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In Vitro Immortalization of Marmoset Cells with Three Subgroups of Herpesvirus Saimiri

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Sequences within the rightmost 7 kilobases of the unique L DNA of herpesvirus saimiri are required for oncogenicity of the virus. The same DNA region has been found to be highly variable among different strains of herpesvirus saimiri. On the basis of this variability, herpesvirus saimiri strains were classified into groups A, B, and non-A, non-B. Herpesvirus saimiri strains representing the three groups were used successfully for in vitro immortalization of phytohemagglutinin-activated, interleukin 2 (IL-2)-expanded peripheral blood lymphocytes of common marmosets (*Callithrix jacchus*). Peripheral blood leukocytes could be immortalized from only a subset of common marmosets (5 of 13). All of the immortalized cell lines contained covalently closed circular viral DNA molecules and initially showed a low level of virus production. Cells immortalized by group A and group non-A, non-B strains did not require IL-2 in the medium. However, the only group B immortalized cell line, 473-SMHI, did not grow well in the absence of IL-2. The different characteristics of cell lines immortalized by herpesvirus saimiri strains belonging to different groups may help to elucidate some functions coded by the highly variable DNA region which is involved in the oncogenic process.

Herpesvirus saimiri, a common passenger of healthy squirrel monkeys, is highly oncogenic in several other New World primates and certain strains of laboratory rabbits, causing rapidly progressing T-cell lymphomas (4, 13, 14, 19). As we have shown before, two attenuated strains of herpesvirus saimiri, 11-att and SMHI-att, which have lost their oncogenic properties, have sizeable deletions within the rightmost 7-kilobase (kb) DNA segment of the 110-kb unique L DNA of the genome (5, 12). (For better alignment among the genomes of herpesvirus saimiri, herpesvirus ateles, and Epstein-Barr virus, a decision has been made at the Eleventh International Herpesvirus Workshop [Leeds, United Kingdom, July 21–26 1986] to change the orientation of the herpesvirus saimiri physical map. Therefore, the rightmost 7-kb region of L DNA mentioned here corresponds to the leftmost 7 kb of L DNA in previous literature [9, 12, 16].) Furthermore, in vitro-constructed deletions mapping in this rightmost 7 kb of the L DNA render the virus nononcogenic (6). These data indicate that the sequences located within the rightmost 7 kb of the L DNA are required for oncogenicity of herpesvirus saimiri.

Although the numerous isolates of herpesvirus saimiri are easily distinguishable from each other by restriction endonuclease site polymorphisms (7, 9), they show good homology throughout the genome (judged by blot hybridization), except in the region required for oncogenicity. Based on lack of cross-hybridization of the rightmost 7-kb region of L DNA, herpesvirus saimiri isolates can be classified into three groups: A, B, and non-A, non-B (16). It is not clear whether all virus strains of group non-A, non-B are homologous to each other or whether they can be further subdivided. For the sake of simplicity, however, we call these non-A, non-B virus strains group N viruses. Data available in the literature indicate that there are differences between the in vivo oncogenic potentials of some group A and B virus strains. In the most susceptible species, the cottontopped marmoset (*Saguinus oedipus*), both group A and B virus strains induce fatal T-cell lymphomas in 100% of the animals. In common marmosets, however, group A strains (11 and OMI) cause tumors but group B strains (SMHI and S295C) do not (13, 14, 22). The in vivo oncogenic potential of the different herpesvirus saimiri isolates classified into group N has not been tested. The possible function coded for by the rightmost 7 kb of the L DNA of herpesvirus saimiri is not known. As we have shown before, the only transcripts from this region in tumor cells (1670 and 70N2) are several small poly(A)$^-$ RNAs (P. Medveczky and C. Mulder, unpublished data). It was reported that four of these RNAs map between 94 and 100 map units within the variable right-end region of strain 11 L DNA (17). Preliminary data from our laboratory indicate that similar small RNA molecules can also be detected in cells from a group B-induced tumor cell line (J. Wrobel, P. Medveczky, and C. Mulder, unpublished data) and a lymphoblastoid cell line immortalized by herpesvirus ateles (D. DeGrand and C. Mulder, unpublished data).

Cell lines derived from tumors induced by herpesvirus saimiri in vivo (1670, 70N2, and 7710), as well as H1591 cells immortalized in vitro by herpesvirus saimiri, contain multiple copies of covalently closed circular viral DNA. Analysis of these viral episomes revealed rearrangements and large deletions within the L DNA region; however, the leftmost and rightmost 15-kb DNA region is always preserved in the tumor cell lines, suggesting that these DNA sequences are required for maintenance of the transformed state (1, 11, 21).

Until recently, the lack of a reliable in vitro immortalization system for herpesvirus saimiri was a major obstacle for progress in this field. The ability to immortalize cells in vitro would greatly facilitate studies of the events involved in the immortalization process, and it may help to elucidate the connection between cell immortalization and oncogenic transformation.

Comparison of the in vitro immortalizing ability of different herpesvirus saimiri isolates belonging to groups A, B, and N and characterization of the resulting cell lines might reveal some basic differences between the cells immortalized by representatives of the three groups and therefore could

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provide insight into the role of the hypervariable right-terminal sequences in oncogenesis.

In the past, numerous attempts have failed to immortalize in vitro peripheral blood leukocytes (PBL) of several primate species which are susceptible to herpesvirus saimiri infection in vivo (cottontopped marmoset, owl monkey, and spider monkey), although one attempt with cottontopped marmoset PBL and strain OMI has resulted in a continuously growing cell line, H1591 (21), and immortalization of phytohemagglutinin (PHA)-activated, interleukin 2 (IL-2)-expanded owl monkey PBL by strain S295C has also been reported (18). Recently, a more efficient in vitro immortalization system has been reported: infection of fresh PBL with herpesvirus saimiri (8). This technique appears to apply only to PBL from some common marmosets. We modified this system for our studies to compare the in vitro immortalizing abilities of different herpesvirus saimiri isolates representing groups A, B, and N and two attenuated strains.

In this study, we show that virus strains belonging to all three subgroups of herpesvirus saimiri (groups A, B, and N) can immortalize PBL from some common marmosets. Virus strains belonging to groups A and N scored reproducibly positive in this in vitro immortalization assay; the frequency of successful immortalization with the group B strain, SMHI, however, was much lower. Furthermore, cell lines immortalized by group A and N viruses did not need IL-2 in the medium for rapid growth, whereas the only cell line analyzed which was immortalized by a group B virus did require IL-2 for optimal growth. All in vitro-immortalized cell lines contained covalently closed circular viral DNA sequences, which could be detected as early as 3 weeks after infection. Episomal herpesvirus saimiri molecules were not detected in cells infected but not immortalized with the virus.

MATERIALS AND METHODS

Viruses and cell culture. Viruses were propagated on owl monkey kidney (OMK) cell monolayers in Dulbecco modified minimal essential medium supplemented with 10% fetal calf serum and 15 μg of gentamicin per ml (DMEM) at 37°C in air–5% CO_2. Strains SMHI and SMHI-att were obtained from M. D. Daniel, strains 11 and 11-att were from B. Fleckenstein, and strains 484-77 and 487-77 were obtained in our laboratory by transfecting OMK cells with 484-77 and 487-77 DNAs (gifts of R. C. Desrosiers).

For immortalization assays, PBL were isolated on Ficoll (Ficoll-Paque; Pharmacia Fine Chemicals, Piscataway, N.J.) from 4 ml of heparinized common marmoset blood and 4×10^6 cells were suspended in 1 ml of virus suspension in DMEM (10^6 to 10^7 PFU/ml) and incubated for 2 h in air–5% CO_2 at 37°C. Subsequently, 3 ml of RPMI 1640 medium supplemented with 20% fetal bovine serum and gentamicin was added to the cultures, and the cultures were kept in air–5% CO_2 at 37°C in a slightly slanted position. The cultures were fed once weekly with the same medium. In some experiments, Ficoll-separated common marmoset PBL (10^6 cells per ml in RPMI 1640–10% fetal bovine serum plus antibiotic) were activated with 0.5 μg of PHA (Burroughs Wellcome Co., Research Triangle Park, N.C.), and 20 U of recombinant human IL-2 (a gift of S. Reich, Biogen, Cambridge, Mass.) per ml was added to the cultures. The cultures were fed twice weekly with RPMI 1640–10% fetal calf serum–20 U of IL-2 per ml, and the expanded cultures were used for immortalization assays 2 to 3 weeks after PHA activation.

Plasmid. pH24, herpesvirus saimiri strain 11 H units cloned in the vector pAGO (2, 3) was kindly provided by F. Colbère-Garapin.

[^{3}H]TdR incorporation assay. Fifty microliters of RPMI 1640–20% fetal calf serum containing 0.5 μCi of [^{3}H]thymidine (TdR; specific activity, 10 to 20 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to 7×10^4 cells suspended in 50 μl of medium, and the cells were cultured in air–5% CO_2 at 37°C in 96-well plates for 24 h. The samples were precipitated with ice-cold trichloroacetic acid, filtered on GF/C filter disks (Whatman, Inc., Clifton, N.J.), and washed, and the radioactivity was counted in Optifluor solution (Packard Instrument Co., Inc., Rockville, Md.).

Virus production. The production of infectious herpesvirus saimiri by the immortalized cell lines was checked by cocultivation of the lymphoblastoid cells with OMK monolayers in DMEM without additional IL-2 in 16-mm (diameter) wells. Duplicate 10-fold dilutions of cells were checked for cytopathic effect 2 weeks after inoculation.

Gel electrophoresis and blot hybridizations. To detect viral DNA in the immortalized cell lines and to determine the physical state of the viral DNA, we used the method of Gardella et al. (10). Briefly, 10^6 viable cells were washed twice with RPMI 1640 and then suspended in 100 μl of sample buffer containing 20% Ficoll and 0.01% bromphenol blue in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA [pH 8.25]). This cell suspension was loaded on a vertical 0.75% agarose gel made in TBE buffer, 100 μl of 4% Ficoll–2% sodium dodecyl sulfate–0.05% xylene cyanol green–2 mg of pronase solution per ml was carefully layered on top of the cells, and then electrophoresis was started at 0.8 V/cm for 3 h and continued at 6.5 to 8 V/cm for an additional 14 h. The gels were transferred to nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) under partial vacuum (15) and hybridized with ^{32}P-labeled DNA obtained by nick repair (20) with [α-^{32}P]dATP or [α-^{32}P]dCTP from Amersham Corp. (Arlington Heights, Ill.). The conditions for prehybridization, hybridization, rinsing of the nitrocellulose filter, and autoradiography have been described previously (16, 23).

RESULTS

Immortalization of common marmoset PBL. In vitro immortalization experiments were performed in either of two ways. (i) After Ficoll separation, the PBL were immediately infected with herpesvirus saimiri and maintained in growth medium without IL-2 (8). (ii) To increase the amount of marmoset cells available for immortalization, the separated PBL were activated with PHA, expanded in IL-2-containing medium for 2 to 3 weeks (6 to 8 duplications), and then infected with herpesvirus saimiri (18). After infection, the cells were fed with either medium without IL-2 or medium containing 20 U of IL-2 per ml.

Infection of fresh PBL resulted in immortalization of cells from only 1 of 13 animals (Table 1). This animal, 1213, was no longer available for subsequent experiments. PBL from all 12 of the other common marmosets were also infected by using protocol ii. This immortalization protocol, applying short-term IL-2-expanded PBL, was more successful; PBL from 4 of 12 animals tested could be immortalized in vitro with one or more herpesvirus saimiri strains (Table 1). We cannot explain why PBL from only a subset of the monkeys could be immortalized. Immortalization was not affected by the presence or absence of IL-2 after infection. Herpesvirus saimiri infection of common marmoset PBL maintained in

IL-2 for longer than 6 weeks following PHA activation never resulted in establishment of an immortalized cell line. Infection of common marmoset PBL with herpesvirus saimiri usually was followed by a dramatic decrease in the number of viable cells during week 1; however, 3 to 4 weeks postinfection, increased cell growth and clumping were observed in those cultures which later were found to be immortalized.

The results of the in vitro immortalization assays with cells from the five susceptible common marmosets are displayed in Table 1. Herpesvirus saimiri strains 11 (group A), 484-77 (group N), and 487-77 (group N) regularly scored positive in the immortalization assay. Strain SMHI (group B) could immortalize IL-2-expanded common marmoset PBL in only one experiment, when the SMHI-infected culture was maintained in growth medium with no added IL-2. The resulting cell line, 473-SMHI, showed IL-2-independent growth for 7 to 8 weeks postinfection but subsequently grew very poorly in IL-2-free medium. Despite continued dependence on IL-2, the 473-SMHI cell line also had to be regarded as immortalized because it displayed all of the other characteristics of herpesvirus saimiri-immortalized cell lines (ability to grow in culture for more than 6 months, typical cell clumping, and presence of circular viral DNA). Infection of common marmoset PBL with the in vivo nononcogenic attenuated herpesvirus saimiri strain 11-att never led to immortalization of the cells. These results are consistent with data from another laboratory indicating that nononcogenic deletion mutants derived from strain 11 do not immortalize common marmoset PBL (8).

Table 2 shows the characteristics of the cell lines described in this report.

Growth properties of herpesvirus saimiri-immortalized cell lines. The cell lines immortalized in vitro with herpesvirus saimiri were in culture for several months (Table 2); they were maintained in RPMI–20% fetal bovine serum and were independent of external IL-2 for continuous growth, with one exception: cell line 473-SMHI grew very poorly in the absence of IL-2 (see below).

To monitor cell growth in cultures of herpesvirus saimiri-immortalized cell lines, [^{3}H]TdR incorporation was measured in the presence or absence of recombinant human IL-2. Figure 1A shows the results of such an experiment. Cell lines 251-11 (group A immortalized), 251-487, 252-487 (group N immortalized) showed high levels of [^{3}H]TdR incorporation, which were approximately doubled by addition of IL-2. 473-SMHI cells, immortalized by a group B virus, had a very slow rate of DNA synthesis in the absence of IL-2, but addition of IL-2 resulted in a larger than 10-fold increase in [^{3}H]TdR incorporation, while noninfected, PHA-activated PBL from the same animal, 473, responded to IL-2 to a much smaller degree. The IL-2 dose-response curves of 473-SMHI and the group A- and N-immortalized cell lines showed considerable differences. 473-SMHI cells showed only a small increase in the TdR incorporation at low IL-2 concentrations, and the curve was linear until 20 U of IL-2 per ml. In cell line 251-11, however, a large increase in TdR incorporation could be observed at low IL-2 concentrations (e.g., 1 U/ml), and the maximum value was reached at 5 U of IL-2 per ml (Fig. 1B). In separate experiments with the group N virus-immortalized cell line 473-487, curves very similar to those obtained with cell line 251-11 were obtained (data not shown).

TABLE 1. Immortalization of common marmoset PBL in vitro with different strains of herpesvirus saimiri

Animal no.	PHA–IL-2	No. of cultures immortalized/no. infected with strain (group):				
		11 (A)	SMHI (B)	484 (N)	487 (N)	11-att
1213	–[a]	1/1	0/1	1/1	ND[b]	ND
473	+[c]	1/2	1/4	3/6	5/5	0/1
252	+[c]	2/2	0/2	1/2	1/1	0/2
251	+[c]	0/1	0/1	1/1	1/1	0/1
313	+[c]	1/1	0/1	1/1	ND	0/1

[a] No IL-2-expanded PBL were infected.
[b] ND, Not done.
[c] Infected fresh PBL (without IL-2 expansion) did not yield immortalized cells.

TABLE 2. Common marmoset cell lines immortalized in vitro by herpesvirus saimiri

Cell line	PBL from marmoset	PBL expanded in IL-2	Virus strain	Subgroup	No. of mo in culture	Virus production[a]
1213-484	CJ 1213	–	484-77	N	14	+
1213-484	CJ 1213	–	484-77	N	20	–
473-SMHI	CJ 473	+	SMHI	B	7	+
473-487	CJ 473	+	487-77	N	5	+
251-487	CJ 251	+	487-77	N	5	+
252-487	CJ 252	+	487-77	N	5	+
251-11	CJ 251	+	11	A	3	+

[a] Serial dilutions of the cultures were seeded on sparse OMK cell monolayers. Virus production was monitored by cytopathic effects on the OMK cells at day 14 of cocultivation.

The results described above indicate that (i) Cells immortalized by group A and N strains of herpesvirus saimiri could grow well in the absence of exogenous IL-2, and (ii) the group B herpesvirus saimiri-immortalized cell line 473-SMHI showed poor growth in medium lacking IL-2 but did respond to IL-2. The dose-response curves of cell line 473-SMHI and those of cell lines immortalized by group A and N herpesvirus saimiri strains were significantly different.

In each set of in vitro immortalization experiments, we used noninfected PBL from the same animal as a negative control. We were never able to maintain the noninfected cultures for 8 weeks or longer by using conditions identical to those of the infected culture. Therefore, the experiments comparing the response of the 473-SMHI cell line with that of PHA-activated, noninfected common marmoset PBL were performed with PBL obtained more recently from animal 473.

Virus production. To detect the production of infectious virus from immortalized cell lines, cells were seeded on sparse monolayers of OMK cells; OMK cells could support a productive cycle of herpesvirus saimiri. Two well-characterized nonproducer cell lines derived from herpesvirus saimiri-induced tumors, 1670 and 70N2, served as a negative control, and OMK cells infected with herpesvirus saimiri strain 484-77 as a positive control. Cell line 1213-484 (18 months after its establishment) was the only immortalized cell line tested which showed no sign of virus production (Table 2). We knew, however, from earlier experiments that this cell line also produced infectious herpesvirus saimiri for more than 1 year after its establishment (Table 2). It was reported that cell lines derived from herpesvirus saimiri-induced tumors and one in vitro-immortalized cottontopped marmoset cell line converted to the nonproducer state 1 or more years after their establishment, and this change was accompanied by the appearance of large deletions and rearrangements of the viral genome (21). Experi-

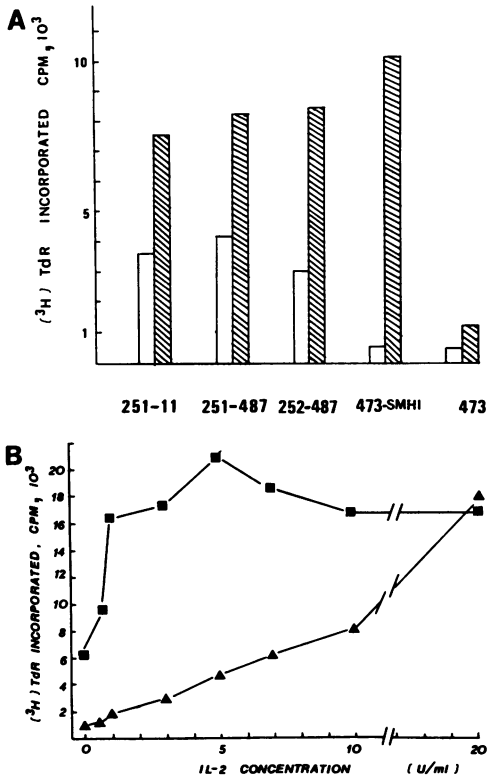

FIG. 1. TdR incorporation of herpesvirus saimiri-immortalized cell lines with and without IL-2. Cells (0.1 ml) were cultured in the presence of [^{3}H]TdR at a cell density of 7×10^5 cells per ml. After 24 h, the cells were precipitated with trichloroacetic acid and filtered on GF/C filters. The results shown here are averages of duplicate samples. (A) The unshaded bars represent [^{3}H]TdR incorporation in RPMI 1640–20% fetal bovine serum; the shaded bars show [^{3}H]TdR incorporation in the same medium supplemented with 50 U of recombinant human IL-2 per ml. The numbers on the abscissa represent the cell lines; 473 represents noninfected PBL of animal 473. (B) Cells were incubated in the presence of various concentrations of IL-2. Symbols: △, cell line 473-SMHI; □, cell line 251-11.

ments are in progress to analyze the viral genomes in 1213-484 cells. All of the other herpesvirus saimiri-immortalized cell lines were virus producers (Table 2), although the degree of virus production varied.

Viral DNA in herpesvirus saimiri-immortalized cell lines. To detect viral DNA in the immortalized cell lines and to determine the physical state of the viral sequences, we used the agarose gel method of Gardella et al. (10), since this method allowed us to differentiate between the covalently closed circular and linear genomes. One million cells were applied to each well of the gel; after electrophoresis, the DNA in the Gardella gels was transferred to nitrocellulose filters and hybridized with pH24 (cloned H DNA of herpesvirus saimiri strain 11). All of the herpesvirus saimiri-immortalized cell lines tested (Fig. 2A, lanes 1 through 6) contained a band migrating at the position of covalently closed circular DNA (arrow). The intensity of hybridization of the episomal DNA bands was slightly different in different cell lines. Comparing them with control lanes with linear viral DNA corresponding to 0.1, 1, and 10 copies per cell (Fig. 2A, lanes 7, 8 and 9), we estimated that the cell lines contained between 1 and 10 copies of covalently closed circular herpesvirus saimiri DNA. Hybridization of the same nitrocellulose filters with cloned *Kpn* E fragment of strain 11 as a probe gave an essentially identical picture (data not shown), indicating that the episomes contained approximately the same amount of repetitive H DNA as did the linear viral genome.

To test whether the episomal viral DNA could be observed in cells infected, but not immortalized by herpesvirus saimiri, we infected PHA-activated, short-term IL-2-expanded common marmoset PBL from two different animals:marmoset 473, cells of which had previously been used successfully for immortalization, and marmoset 8,

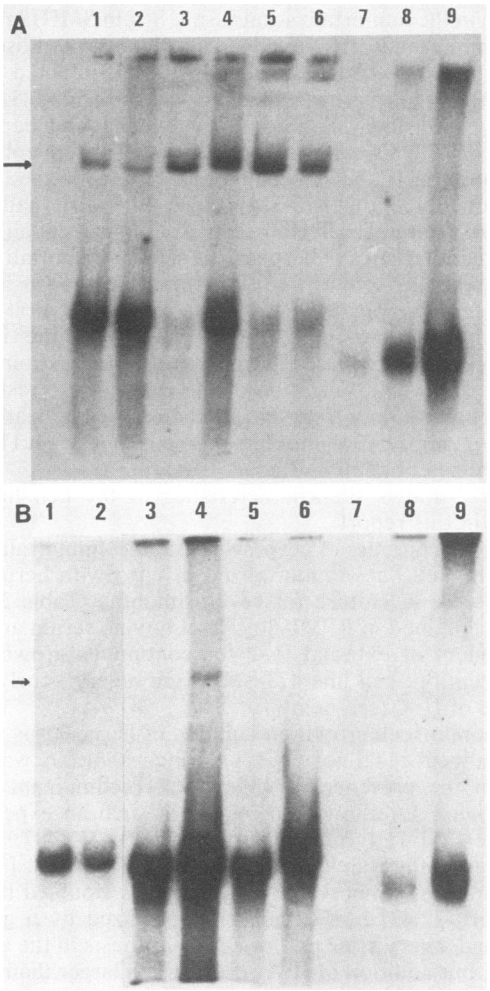

FIG. 2. Viral DNA in cell lines immortalized in vitro with herpesvirus saimiri. Cells (10^6) were washed twice with RPMI 1640, suspended in 100 μl of sample buffer and subjected to electrophoresis through a 0.75% agarose Gardella gel (8). The DNA in the gel was transferred to a nitrocellulose filter, which was subsequently hybridized with [^{32}P]-labeled pH24 DNA (cloned herpesvirus saimiri H DNA). (A) Lanes: 1, cell line 473-SMHI; 2, 251-11; 3, 1213-484; 4, 473-487; 5, 251-487; 6, 252-487; 7, 8, and 9, strain 484-77 M DNA equivalent to 0.1, 1, and 10 copies per cell, respectively. (B) Cells were analyzed 1 week (lanes 1 and 2) and 3 weeks (lanes 3 to 6) after infection with the strains indicated. Lanes: 1, PBL of animal 473 infected with strain 484; 2, PBL of animal 8 infected with strain SMHI; 3, PBL of animal 473 infected with strain SMHI; 4, PBL of animal 473 infected with strain 484-77; 5, PBL of animal 8 infected with strain SMHI; 6, PBL of animal 8 infected with strain 484-77; 7 to 9, strain 484-77 M DNA equivalent to 0.1, 1, and 10 copies per cell, respectively. The arrow indicates the position of circular viral DNA on the gel.

which had not been susceptible. Cells from both monkeys were infected with strain 484-77 (group N), which was a strongly immortalizing strain, and strain SMHI (group B), which usually gave negative results. The cells were kept in IL-2-containing medium and checked weekly on Gardella gel and hybridized with the herpesvirus saimiri H DNA-specific probe pH24. No circular viral DNA could be detected 1 and 2 weeks after infection, although the method was sensitive enough to detect 0.1 copy of viral DNA per cell. Three weeks following infection, however, a band could be detected at the position of covalently closed circular viral DNA in 473 cells infected with strain 484-77 (Fig. 2B, lane 4) but not in any of the other cells. Figure 2B, lanes 5 to 7, contained linear viral DNA equivalent to 0.1, 1, and 10 copies per cell, respectively, which allowed us to quantitate the amount of circular DNA detected as 1 to 2 copies per cell. Four weeks after infection, 473 cells infected with 484-77 virus showed increased cell growth and clumping, and the cells were able to grow in the absence of IL-2. The 473 cells infected with SMHI or mock-infected and the animal 8 cells infected with any of the virus strains gradually stopped replicating and subsequently died.

This experiment indicated that episomal viral DNA could be observed only in immortalized cells and could first be detected in these cells 3 weeks postinfection. It is not known at this time whether most of the cells contained one copy of circular viral DNA per cell or whether only a small part of the cell population harbored a larger amount of the herpesvirus saimiri episomes.

In the course of our studies, cell lines immortalized with herpesvirus saimiri and common marmoset PBL infected with herpesvirus saimiri and maintained for a short time (maximum, 2 months) in IL-2 were frequently tested for episomal viral DNA by the Gardella gel method. We could always detect the presence of covalently closed circular DNA in the immortalized cells; however, we never observed viral episomal DNA in herpesvirus saimiri-infected cells which did not show signs of immortalization.

DISCUSSION

Herpesvirus saimiri isolates classified into groups A, B, and N have DNA sequences lacking any intergroup homology within the DNA region required for oncogenicity (16). Both group A and B strains are highly oncogenic in the most susceptible species, the cottontopped marmoset. However, in another New World primate, the common marmoset, group A strains cause fatal lymphomas but group B strains are reported not to be oncogenic (13, 14, 22).

This study was undertaken to (i) compare the abilities of different herpesvirus saimiri strains to immortalize common marmoset PBL in vitro and (ii) analyze different properties of the resulting cell lines to test whether there are basic differences between group A, B, and N isolates which could indicate functions coded by the rightmost 7-kb sequences.

In this report, we showed that viruses belonging to all three groups (A, B, and N) could immortalize common marmoset PBL in vitro. Previously it has been reported that nononcogenic, attenuated strains, as well as group B virus strains, do not immortalize PBL in vitro (8). We found that PBL from 5 of 13 common marmosets could be reproducibly immortalized with group A and N isolates. Strain SMHI (group B) immortalized PBL of 1 of 13 animals, and on only one occasion. Group A virus strains were reported to cause lymphomas in 100% of infected common marmosets (22), but group B virus strains have never been found to be oncogenic in this species (13, 14). Since the numbers of animals infected with group A strains was very low and no in vivo studies were done with group N strains, direct comparison was not possible between the oncogenicity in vivo and the immortalization ability in vitro of the different strains. Our results and the data available in the literature suggested, however, that the frequency of immortalization of common marmoset PBL in vitro and the induction of lymphoma in the same species in vivo was much higher with herpesvirus saimiri strains of group A than with strains of group B.

473-SMHI, the only cell line immortalized by a group B strain, differed from other group A and N herpesvirus saimiri-immortalized cell lines in two respects: (i) It grew poorly in growth medium without IL-2 but had an enhanced response to exogenous IL-2. (ii) A higher dose of IL-2 (20 U/ml) was necessary to achieve maximal TdR incorporation in 473-SMHI than in the other cell lines tested, which responded to IL-2 at low concentrations. Since 473-SMHI cells had been cultured previously for several months, in growth medium containing IL-2, this decreased response to IL-2 could be due to desensitization of the IL-2 receptors of the 473-SMHI cells. Although cell line 473-SMHI did not show IL-2-independent growth, we consider it to be herpesvirus saimiri immortalized, because (i) control cell cultures similarly activated with PHA, expanded in IL-2, but not infected with herpesvirus saimiri never lived longer than 6 to 8 weeks, whereas 473-SMHI cells were kept in culture for 14 months; and (ii) 473-SMHI cells, similar to all other herpesvirus saimiri-immortalized cells and herpesvirus saimiri-induced tumor cell lines, contain episomal viral genomes.

It has been reported that owl monkey PBL cells immortalized in vitro with another herpesvirus saimiri group B virus strain, S295C, grew poorly without exogenously added IL-2 (18). Cell line 7710, derived from tumors induced by group B strain S295C in New Zealand White rabbits, also showed IL-2 dependency, even after long in vitro passage. However, inoculation of inbred rabbits with these IL-2-dependent 7710 cells caused fatal lymphomas in the animals, indicating that the cells were oncogenic despite their IL-2 dependence (1).

All herpesvirus saimiri-immortalized cell lines analyzed in our study carried covalently closed circular viral DNA. The amount of these episomal DNA molecules varied from 1 to 10 copies per cell in the different cell lines immortalized by herpesvirus saimiri. However, in ongoing infections not leading to immortalization, no covalently closed circular viral DNA was found. This suggests that the occurrence of viral episomes is essential for the oncogenic process and immortalization.

In a comparison of the two methods for in vitro immortalization used in our experiments, the assay using PHA-activated, IL-2-expanded PBL offers several advantages: (i) PBL of a larger subpopulation of common marmosets can be used successfully, (ii) the number of cells available for experiment is higher, and (iii) PBL of certain other New World monkey species can also be immortalized with herpesvirus saimiri. By use of PHA-activated IL-2-expanded PBL, owl monkey cell lines have been established by strain S295C (18), and recently, cottontopped marmoset cell lines have been immortalized with strains 11 (group A) and 484-77 (group N) (D. DeGrand and C. Mulder, unpublished data), although previous attempts to immortalize freshly isolated PBL from the same species had failed repeatedly, with one exception (21).

The hypervariable region of herpesvirus saimiri supposedly specifies important functions influencing the oncogenic

process. Characterization of cell lines immortalized in vitro by herpesvirus saimiri strains of different groups may be a valuable tool in elucidating these functions.

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