Oncogenicity in Marmosets of HL-23V, a Type C Oncornavirus Isolated From Human Leukemic Cells, and Comparison With Simian Sarcoma Virus Type 1 (SSV-1/SSA V-1)1,2

C. M. Bergholz,3 L. G. Wolfe,3,4 F. Deinhardt,3,4 B. Thakkar,3 and B. Marczynska3,4

ABSTRACT—Type C virus produced by dog thymus cells (A7573) that were infected with virus (HL-23V), isolated from cultured leukocytes of an acute myelogenous leukemia patient, transformed marmoset and horse cells in vitro and induced virus-producing fibromas in marmosets. The tumors and transformed foci were indistinguishable morphologically from those induced by simian sarcoma virus, type 1 (SSV-1/SSA V-1). HL-23V was indistinguishable from SSV-1/SSA V-1 by immunofluorescence and neutralization tests, and the nontransforming virus associated with HL-23V completely inhibited SSV-1 focus induction in interference tests. Cell cultures established from a marmoset fibroma produced transforming and nontransforming virus biologically and antigenically indistinguishable from HL-23V and SSV-1/SSA V-1.—J Natl Cancer Inst 58: 1041-1046, 1977.

Oncornaviruses of type C morphology cause leukemias, lymphomas, or sarcomas in several vertebrates and have been isolated from many different species (1, 2), including nonhuman primates (3-8). Although an etiologic association between oncornaviruses and human cancer has not been proved, high molecular weight RNA (9-14), reverse transcriptase (9-19), and viral structural proteins (20) similar or identical to those of known mammalian oncornaviruses, particularly the SSV-1/SSA V-1 complex (12-14, 16, 18-24), have been demonstrated in human malignant tissue. Isolation of type C virus from cultures of human tumor cells (21-23, 25, 26) and normal embryonic cells (24) has been described. A type C virus (HL-23V) was isolated from cultured peripheral leukocytes and bone marrow cells of an AML patient by Gallagher et al. (21). Previous studies showed that the reverse transcriptase (21) and the p30 (20), p12, and gp 71 (27) structural proteins of HL-23V were closely related or identical to those of SSV-1 and SSA V-1, both isolated from a naturally occurring fibrosarcoma of a woolly monkey (4). In addition, it was found that HL-23V-infected cell lines contained a second type C virus, indistinguishable from BaEV (28, 29). Examination of uncultured leukemic blood cells and postmortem tissue from patient HL-23 revealed RNA sequences in cytoplasmic virus-like particles homologous to SSV-1/SSA V-1 and BaEV (30). Identification of BaEV-related DNA proviral sequences in HL-23 spleen (30), as well as peripheral blood cells of 7 other leukemia patients (31), was also reported, but attempts to demonstrate SSV-1/SSA V-1 proviral DNA in HL-23 tissues have been unsuccessful. The studies reported here demonstrate that HL-23V-infected cell lines, like SSV-1/SSA V-1-infected cell lines, produce both transforming and nontransforming virus antigenically indistinguishable from SSV-1/SSA V-1 and induce tumors in marmoset monkeys.

MATERIALS AND METHODS

Cells.—Dog thymus cells (FC12th-A7573; Naval Biomedical Research Laboratory, Oakland, Calif.) and horse skin cells (R-1042; supplied by Dr. J. Rhim, Microbiological Associates, Bethesda, Md.) were maintained in Eagle's basal medium (Gibco, Grand Island, N.Y.) improved with nonessential amino acids and supplemented with 10% (heat-inactivated) FCS (Reheis Chemical Co., Phoenix, Ariz.). Marmoset skin (HF) and lung (1283) cell lines were maintained in RPMI-1640 (Gibco) with 10% FCS. We established the marmoset tumor cell culture by mincing excised tissue and explanting 1 mm³ pieces in 25-cm² flasks with 2 ml of RPMI-1640 supplemented with 20% FCS. When sufficient outgrowth occurred, explant cultures were treated with 0.25% trypsin-EDTA and plated in new flasks.

Virus.—A dog thymus cell line infected with virus produced by HL-23 AML cells in culture (28) was supplied by Dr. R. Gallo (National Cancer Institute, Bethesda, Md.) and was the source of HL-23V. This cell line, designated A7573(HL-23V), and other HL-23V-infected cultures were handled and incubated in a laboratory remote from other oncornavirus work, and all possible precautions were taken in use of media and materials to avoid contamination. An SSV-1-transformed marmoset fibroblastic cell line, HF(SSV-1/SSA V-1), was the source of SSV-1/SSA V-1. Virus-producing cells were plated at 1-2×10⁶ cells/flask (75 cm²) on day 1. Supernatants were collected on day 4, passed through a 0.45 µm Millipore filter (Millipore Corp., Bedford, Mass.), and stored at -70°C.

Animals.—Four white-lipped marmosets (Saguinus fuscicollis nigrifrons, S. f. illigeri), born in our breeding colony and hand-reared by established procedures, were inoculated when 3-5 days old im or ip with 3-7×10⁶ cells of A7573(HL-23V).

Focus assay.—Normal marmoset 1283 cells were seeded at 5×10⁵ cells/25-cm² flask and on the following day were treated with DEAE-dextran (20 µg/ml) for 1 hour, washed twice with HBSS, and infected with 0.5 ml of virus inoculum. Virus was adsorbed for 90 minutes at 37°C. Foci were counted 14 days post infection.

XC test.—For assay of nontransforming virus, normal marmoset cells were inoculated with tenfold dilutions of

ABBREVIATIONS USED: SSV-1 = simian sarcoma virus, type 1; SSA V-1 = simian sarcoma-associated virus, type 1; AML = acute myelogenous leukemia; BaEV = baboon endogenous virus; FCS = fetal calf serum; HBSS = Hanks' balanced salt solution; PBS = phosphate-buffered saline; MC = mixed culture cytopathogenicity; PI = post inoculation; FFU = focus-forming units; GAL V = gibbon ape lymphoma virus; FeSV = feline sarcoma virus.

1 Received June 21, 1976; accepted September 27, 1976.
2 Supported by Public Health Service contract NO1 CP39219 from the Division of Cancer Cause and Prevention, National Cancer Institute.
3 Department of Microbiology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Ill. 60612.
4 Department of Microbiology, University of Illinois Medical Center, Chicago, Ill. 60612.
virus as described above. Cultures infected beyond the end point of focus induction were subcultured twice. A mixed culture cytopathogenic test with XC cells was performed as described by Rangan et al. (32). The titer is expressed as the reciprocal of the highest dilution that induced syncytia (≥8 nuclei/syncytium).

Indirect immunofluorescence.—Acetone-fixed cell monolayers were treated with marmoset plasma diluted with 2.5% bovine serum albumin in PBS for 30 minutes at 37°C. After two washes with PBS, monolayers were reacted with fluorescein-conjugated goat antihuman IgG (Hyland Laboratories, Los Angeles, Calif.) for 30 minutes at 37°C, washed two times, and mounted.

Neutralization tests.—Twofold dilutions of serum or plasma in a final volume of 0.6 ml were incubated at 4°C for 4 hours with 0.6 ml virus at a dilution calculated to produce 50 foci/flask. Dilutions were made with HBSS containing 2% agammaglobulin FCS. Neutralization of focus-forming virus was determined by focus assay.

Chromosome preparations.—Chromosomes were prepared according to the method of Moorhead and Nowell (33). Slides were stained with Giemsa diluted in Sorenson's buffer at pH 6.8-7.4 at a concentration of one Pasteur pipette drop of dye to 1 ml of buffer. Mitoses arrested in metaphase were photographed with a Zeiss photomicroscope on Kodak high-contrast copy film. Prints were made on 8x10-inch paper. Prints of chromosomes from the best mitoses were cut out, paired, and analyzed.

RESULTS

Filtered cellfree supernatants of a dog thymus cell line infected with HL-23V [A7573(HL-23V)] were tested for transforming virus by inoculation of partially confluent monolayers of marmoset skin (HF) or fetal lung (1283) cells, either untreated or pretreated for 1 hour with 20 μg/ml of DEAE-dextran according to procedures routinely used for SSV-I focus assays (34). Foci of transformed cells appeared within 5-7 days in pretreated cultures, whereas no foci appeared in untreated cultures within 21 days. The foci of transformed cells consisted of densely packed fibroblast-like cells and were indistinguishable from those induced by SSV-I (figs. 1, 2). Infection with cellfree A7573(HL-23V) supernatants also induced transformation in 14 additional marmoset fibroblastic cell lines derived from adult skin or fetal tissues (Johnson L: Personal communication), horse skin cells (R-1042), and rat kidney cells (NRK; Ting RC: Personal communication). The morphology of A7573 cells infected with either HL-23V or SSV-1/SSAV-1 was indistinguishable from that of uninfected cells.

HL-23V virus pools were tested for the presence of excess nontransforming associated virus by the MC test with XC cells (table 1). Normal marmoset cells infected with HL-23V at 10- to 100-fold dilutions beyond the end point of focus induction were positive for syncytial formation in the MC test; this indicated that HL-23V stocks, like SSV-1 (34), contained a 10- to 100-fold excess of nontransforming virus. Our results (table 1) indicated that dog thymus cells chronically infected with HL-23V and marmoset cells transformed by HL-23V produce levels of both transforming and nontransforming virus comparable to those of SSV-1/SSAV-1-infected cell lines. Normal dog thymus cells infected with SSV-1-induced syncytia with XC cells but did not produce detectable transforming virus; this suggested that transforming HL-23V did not arise by rescue of a sarcoma genome present in normal dog thymus cells. Karyotype analyses of A7573(HL-23V) and A7573 cells received from Dr. Gallo confirmed their canine origin.

Interference between HL-23V and SSV-1 was tested by injection of normal marmoset cells with tenfold dilutions of HL-23V, subculture of focus-free cultures, and then challenge of the subcultures with SSV-1 or HL-23V. SSV-1 and HL-23V focus formation was inhibited in cultures infected with HL-23V at 10- and 100-fold dilutions beyond the focus end point (table 2). Parallel cultures infected with HL-23V and not challenged were positive for syncytial formation in the MC test and for type C virus by electron microscopy through dilutions 100-fold beyond the focus end point, corresponding to the interference end point. Cultures infected with virus at greater than 100-fold dilutions beyond the focus end point were negative by all three tests.

**Table 1.**—Titers of transforming and nontransforming virus produced by cell lines chronically infected with HL-23V or SSV-1/SSAV-1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Designation</th>
<th>Virus</th>
<th>Transforming virus</th>
<th>Non-transforming virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog thymus</td>
<td>A7573</td>
<td>HL-23V</td>
<td>3×10⁵</td>
<td>10⁴</td>
</tr>
<tr>
<td>Marmoset skin</td>
<td>HF</td>
<td>SSV-1/SSAV-1</td>
<td>7×10⁵</td>
<td>NT</td>
</tr>
<tr>
<td>Marmoset lung</td>
<td>1283</td>
<td>SSV-1</td>
<td>1×10⁴</td>
<td>10⁹</td>
</tr>
<tr>
<td>Horse skin</td>
<td>R-1042</td>
<td>HL-23V</td>
<td>6×10⁴</td>
<td>10⁴</td>
</tr>
</tbody>
</table>

* FFU/ml assayed on normal marmoset 1283 lung cells.
* The titer is expressed as the reciprocal of the highest dilution of virus that induced syncytia (≥8 nuclei/syncytium) in an MC test with XC cells.
* Not tested.

**Table 2.**—Demonstration of nontransforming, interfering HL-23V beyond focus end point

<table>
<thead>
<tr>
<th>Virus dilution</th>
<th>FFU/ml</th>
<th>XC test</th>
<th>Electron microscopy</th>
<th>Challenge virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>HL-23V</td>
</tr>
<tr>
<td>0</td>
<td>TMTCC</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>-1</td>
<td>135</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>-2</td>
<td>33</td>
<td>+</td>
<td>+</td>
<td>NT</td>
</tr>
<tr>
<td>-3</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>-4</td>
<td>0</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>-5</td>
<td>0</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>-6</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>43</td>
</tr>
<tr>
<td>Uninfected</td>
<td></td>
<td>-</td>
<td>-</td>
<td>57</td>
</tr>
</tbody>
</table>

* Normal marmoset cells were infected with tenfold dilution of HL-23V according to the method described for focus assay. Focus-free cultures were subcultured and examined for presence of nontransforming virus by electron microscopy, XC test, and challenge with transforming virus.
* FFU/ml.
* Too many to count.
Oncogenicity of HL-23V was investigated by inoculation of 4 newborn marmosets with 3–7×10^7 A7573(HL-23V) cells (table 3). Of 3 marmosets inoculated im, 2 developed palpable tumors by 4 weeks PI; the tumor in 1 animal had regressed completely by 6 weeks, and the tumor in the other animal, 75-BF-1, was partially excised 6 weeks PI when undergoing regression. After surgery, the residual mass slowly enlarged for 12 weeks and then regressed; a small mass was still present 28 weeks PI when the animal died of acute pneumonia. Both tumor specimens from 75-BF-1 were fibromas composed of fibroblasts, abundant collagen, and some lymphoid cells; virus particles budding from fibroblasts and extracellular type C virus were observed by electron microscopy. Total and differential leukocyte counts, monitored monthly in all 4 animals, remained normal throughout the observation period. Two animals that never developed detectable tumors and the 1 animal that developed a regressor tumor were clinically normal 10 months PI. The behavior and morphology of HL-23V-induced marmoset tumors were similar or identical to those of SSV-1-induced tumors (4–6). All 4 marmosets developed antibodies that reacted with HL-23V antigens in indirect immunofluorescence tests (table 2). Peak antibody titers were highest, 1:4,096, in the animal with the prolonged clinical course (75-BF-1).

Cell cultures were established from 75-BF-1 tumor tissue obtained at biopsy and autopsy; they were identified as marmoset cells by karyotype analysis. The morphology and growth pattern of the cultures closely resembled those of the cells transformed in vitro by HL-23V or SSV-1 (34); budding particles and extracellular type C virus were readily apparent by electron microscopy. Filtered supernatant, collected from passage 7 of the biopsy culture and from passage 2 of the autopsy culture, contained 1×10^4 and 1×10^3 FFU/ml respectively, of a transforming virus and a tenfold excess of nontransforming virus. Nonidet P-40 disrupted virus from cultured tumor cells reacted with goat anti-SSV-1/SSAV-1 p30 in an immunodiffusion test with a line of complete identity; p30 antigen indistinguishable from SSV-1/SSAV-1 p30 was identified in 75-BF-1(HL-23V) cultured cells by competition radioimmunoassay, but no BaEV p30 was detected by this technique (Parks W: Personal communication).

HL-23V and SSV-1/SSAV-1 were compared by titration of marmoset anti-SSV-1/SSAV-1 serum and anti-HL-23V plasma by the indirect immunofluorescence technique, with the use of SSV-1/SSAV-1- and HL-23V-infected cell monolayers (table 4). The anti-SSV-1 serum and HL-23V plasma gave the same end points whether titered on SSV-1SSAV-1- or HL-23V-infected marmoset skin or dog thymus cells.

The antigenic properties of HL-23V produced by dog thymus cells (A7573) or the marmoset tumor cell culture (75-BF-1) were compared with those of SSV-1 by neutralization tests (table 5). Marmoset SSV-1/SSAV-1 and GALV antisera neutralized SSV-1 and HL-23V equally well, whereas a marmoset antiserum to FeSV (Snyder-Theilen strain) failed to neutralize either virus. HL-23V produced by 75-BF-1 cells at passage 4 was neutralized by the goat anti-SSV-1/SSAV-1 serum as effectively as SSV-1, and the neutralizing titer of the marmoset antiserum was expressed as the reciprocal of the highest plasma dilution with which virus-specific cytoplasmic fluorescence was observed.

Table 4.—Antigenic comparison of HL-23V and SSV-1/SSAV-1 by immunofluorescence tests

<table>
<thead>
<tr>
<th>Cells(virus)</th>
<th>Anti-HL-23V</th>
<th>Anti-SSV-1/SSAV-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF(SSV-1/SSAV-1)</td>
<td>512</td>
<td>64</td>
</tr>
<tr>
<td>HF(HL-23V)</td>
<td>512</td>
<td>64</td>
</tr>
<tr>
<td>A7573(HL-23V)</td>
<td>512</td>
<td>NT</td>
</tr>
<tr>
<td>HF(FeSV)</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

* For description of cell lines, see table 1.
* The titer is expressed as the reciprocal of the highest dilution of serum or plasma showing virus-specific fluorescence in an indirect immunofluorescence assay. NT = not tested.
* Sera from 3 marmosets with SSV-1-induced tumors.

Table 5.—Comparison of HL-23V and SSV-1 in neutralization tests

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Cells(virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HF(SSV-1)</td>
<td>256</td>
</tr>
<tr>
<td>A7573-(HL-23V)</td>
<td>256</td>
</tr>
<tr>
<td>75-BF-1(HL-23V)</td>
<td>NT</td>
</tr>
</tbody>
</table>

* For description of cell lines, see table 1.
* Neutralization titer, expressed as the reciprocal of the serum dilution producing ≥50% inhibition of focus formation relative to virus incubated without test serum. Data shown for each serum represent results obtained in one representative experiment with the use of the viruses indicated. All assays were repeated two to four times with similar results.
* GALV antiserum.
* Marmoset 75-BF-1 plasma, 2 mo PI.
* Antiserum to FeSV (Snyder-Theilen strain); neutralization titer, 1:128.

Table 3.—Antibody response and tumor induction in marmosets inoculated with A7573(HL-23V) cells

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Number of cells inoculated</th>
<th>Route of inoculation</th>
<th>Antibody titer, mo PI</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>75-BF-1</td>
<td>3×10^7</td>
<td>im</td>
<td>32 512 4,096 2,048 512 1,024</td>
<td>+</td>
</tr>
<tr>
<td>75-BC-1</td>
<td>3×10^7</td>
<td>ip</td>
<td>NT&lt;4 256 128 128 64</td>
<td>-</td>
</tr>
<tr>
<td>75-CN-1</td>
<td>7×10^7</td>
<td>ip</td>
<td>NT&lt;4 256 128 128 64</td>
<td>+</td>
</tr>
<tr>
<td>75-CP-1</td>
<td>7×10^7</td>
<td>im</td>
<td>&lt;4 256 128 128 64 128</td>
<td>-</td>
</tr>
</tbody>
</table>

* The titer is expressed as the reciprocal of the highest plasma dilution with which virus-specific cytoplasmic fluorescence was observed.
* Not tested.
plasma (75-BF-1) was the same against SSV-1 or HL-23V produced by 75-BF-1 tumor cells and was only twofold lower against HL-23V produced by dog thymus cells. In addition, we observed the appearance of 2–8 foci when HL-23V produced by dog thymus or marmoset cells was reacted with SSV-1/SSAV-1 antisera or anti-HL-23V marmoset plasma at low dilutions. The breakthrough of focus-forming virus at low serum dilutions was never observed in neutralization tests with SSV-1/SSAV-1.

DISCUSSION

Studies reported here demonstrated that HL-23V-infected cells produced not only SSAV-1, as reported previously (28, 29), but also the sarcomagenic component of the simian sarcoma virus complex. HL-23V readily transformed marmoset cells in vitro and the transformed cell lines produced levels of transforming and nontransforming virus comparable to those produced by the SSV-1-transformed marmoset cell lines. The HL-23V-transforming activity, independently confirmed in another laboratory (Ting RC: Personal communication), was detected when first tested in our laboratory 1 week after receipt of the A7573(HL-23V) cell line and was reproducible with A7573(HL-23V) frozen culture fluids stored at that time, as well as with fresh culture fluids. In contrast to our findings, Teich et al. (28) did not report transformation of marmoset cells by HL-23V and, furthermore, cited the poor growth of HL-23V in marmoset cells, as measured by XC plaque assay, as evidence for the nonidentity of HL-23V and SSAV-1. To the best of our knowledge, the protocol for assay of focus-forming virus, as first described by this laboratory (34), was not performed in that study.

HL-23V-infected dog thymus cells, like SSV-1-transformed marmoset cells, induced fibromas when inoculated into marmosets, and the tumor cells established in culture produced virus indistinguishable from SSV-1/SSAV-1 and HL-23V grown in dog thymus cells. Tumor induction in marmosets by inoculation of cellfree preparations of HL-23V was not attempted due to the low levels of transforming virus produced by HL-23V-infected cells and to the difficulty experienced in the demonstration of oncogenicity in marmosets with cellfree SSV-1 previously, where only 1 of 6 inoculated monkeys developed tumors (5). Because HL-23V produced directly by the human myeloblasts was not available, it was impossible to test the oncogenicity of such preparations. Induction of leukemia in marmosets by SSAV-1 has not been tested directly; however, leukemia has never been observed in monkeys inoculated with cellfree SSV-1/SSAV-1 or cells infected with SSV-1/SSAV-1 or HL-23V, both of which produce excess nontransforming associated virus.

Though studies reported previously demonstrated the presence of a BaEV component in A7573 cells infected with HL-23V (28, 29), no BaEV p30 was detected in the cultured marmoset tumor cells, and the tumor cells did not induce syncytial formation in MC tests with KB cells. This finding does not eliminate the possibility of BaEV information in HL-23V-infected marmoset cells; however, attempts to infect marmoset cells in vivo and in vitro with BaEV were unsuccessful in previous studies performed in our laboratory (unpublished data).

Neutralization and interference tests, in which inhibition of HL-23V focus induction was measured, failed to distinguish between HL-23V and SSV-1, in agreement with results reported by Teich et al. (28) who used an HL-23V pseudotype of murine sarcoma virus. The twofold differences of serum antibody titers observed in some neutralization tests are generally not considered significant, and though these tests were repeated several times with similar results, it is doubtful that they indicate true antigenic differences between SSV-1/SSAV-1 and HL-23V. We are, however, investigating the possibility that the few transforming virus particles not neutralized by low dilutions of high-titered anti-HL-23V plasma or anti-SSV-1 serum may represent hybrid viruses, i.e., a phenotypic mixture of hybrid viruses, resulting from interaction of HL-23V and another virus, possibly the BaEV component detected previously in HL-23V preparations.

Inasmuch as our results agreed with earlier reports of the genetic and antigenic identity of HL-23V and SSV-1/SSAV-1 (27, 29) and, in addition, demonstrated that the biologic and oncogenic properties of HL-23V and SSV-1/SSAV-1 were indistinguishable, the question of the true origin of these viruses remains open to further experimental analysis and speculation. Viral contamination of the AML cells in culture is a possibility, and a difficult one to disprove. The association of both sarcomagenic (SSV-1/SSAV-1) and endogenous (BaEV) viruses with a human leukemia is surprising, however, because neither of these viruses has been associated with leukemias in their natural or experimental hosts. Nevertheless, it is of interest that the isolation of both BaEV- and SSV-1-related viruses from human embryonic tissues was reported recently by Panem et al. (26, 35), and nucleic acid sequences related to these viruses have been demonstrated in uncultured leukocytes and postmortem spleen tissues of patient HL-23V (30). But even if it can be confirmed that these viruses are really present in the human population, the role of SSV-1, SSAV-1, or BaEV-related viruses in human neoplasia has yet to be proved.

REFERENCES

ONCOGENICITY AND TRANSFORMING ACTIVITY OF HL-23V


**FIGURE 1.**—Focal transformation of marmoset skin cells 10 days after infection with SSV-1. Unstained in plastic flask. × 50

**FIGURE 2.**—Focal transformation of marmoset skin cells 7 days after infection with HL-23V. Unstained in plastic flask. × 50