Selective Killing of Simian Virus 40-transformed Human Fibroblasts by Parvovirus H-1

Yong Quan Chen, Françoise de Foresta, Jacqueline Hertoghs, Bernard L. Avalosse, Jan J. Cornelis, and Jean Rommelaere

ABSTRACT

A normal strain of human foreskin fibroblasts, two SV40-transformed derivatives with finite and infinite life spans, and an established line of SV40-transformed newborn human kidney cells are compared for their susceptibility to infection with parvovirus H-1. H-1 inocula, which do not detectably alter the growth of normal cells, cause a progressive degeneration of all three SV40-transformed cultures. The resistance of normal cells is not a membrane phenomenon since they adsorb and take up H-1 as efficiently as the transformants. Moreover, the fraction of infected cells supporting the synthesis and nuclear migration of H-1 proteins is similar in normal and SV40-transformed cultures. On the other hand, the enhanced H-1 sensitivity of transformed cells correlates with a 5- to 30-fold increase in their accumulation of newly synthesized parvoviral DNA, as compared with normal cultures. This stimulation of H-1 DNA replication is most pronounced for the amplification of duplex replicative forms, although the conversion of parental single-stranded DNA to replicative forms is also enhanced to a smaller extent. In addition, SV40-transformed cells support productive H-1 infection and release a burst of infectious virus, whereas no H-1 production can be detected in the normal cell strain. The latter difference was confirmed for another series of 7 normal and 16 SV40-transformed strains of human skin fibroblasts. Altogether, these results indicate that intracellular limitations on H-1 DNA replication are associated with the abortive nature of the parvoviral life cycle in normal human fibroblasts and are overcome after SV40 transformation, resulting in the selective killing of the transformants. This observation raises the possibility that oncolysis might contribute to the oncosuppressive activity displayed by parvoviruses in vivo.

INTRODUCTION

Parvoviruses are nuclear replicating, single-stranded DNA viruses of small size and low genetic complexity, which have been isolated, in particular, from a number of avian and mammalian species including humans (1). Parvoviruses have a lytic replicative life cycle and are devoid of detectable oncogenic activity, but they have the intriguing ability to suppress cancer formation in laboratory animals and there is speculation about their similar effect in humans (for reviews, see Refs. 2 and 3). Vertebrate parvoviruses can be divided into two subgroups, nondefective (so-called autonomous) and defective (so-called adeno-associated) members, respectively. The antineoplastic activity, but they have the intriguing ability to suppress cancer formation, whereas adeno-associated viruses specifically inhibit helper virus oncogenicity (2). The cellular and molecular bases of oncosuppression by parvoviruses are poorly understood. Parvoviruses might interfere with complex physiological processes conditioning tumor development (5). On the other hand, adeno-associated (6, 7) and autonomous (8) parvoviruses have both been shown to inhibit in vitro transformation of cells in culture, although they might act by different mechanisms. Thus, direct interactions between parvoviruses and transformed cells or their precursors, as occurring in vitro, might also contribute to oncosuppression.

We reported previously that the autonomous parvovirus MVM3 prevents the tumor virus SV40 from transforming in vitro mouse cells which had been selected for their resistance to MVM infection (8). This inhibition could be ascribed to the specific lysis of SV40 transformants by MVM. Together with the known requirements of parvoviruses for the proliferation (3) and appropriate differentiation (1) of their host cells, this observation led us to hypothesize that malignant transformation may render normally resistant cells permissive to the replication of these viruses. Hence, transformed cells would be a preferential target for the lytic action of autonomous parvoviruses. The possible involvement of such an oncolysis in cancer suppression by nondefective parvoviruses remains to be determined.

The work presented in this paper was aimed at analyzing the mechanism by which SV40 transformation sensitizes cells to the killing effect of autonomous parvoviruses and at determining the generality of this phenomenon. Since it was known that the nondefective parvovirus H-1 can be propagated in an established line of SV40-transformed newborn human kidney cells (9), it was decided to compare a normal diploid strain of human foreskin fibroblasts and two SV40-transformed derivatives with finite and infinite life spans for their susceptibility to H-1 infection. Consistent with the oncolysis hypothesis, SV40 transformation was found to correlate with an enhanced cell permissiveness to H-1.

MATERIALS AND METHODS

Cells. Human cell strains were grown as monolayers in Eagle's minimal essential medium supplemented with 10% fetal calf serum. NB-E is an established line of SV40-transformed newborn human kidney cells (10). VH-10 is a normal finite-life strain of foreskin human fibroblasts from which nonestablished (VH-10 ISV) and established (VH-10 SV), SV40 T-antigen-positive clones were independently derived by transformation with the early region of SV40 6-17 mutant deleted within the replication origin. The VH-10 series was prepared by B. Klein and kindly provided by A. van der Eb (Leiden University, Leiden, The Netherlands). Cells were counted with a hemocytometer after trypsinization and their viability was determined by their ability to exclude 0.07% (w/v) trypan blue stain.

Virus. H-1 parvovirus (wild type) was propagated in NB-E cells and purified according to the method of Tattersall et al. (11). 3H- and 32P-labeled virus was produced by incubating infected cells with [3H]thymidine (5 Ci/mmol, 25 μCi/ml) and [32P] (carrier free, 0.1 mCi/ml), as described by Rhode (12) and Rommelaere and Ward (13), respectively. Conditions for cell infection, virus harvesting, and titration by plaque assay have been described previously (14).

H-1 Uptake. Cells were incubated for 60 min at 37°C or 4°C in the

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: MVM, minute virus of mice; SS, single-stranded; RF, replicative form.
presence of ³H-labeled H-1. Virus binding and uptake were measured as described by Linser and Armentrout (15), except that cells were released from the surface of the dish by treatment with 1 mM EDTA and 0.05 g trypsin per 100 ml. The recovered radioactivity (cpm) was measured in a liquid scintillation counter and reconstituted more than 97% of the inoculum. Cell-associated radioactivity prior to and after EDTA elution was taken as measurement of virus binding and uptake, respectively.

H-1 DNA Replication. The conversion of parental SS DNA to double-stranded monomeric and oligomeric RF was monitored in cells infected with ³²P-labeled H-1. Intracellular viral DNA was isolated by a modification of the Hirt procedure, purified, and fractionated by electrophoresis through 1% agarose gels for 240 V × h as described previously (13). Bands were localized in dried gels by autoradiography, cut out, dissolved in 10 μl of pure formamide, and counted by scintillation spectrometry in HP/β liquid.

Total viral DNA synthesis was measured either in whole cell lysates trapped onto nitrocellulose filters (dispersed cell assay) (16) or in blots of DNA fractionated by agarose gel electrophoresis and transferred to nitrocellulose sheets (17). DNA was quantitated by hybridization to a ³²P-labeled specific probe, essentially as described by Winocour and Keshet (18). The probe was cloned DNA of MVM (19), a parovirus displaying over 70% sequence homology with H-1 (20), and was kindly provided by Dr. D. C. Ward (Yale University). The probe was labeled with [α-³²P]dATP by nick translation according to the method of Rigby et al. (21) (2–3 × 10⁶ cpm/μg) and was used at 1–2 × 10⁶ cpm/ml. Hybridized radioactivity was quantitated by scintillation spectrometry or revealed by exposure to X-ray film for autoradiography (~70°C, with intensifying screen).

H-1 Protein Synthesis. Viral protein synthesis was monitored by the binding of anti-H-1 mouse serum (Microbiological Associates) recognizing capsid proteins to infected cell (lysates). Bound antibodies were revealed by the sandwich technique with a second peroxidase-coupled (immunoenzymatic assay) or radioactive (immunoradiometric assay) anti-mouse immunoglobulin.

The immunoenzymatic assay has been described elsewhere (22). Integrated absorbance of individual nuclei was measured with an M 85 Vickers scanning densitometer (λ = 570 nm).

For the immunoradiometric assay, nitrocellulose filters loaded with repeatedly frozen and thawed cells (10⁶ cells/filter) were coated with 10% fetal calf serum and incubated for 3 h at room temperature with a 1:100 dilution of anti-H-1 serum in 10 mM Tris, 0.15 M NaCl, 1 mM EDTA, 10% fetal calf serum, and 0.2% Tween 80 (pH 7.5). Stock anti-H-1 serum was pseudosorbed for 1 h with 10 volumes of a suspension of 10⁶ uninfected cells/ml. Filters were washed and further incubated for 1 h at room temperature with ³²P-labeled anti-mouse immunoglobulins (Amersham, 18 μCi/μg, 0.1 μCi/ml) in the same buffer. After washing and drying, filters were either dissolved and counted in scintillation fluid or exposed to X-ray films for autoradiography (~70°C, with intensifying screen).

RESULTS

Sensitivity of Human Fibroblasts to the Killing Effect of Parvovirus H-1

The nondefective parvovirus H-1 was tested for its effects on the growth of a culture of normal diploid human fibroblasts (VH-10) and two derived SV40-transformed strains with a finite (VH-10 ISV) and an infinite (VH-10 SV) life span. NB-E, an established line of SV40-transformed newborn human kidney cells, is used routinely to produce and to plaque H-1 and was included for comparison. Cells growing in monolayers were harvested at intervals postinfection and counted after vital staining. Inoculation of H-1 (1.0 plaque-forming unit/cell) had no significant effect on the proliferation of normal fibroblasts (Fig. 1). The viability of normal cells, as measured by the trypan blue exclusion method, was greater than 95%, irrespective of whether cultures were infected or not (data not shown).

In contrast, under those same conditions, H-1 killed SV40-transformed cells and caused the degeneration of corresponding cultures, as was evident from a time-dependent decrease in the number of residual viable cells (Fig. 1). The greater H-1 sensitivity of SV40 transformants did not correlate with an enhanced growth rate compared with normal cells. Cell sensitization by SV40 did not require that transformation led to the establishment of a continuous line. Indeed, for the pair of SV40-transformed VH-10 cells tested, immortalization did not coincide with any increase in susceptibility to H-1.

H-1 Virus Uptake

With the aim of identifying the barrier(s) which protect normal cells against H-1 attack and are overcome as a result of SV40 transformation, the ongoing H-1 life cycle was analyzed comparatively in the different cultures. The primary resistance that a cell can offer to paroviruses occurs at its surface where virions first adsorb onto specific membrane receptors and are subsequently taken up. Virus binding and uptake can be distinguished by the fact that the former process can take place at low temperatures and be reversed by treatment with EDTA, whereas the latter cannot (15). As shown in Table 1, H-1 adsorption and uptake were as efficient in normal as in trans-
formed cells, suggesting that the sensitizing effect of SV40 transformation is not mediated by an increase in H-1 penetration.

**Intracellular H-1 Replication**

It therefore appears likely that normal VH-10 cells survive H-1 infection because of some intracellular defect in virus replication. It has been shown that such intracellular restrictions on parovirus replication can be overcome partially by increasing the multiplicity of infection (plaque-forming units/cell) but that the productive infection obtained under those conditions rapidly out (23). Consistently, some killing of primarily infected VH-10 cells could be detected at higher than 150 plaque-forming units/cell, although it did not affect the survival of the culture (data not shown).

**H-1 DNA Replication.** The total amount of H-1 DNA present in infected cultures late during the primary virus cycle, was measured by hybridization, using a dispersed cell assay. As shown in Table 2 (Column 5), overall H-1 DNA synthesis was increased 5- to 30-fold as a result of SV40 transformation of host cells. This stimulation was greatest in NB-E cells which were also most sensitive to the viral killing effect (Fig. 1), suggesting that the dependence of H-1 DNA synthesis on cell transformation might constitute a determinant of oncogenesis. The replication of paroviral DNA involves three successive steps, namely, the conversion of parental SS DNA to duplex RF, the replication of RF DNA, and the displacement synthesis of progeny SS DNA (24). SV40 transformation markedly increased the production of RF DNA (Fig. 2), to an extent similar to that of total H-1 DNA synthesis (Table 2). Only a small part of the enhancement of RF DNA formation in transformed cells could be ascribed to the stimulation of parental H-1 DNA conversion (Table 2). These results therefore locate the major transformation-sensitive step of H-1 DNA synthesis at the level of RF replication.

**H-1 Protein Synthesis.** The amount of H-1 proteins synthesized by infected cells was quantitated immunologically, using anti-H-1 serum recognizing viral capsid proteins. The binding of anti-H-1 immunoglobulins was measured either in the whole culture by an immunoradiometric filter assay (Fig. 3a) or in individual cells by immunoenzymatic staining (Fig. 3, b and c). The number of infected cells synthesizing detectable amounts of H-1 proteins was similar for the four cultures tested (Table 3) and in all cases viral proteins accumulated into the cell nucleus (Fig. 3). These observations suggest that the defect responsible for the abortive nature of the lytic H-1 life cycle in normal cells does not prevent the expression of structural genes and does not concern protein translocation from the cytoplasm to the nucleus. SV40-transformed cells supported a higher level of H-1 protein synthesis than normal fibroblasts (Table 3; Fig. 3). Transformation-induced stimulation of viral protein (Table 3) and DNA (Table

### Table 2  H-1 DNA replication in normal and SV40-transformed human fibroblasts

<table>
<thead>
<tr>
<th>Cells</th>
<th>Parental DNA conversion</th>
<th>Total viral DNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>Relative conversion</td>
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<tr>
<td>VΗ-10</td>
<td>314</td>
<td>1.0</td>
</tr>
<tr>
<td>VΗ-10 SV</td>
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<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>VΗ-10 ISV</td>
<td>399</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>NB-E</td>
<td>583</td>
<td>1.9 ± 0.2</td>
</tr>
</tbody>
</table>

**Fig. 2.** H-1 RF DNA replication in normal and SV40-transformed human fibroblasts. Cultures (10⁵ cells) were infected with virus H-1 (multiplicity of infection, 1) and further incubated for 12 (Lanes 1, 4, 7, and 10), 24 (Lanes 2, 5, 8, and 11), or 36 (Lanes 3, 6, 9, and 12) h. Viral DNA was extracted, fractionated by agarose gel electrophoresis, and blot-hybridized with a ³²P-labeled probe. Autoradiographic exposure, 4 h (NB-E) and 24 h (VΗ-10 series). RF1, monomer length double-stranded RF; RF2, dimeric double-stranded RF; M, marker (in vitro converted H-1 virion DNA).

**Fig. 3.** H-1 protein synthesis in normal and SV40-transformed human fibroblasts. Cultures were infected with H-1 (multiplicity of infection, 1), incubated for 24 h, and analyzed for viral protein synthesis by immunoradiography (a) or immunoenzymatic staining (b, c), using ¹²⁵I-labeled (a) or peroxidase-coupled (b, c) immunoglobulins. a, autoradiogram of lysates of 10⁵ H-1-infected cells (exposure time, 40 h); mock-infected samples were indistinguishable from background and are not shown. b and c, stained H-1-infected cells; +, positively staining cells; −, negatively staining cell; bar, 10 μm.
Abortive Interaction between Parvovirus H-1 and Normal Human Fibroblasts

A series of nonpermanent cultures of normal human skin fibroblasts were found to resist productive infection with parvovirus H-1. Resistance could be ascribed to the failure of an intracellular stage of the replicative life cycle of H-1, resulting in a lack of amplification of the virus inoculum. Similar results have been reported for human amnion, embryo kidney (25), and lung (9) cells. A quantitative comparison of H-1 replication was undertaken between normal skin fibroblasts and corresponding permissive cultures, namely SV40 transformants derived therefrom (see below). A striking difference was observed at the level of the replication of RF DNA, which was much enhanced in transformed cultures and correlated with their ability to produce infectious virus. The defect of the H-1 life cycle in diploid human fibroblasts might concern RF DNA replication itself. Alternatively, another step of the viral cycle conditioning the extent of RF DNA replication might be impaired, although our data suggest that virus uptake, nuclear translocation, and parental DNA conversion are unlikely to be primarily involved.

The inability of normal human fibroblasts to support RF DNA replication efficiently might result from their failure to express nonessential host functions usurped by H-1 for its own growth. In addition, H-1 DNA replication appears to involve virus-encoded functions (26). Nonpermissive cells might also fail to synthesize or modify these viral products, although no major impairment of H-1 capsid gene expression was detected (28). The inability to produce infectious virus. The defect of the H-1 life cycle in diploid human fibroblasts might concern RF DNA replication itself. Alternatively, another step of the viral cycle conditioning the extent of RF DNA replication might be impaired, although our data suggest that virus uptake, nuclear translocation, and parental DNA conversion are unlikely to be primarily involved.

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ical states can lead to the arrest of the parvoviral life cycle at different steps, both on the surface and on the inside of the cell (32). Therefore, there appear to be multiple conditional host factors which determine permissiveness to parvoviruses. It follows that the specific abortive interaction reported here for diploid human fibroblasts cannot be necessarily generalized to other cell types.

Induction of H-1 Sensitivity by SV40 Transformation

The acquisition of permissiveness to H-1 replication by SV40 transformants correlated with their sensitization to parvoviral lytic action. H-1 inocula which had little effect on the growth of normal cultures caused a progressive degeneration of transformed cells. Thus, the preferential killing of transformed cells is likely to result from their propensity to replicate H-1 rather than their lower resistance to its cytopathic effect. A similar sensitization of SV40 transformants to the autonomous parvovirus MVM was reported previously for mouse cells which had been selected in vitro for their resistance to this virus (8). Therefore, SV40 transformation overcomes both natural and selected cellular protections against parvoviral attack.

The enhanced susceptibility of SV40 transformants to H-1 infection does not require their establishment into a permanent culture. Thus, SV40 transformation appears to act directly and not merely by facilitating cell immortalization. Since the expression of the SV40 genome in transformants is restricted to the early region-encoding T-antigens (33), the latter protein(s) is likely to mediate sensitization to H-1. Large T-antigen is known to activate cellular gene expression (33, 34). As stated above, cell permissiveness to parvoviruses involves host functions, some of which are not permanently expressed and might conceivably be induced by SV40. On the other hand, a direct interaction between the H-1 genome and SV40 T-antigen(s) might also potentiate H-1 replication. Another tumor virus, adenovirus, was found to provide helper functions to H-1 in coinfected human cells (35) and encodes a tumor antigen which could possibly interact directly with the DNA of the defective parvovirus adeno-associated virus (7). Whatever mechanism it involves, sensitization to H-1 is apparently not specific for tumor virus-transformed cells. Indeed we have recently extended the present observation to human diploid fibroblasts transformed by γ-irradiation (36). Nonvirally transformed cells therefore appear to supply helper functions similar to those encoded or induced by SV40.

Altogether, our data point to a positive interrelation between two complex cellular phenotypes each composed of multiple components, i.e., permissiveness to autonomous parvoviruses and in vitro malignant transformation. This result raises two intriguing possibilities. (a) The lytic life cycle of parvoviruses may provide specific markers for cell transformation. We report here that SV40 transformation bypasses a limitation to H-1 DNA replication. In contrast, a postreplicative step of the growth of parvovirus MVM was found to be stimulated as a result of the transformation of rat cells with the retrovirus avian erythroblastosis virus (37). An interesting issue is whether the overcoming of distinct barriers to parvovirus replication requires the expression of different transformation traits. (b) The suppression of cancer development by autonomous parvoviruses may result, at least in part, from oncology. The preferential cytolysis of autonomous parvoviruses in transformed cells accounts for the ability of these viruses to specifically inhibit in vitro malignant transformation (8). Whether cancer cells provide a similar target for destruction by parvoviruses in vivo remains to be determined.

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The authors are greatly indebted to Drs. A. van der Eb and B. Klein for their generous gift of VH-10 and derived cells, to C. Kumps for technical assistance, and to Dr. P. Tattersall for critical reading of the manuscript. Some human mutant cells were kindly provided by Drs. C. Arlett, A. Lehmann, and D. Bootma.

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Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O₂ consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[0.65 \cdot (+0.27) + 0.35 \cdot (-0.16) = +0.12\]

a figure identical to the observed +0.12 for normal leukocytes.
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