutant frequencies 5.2-fold and 2.9-fold in V79 and BHK cells respectively, in comparison to the pBR322-infection. Obviously JCV (DNA) did not differ essentially from BKV and SV40 (DNAs) in the ability to mutate these cells. Similar results were obtained after selection for Oua^r mutants (not shown here).

Mutation induction in human cell lines

When inferring possible genotoxic effects of polyomaviruses such as BKV and JCV to exist in the human population, we have to consider that replication, transforming potential, and pathology of the viruses seem to depend on host cell factors needed for viral expression [15, 23]. To test, first of all, the sensitivity of any human cells to human polyomavirus-induced mutagenesis cultures of

Table 2. Mutations of human cells induced by infection with human polyomavirus (DNA)

Cell line	Type of mutant selection Resistance to[a]	Infecting agent[b]	Plating efficiency (%)	Mutant frequency ($\times 10^{-5}$)	Increase in mutant frequency[c]
HEp-2	TG	mock infection	0.55	0.16	–
HEp-2	TG	BKV	0.39	1.28	8.0
HEp-2	TG	pJCV	0.55	1.83	11.4
LNSV-R31	Oua	mock infection	0.69	1.16	–
LNSV-R31	Oua	BKV	0.43	40.10	34.6
LNSV-R31	Oua	pBKV[d]	0.40	7.50	5.0
LNSV-R31	Oua	pJCV[d]	0.57	14.75	6.7
LNSV-R31	AP	mock infection	0.69	1.45	–
LNSV-R31	AP	BKV	0.43	8.87	6.1
XP25RO-SV	TG	mock infection	0.41	0.49	–
XP25RO-SV	TG	pJCV	0.52	2.66	5.4

[a] TG, 6-thioguanine (2.5×10^{-6} M for selection of HEp-2 mutants, 5×10^{-6} M for selection of XP25RO-SV mutants); Oua, ouabain (5×10^{-7} M); AP, aminopterin (5.5×10^{-8} M)

[b] Infection with BKV was performed at a ratio of 1×10^{-2} HAU/cell, infection with DNA at a final concentration of 2.0 µg viral DNA per ml

[c] Increase in mut. freq. is defined as mut. freq. after infection/mut. freq. after mock infection

[d] In experiments with pBKV and pJCV the corresponding frequencies of Oua resistant mutants after mock infection were 1.50×10^{-5} and 2.20×10^{-5}

different established cell lines previously shown to be suitable for mutant selection after chemical mutagenesis (unpublished results) were subjected to infection in the same way as were hamster cells (see Table 2). In the case of the LNSV-R31 cell line, which was derived from HPRT deficient fibroblasts, both the Oua and the AP resistance marker were used instead of the TG resistance marker for selection. We found that, in the three human cell lines chosen by chance, infection with human polyomavirus (DNA) led to a rise in the frequency of mutants ranging generally from 5-times up to more than 10-times the control value. The highest effect, a 34.6-fold increase in the mutant frequency, was obtained by testing the Oua resistance marker in LNSV-R31 cells infected with BK virus.

The validity of these cell lines as models for in vivo processes, however, seems to be limited, especially since cellular alterations associated with cell line establishment, viral transformation and deficiency in excision repair may implicate changes of genetic stability [12, 39, 42]. Normal human cells such as early passage fibroblasts would be more closely related to cells in the human body but they can be worked only with some difficulty in quantitative mutagenesis experiments [14].

Table 3. Variant frequency of human peripheral blood lymphocytes infected with BK virus

Expression time (days)	Cultures without TG		Cultures with TG		Variant frequency ($\times 10^{-5}$)	Increase in variant frequency	T antigen positive cells (%)
	Labeled cells[a]	LI[b]	Labeled cells[a]	LI[b] ($\times 10^{-5}$)			
Uninfected cells							
1	404 (500)	0.808	14 (7.2×10^5)	1.944	2.4	–	–
4	420 (486)	0.864	9 (5.0×10^5)	1.800	2.1	–	–
7	140 (372)	0.376	6 (8.0×10^5)	0.750	2.0	–	–
Infected cells[c]							
1	376 (524)	0.718	36 (6.0×10^5)	6.000	8.4	3.5	20–25
4	242 (324)	0.708	63 (4.6×10^5)	13.096	19.4	9.3	5–10
7	76.5 (374)	0.204	55.5 (12.0×10^5)	4.625	22.6	11.3	<1

[a] The numbers of counted nuclei are given in parentheses, for details see Material and methods

[b] *LI* Labeling index

[c] Infection with BKV was performed at a ratio of 6×10^{-4} HAU/cell

Detection of 6-thioguanine resistant human lymphocytes

Since it was suggested that human lymphocytes play a role in the mechanism of natural infection by human polyomaviruses [13, 31] and, on the other hand, human lymphocytes cultured in vitro are used successfully in mutagenesis studies [25], we decided to test whether an infection of peripheral human lymphocytes taken from healthy donors with BKV gives rise to a production of TG^r cells indicating a mutagenic effect.

In these experiments lymphocytes infected with BKV at a ratio of 6×10^{-4} HAU/cell were cultivated in LM for different periods prior to selection and determination of variant frequencies (VFs) by the autoradiographic method [1]. As is shown in Table 3, while the VFs of noninfected cells rather declined during the cultivation periods, the VFs of infected cells increased exceeding the former ones by about one order of magnitude in the four- and the seven-day cultures. Culturing infected lymphocytes for only 1 day before onset of selection obviously did not permit a full expression of TG^r variants. The level of T antigen expression in infected cells fell from initially 20–25% on day 1 to <1% on day 7, an outcome which principally has been observed also with other BKV-infected

cell systems [46], but, nevertheless, was accompanied by an increase of presumably virus-induced TG^r variants in the human lymphocytic cultures.

Discussion

The results presented here show that infection of human or rodent cells with human polyomavirus (DNA) causes the appearance of drug resistant mutants. When selecting for TG^r mutants, this effect was found to depend on both the applied virus dose and the expression time chosen, suggesting an induction of HPRT gene mutations by the virus. The higher yield of mutants found to be caused by infection of rodent cells with BKV-IR as compared to the wild-type virus, might be attributed to mutational changes of the cellular genome being induced by the putative transposable element present in the variant's genome [7, 27]. This possibility was proposed already by the authors mentioned when discussing an association of this virus and similar variants with certain human tumors found by them.

Since infection with both, BKV and JCV, is widespread in human population these viruses should be considered genetic risk factors, especially under conditions leading to an activation of latent virus in the organism or if additional environmental genotoxic agents become effective. Continuing our experiments on BKV-induced mutagenesis we found a clearly icreased level of TG^r variant lymphocytes in Syrian hamsters infected with BKV, indicating a viral mutagenicity in vivo (G. Grabowski and M. Theile, in prep.).

The results obtained with the TG selection system were confirmed by using the Oua resistance and AP resistance markers. Furthermore, though a detection of induced mutations might have been influenced by cell line-specific conditions such as the portion of cells surviving infection, the nature of infection being primary or a superinfection of a transformed line, or the mutability of the specific locus in question, none of the cell lines tested so far has failed to respond positively.

It cannot be excluded that expecially in the autoradiographic short-term assay a number of phenocopies were scored together with true TG^r mutants (the designation "variants" instead of "mutants" usually makes allowance for this) [2]. However, as was shown by the authors quoted, the autoradiographic assay, besides the T-cell cloning assay, can serve as a useful measure of TG^r T-cell mutant frequencies [2]. The procedure of selection TG^r lymphocytes, which had been optimized by us in preliminary experiments, implicated highly stringent conditions along the plateau of the TG response curve. In contrast to a conclusion reported by Lecatsas et al. [19], our results did not indicate a possible stimulation of lymphocyte proliferation by BK virus. However, the experimental conditions they used do not appear to be suitable to detect well-defined information. Our findings shown by the corresponding LI data for TG-free cultures (Table 3) were confirmed in experiments with parallel cultures where the radioactive material was precipitated by TCA, washed on paper filters and measured in a liquid scintillation counter (results not shown).

At present we do not have definite information on the nature of the induced mutation at the molecular level. When isolated BKV-induced mutants were tested, subsequent to prolonged cultivation in non-selective medium, for their colony forming ability in different selective media there was found a noticeable diversity in the degree of drug resistance, at least for a number of TG^r rodent cell clones (result not shown here). Such an outcome can be reasonably explained with variants of residual HPRT enzyme activity caused by mutations in different loci of the HPRT gene [37]. Earlier results on SV40-induced mutagenesis led to the conclusion of point mutations having occurred in the majority of TG^r mutants, were compatible with the assumption of a structurally altered enzyme dihydrofolate reductase in antifolate drug resistant mutants, or were interpreted by base exchange mutations having led to Oua resistance [11, 38]. But other types of mutations shown to cause these kinds of drug resistance too [20, 32, 43] might also be involved. It may be mentioned in this connection that in normal human lymphocytes a substantial portion of spontaneous mutations at the HRPT or the HLA locus have been associated with gene deletions and further recombinational changes [43, 44]. In cells infected with polyomaviruses, both virus and host undergo genomic rearrangements [3, 26]. Retention of viral sequences was observed in some SV40-induced rodent cell mutant clones [39] (a result which we could not find for a number of BKV-induced mutants tested so far in preliminary experiments). Concerning the mutational mechanism of SV40 activation of error-prone repair processes and/or mutator-like activity of large-T antigen has been discussed [40]. Further work to define the types of mutations that are induced by polyomavirus infection may provide information on the mechanism of mutagenesis.

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