RAPID COMMUNICATION

First Continuous Propagation of B19 Parvovirus in a Cell Line

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The pathogenic human parvovirus B19 has extreme tropism for human erythroid progenitor cells and has resisted cultivation in conventional cell lines. We report first propagation of this virus in an erythropoietin-dependent strain of a megakaryoblastic leukemia cell line called UT-7. Virus protein was present in about 5% of cells after 1 week of culture. Appropriate ratios of major and minor capsid proteins were determined by immunoblot, and newly synthesized capsid protein was detected by immunoprecipitation of radioactively labeled cell lysates. High molecular weight monomer and dimer intermediates were detected by Southern analysis, indicating active viral replication. Approximately 1.000 genome copies were present per infected cell, and at the optimal multiplicity of infection 20- to 50-fold more virus was produced than inoculated. Virus propagation only occurred in UT-7 cells that were adapted to growth in erythropoietin; virus signal was not detected in UT-7 cells adapted for growth in granulocyte-macrophage colony-stimulating factor or interleukin-3, even with exposure to erythropoietin for several days. Infectious virus was detected in cultures as long as 3 months after inoculation. Despite persistence, there was no evidence of viral integration on Southern analysis. This cell line may prove useful for the production of infectious virus and in the analysis of B19 parvovirus persistence, cytotoxicity, and permissivity.

MATERIALS AND METHODS

Cell culture. The source of B19 parvovirus for all experiments was a serum sample obtained from a patient with sickle cell anemia (Gray) during the acute phase of a transient aplastic crisis, previously determined by DNA dot-blot analysis to contain approximately 1 × 10^11 genome copies/mL. UT-7 cells were derived from a patient with acute megakaryoblastic leukemia, UT-7 propagation in vitro is strictly dependent on the presence of interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), or erythropoietin (Epo). By cell surface marker analysis, the majority of UT-7 cells react with monoclonal antibodies (MoAbs) to platelet glycoprotein (GP) Ib (CD42b), GP Ib/IIa (CD41), My7 (CD13), My9 (CD33), and glycophorin A. UT-7 cells have been adapted for growth to higher cell density in Epo by continuous passage in the presence of this hormone. Such adapted cells, termed UT-7/Epo, are phenotypically identical to the parent UT-7 line except that: (1) they reach concentrations of 10^7/mL compared with 10^6/mL in culture and (2) they have a much lower concentration of Epo receptors on their cell surface (N.K., unpublished data, 1991).

For infection, UT-7 cells were inoculated with varying dilutions of parvovirus-containing serum; virus was allowed to absorb to cells at high concentration (5 × 10^7 cells/mL in a total volume of 0.4 mL) for 2 hours at 4°C, and then diluted to a concentration of 2 × 10^6 cells/mL in Iscove’s modification of Dulbecco’s medium (IMDM; Flow Laboratories, McLean, VA), 10% fetal calf serum (FCS; GIBCO Laboratories, Grand Island NY), and recombinant human Epo (1 U/mL; Amgen Biologicals, Thousand Oaks, CA). For some experiments, UT-7 cells had been adapted for growth in GM-CSF (2.5 U/mL; Genzyme, Boston, MA) or IL-3 (2 U/mL; Genzyme). Cultures were performed in 25-cm^2 flasks (Corning Glass Works, Corning, NY) at 37°C, 95% humidity, 5% CO2. Cells were generally washed free of inoculum after 1 day of culture and harvested for analysis at varying times afterwards. Cell morphology was determined by Wrights’ Giemsa staining of cytocentrifuged preparations.

Analysis of B19 parvovirus proteins and DNA. For immunofluorescence, cytocentrifuge preparations were fixed in acetone at −20°C for 30 seconds, washed twice in phosphate-buffered saline (PBS)-0.5% bovine serum albumin, blotted dry, and stored frozen; parvovirus capsid antigen was detected using an MoAb prepared by immunization of mice with empty B19 parvovirus...
capsids produced in Chinese hamster ovary cells. DNA for slot-blot analysis was prepared from whole cells and culture supernatants by treatment with 0.1 mol/L NaCl, Tris-HCl pH 7.5, 0.5% sodium dodecylsulfate, 5 mmol/L EDTA, and 200 μg/mL protease K (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight at 37°C followed by phenol-chloroform extraction. For Southern analysis, DNA was further purified by ethanol precipitation and RNease A treatment, repeated protease K digestion and phenol-chloroform extraction, and a second ethanol precipitation. High and low molecular weight DNA were separated by the method of Hirt.

Slot-blot analysis of DNA was performed after addition of NaOH to DNA extracted from 2 × 10⁶ cells or 1 mL of culture supernatant to a final concentration of 0.3 mol/L and heating to 65°C to destroy RNA and denature DNA. After HCl neutralization, serial dilutions were made in 10X SSC and applied to a minifold slot-blot apparatus (Schleicher and Shuell, Keene, NH). Immobilized DNA on Nitrane (Schleicher and Shuell) was baked at 80°C for 2 hours and then hybridized with a 32P-labeled pYT103c insert probe in 6X SSC, 5X Denhardts solution, 0.5% sodium dodecyl sulfate (SDS) and 100 μg/mL denatured salmon sperm DNA overnight at 65°C, washed three times with 2X SSC-0.1% SDS for 5 minutes at room temperature, two times with 2X SSC-1% SDS for 30 minutes at 65°C, two times with 0.1X SSC-0.1% SDS for 30 minutes at 55°C. Kodak Xomat-AR film (Eastman Kodak, Rochester, NY) was exposed to the filters to develop autoradiographs. The quantity of B19 genome copies was determined by comparison to 1 ng B19 DNA from the cloned plasmid pYT103c.

Southern analysis was performed using appropriate quantities of DNA as determined by slot-blot testing. DNA was assayed undigested or after treatment with EcoRI alone, EcoRI and BamHI, or EcoRI and Hind III. Completion of enzymatic digestion was determined by parallel treatment of 1.0 μg phage DNA with aliquots of the reaction mixtures, followed by agarose gel electrophoresis.

Gene amplification of B19 parvovirus DNA was performed by standard techniques using oligonucleotide primers previously determined to provide optimal sensitivity.

Immunoblotting and immunoprecipitation were performed as previously described. For Western analysis, lysates of infected cells were electrophoresed in 8% SDS-polyacrylamide gels in reducing conditions using Laemmli's discontinuous buffer system. The separated polypeptides were electrophoretically transferred to nitrocellulose sheets in an electrode buffer containing 25 mmol/L Tris-HCl, pH 8.3, 193 mmol/L glycine, and 20% (vol/vol) methanol at 0.2 A overnight. High titer convalescent phase human anti-B19 parvovirus serum was reacted with the antigen on the nitrocellulose for 1 hour at room temperature, followed after washing with 0.1% Tween-PBS by treatment with biotinylated antihuman IgG (Vector tain ABC kit; Vector Laboratories, Burlingame, CA) for 30 minutes and streptavidin-biotinylated horseradish peroxidase complex as an indicator of bound second antibody, also for 30 minutes at room temperature. The reaction was developed with horseradish peroxidase using 4-chloro-1-naphthol (Bio-Rad, Richmond, CA) as substrate.

**Assay for infectious virus.** Infectious virus was assayed by its ability to inhibit erythropoiesis measured by CFU-E-derived colony formation in vitro. Supernatants from infected and control, uninfected cultures were incubated with normal bone marrow mononuclear cells for 4 hours at 4°C to allow absorbance and then diluted in culture medium. 1 × 10⁶ cells suspended in IMDM containing 0.8% methyloculose, 30% FCS, 1% bovine serum albumin, and 10 U/mL recombinant human Epo (Amgen, Thousand Oaks, CA). Erythroid colonies were counted in triplicate plates after 6 to 7 days of incubation at 37°C, 95% humidity, 5% CO₂.

**RESULTS**

Inoculation of UT-7/Epo cells with B19 parvovirus was not associated with a cytopathic effect, nor was cell growth in the cultures slowed (data not shown).

B19 parvovirus capsid protein was detected by immunofluorescence within 2 days of inoculation of UT-7/Epo cells (Fig 1). Staining was predominantly nuclear. At 1 week, approximately 5% of cells demonstrated specific fluorescence (after several weeks of culture, 0% to 2% of cells showed specific fluorescence). Specific capsid proteins were detected by immunoblot of cell lysates obtained on days 2 and 3, with strong signals present on days 4 and later (Fig 2A). The ratio of major to minor capsid proteins was about 10:1 as determined by densitometry. Smaller sized proteins apparent on immunoblot may represent either degradation products or recently described polypeptides encoded by short RNA species from the far right side of the genome, of unknown function. Newly synthesized major capsid protein could be detected by immunoprecipitation of 35S-methionine-labeled cells with antiparvovirus antibodies present in convalescent phase human antisemur (Fig 2B).

The genome of parvoviruses is single-stranded DNA, and replication proceeds through double-stranded intermediates. As shown in Fig 3A, high molecular weight intermediates corresponding to monomer and dimer forms were present on Southern analysis of DNA from infected cultures. Only UT-7/Epo cells that had been adapted to growth dependence on Epo by many months of passage in hormone were permissive to parvovirus replication. UT-7 cells that were adapted to growth in GM-CSF or IL-3 did not support parvovirus replication, even if Epo was added with virus and present throughout the culture period (Fig 3B).

Unexpected bands of approximately 4 kb were present on the initial Southern analyses, suggesting annealing of positive and negative parvovirus DNA strands (Fig 3A). Replication from both positive and negative strands was inferred from the pattern of bands present after double enzymatic digestion of total cellular DNA, using Hind III for digestion of the left side of the genome and BamHI for the right side (Fig 4 and diagram). We were unable to detect evidence of integration of B19 parvovirus into genomic DNA by the presence of anomalous high molecular weight bands on Southern analysis (Fig 4A), and all the virus signal separated with the low molecular weight fraction after Hirt extraction and Southern hybridization (Fig 4B). B19 parvovirus genome was not detected in the high molecular weight fraction even after gene amplification using the polymerase chain reaction (data not shown).

The ratio of output to input parvovirus genomic DNA was determined by dot-blot DNA analysis to determine an optimal multiplicity of infection (m.o.i.) (Fig 5). At an m.o.i. of 0.5, 20- to 50-fold more DNA was present compared with the inoculum, measured at 3, 5, and 7 days postinfection. Almost all the viral DNA was present in cell lysates with...
only a minor proportion released into the supernatant, consistent with the lack of cytotoxicity in this cell line after parvovirus infection (data not shown). Virus was estimated to be present at about 1,000 genome copies/cell.

UT-7/Epo cultures continued to produce B19 parvovirus for long periods of time (Table 1). Virus was detected by dot-blot hybridization in cell culture supernatants after 3 months of continuous culture. Virus derived from culture supernatants was confirmed to be infectious using the CFU-E inhibition assay; inhibition of 50% to 60% observed with these active supernatants corresponded to the infective activity of viremic serum of titer = 10^10/mL.

DISCUSSION

B19 parvovirus production in UT-7/Epo cells can be compared and contrasted with virus replication in normal target populations, bone marrow, and fetal liver. The

Fig 1. Immunofluorescence of UT-7/Epo cells infected with B19 parvovirus. Cells were removed from culture 2 days after inoculation and stained with (A) mouse MoAb specific to parvovirus capsid proteins or (B) isotype specific control MoAb. Original magnification ×1,000.
Fig 2. Detection of parvovirus proteins. (A) Immunoblot of cell lysates harvested on days 1 through 7 after inoculation, developed with convalescent phase human antiserum to parvovirus B19. The location of VP1 (83 Kd) and VP2 (58 Kd) is shown on the right. C = uninfected culture. (B) Immunoprecipitation of $^{35}$S-methionine-labeled parvovirus protein derived from UT-7/Epo cells inoculated 4 days earlier with B19 parvovirus with convalescent phase human antiserum. The location of VP2 is shown on the right.

Fig 3. B19 parvovirus replication in UT-7/Epo cells. (A) Time course of replication. Southern analysis after digestion with EcoRI for B19 parvovirus sequences was performed at different times after inoculation, as indicated in the label at the top of the autoradiograph. Inoculated UT-7/Epo cells had been adapted to growth dependence in Epo. (B) Inability to detect B19 parvovirus replicative forms in UT-7 cells adapted to growth in GM-CSF or IL-3, harvested at day 5. Culture conditions: (a) no Epo added; (b) Epo (1 U/mL) added before inoculation with virus, inoculum washed free at day 1; (c) Epo (1 U/mL) added with inoculation and present throughout the period of culture.
pattern of DNA replication through intermediate monomer and dimer forms is similar in UT-7/Epo and erythroid progenitor cells, although replication from positive and negative template strands cannot be distinguished for technical reasons in marrow cultures; in addition, in both systems replication follows similar kinetics, with appearance of replicative DNA 48 hours after inoculation. Virus is predominantly intracellular in both culture systems. Parvovirus capsid proteins are produced in the appropriate ratios of 10:1 major:minor structural species, approximately that observed in bone marrow culture. The dependence of parvovirus propagation on Epo is another common feature of these systems.

However, in marrow and fetal liver cultures, B19 parvovirus is cytolytic, and with destruction of the erythroid target cells, virus becomes undetectable with time. In contrast, B19 parvovirus is not cytotoxic to UT-7/Epo cells, and virus infection persists in the cell line culture for extended periods of time. Also in contrast, B19 parvovirus production in UT-7/Epo cells is less efficient than in bone marrow cells. While only 5% of cells show immunofluorescence in UT-7/Epo cultures, in bone marrow cultures, usually about 10% of total cells and 30% to 40% of erythroid cells stain specifically at 48 hours after inoculation. The efficiency of infection by parvovirus between UT-7/Epo and marrow cell cultures is not readily compared: a lower m.o.i., 0.05, resulted in maximal relative virus production for UT-7/Epo compared with the best m.o.i. for total bone marrow cells of
virus into UT-7/Epo cells but higher levels of virus production. UT-7/Epo cultures were inoculated with varying quantities of virus containing serum (abscissa) and cell lysates harvested at different times (see key); supernatant was harvested at day 7.

![Graph](Fig 5. Effect of multiplicity of infection on B19 parvovirus production. UT-7/Epo cultures were inoculated with varying quantities of virus containing serum (abscissa) and cell lysates harvested at different times (see key); supernatant was harvested at day 7.)

40, but the relative amount of virus produced at optimal m.o.i. was about fourfold higher for marrow (Fig 5 and ref 5). These data are consistent with more efficient entry of virus into UT-7/Epo cells but higher levels of virus production in marrow erythroid progenitors.

A striking finding in UT-7 cell culture is the necessity of prior adaptation to growth in Epo for parvovirus replication. In bone marrow cultures, B19 parvovirus propagation correlates with the stages of erythroid differentiation. Inability to detect parvovirus replication in similar cells adapted to growth in GM-CSF or IL-3 with shorter periods of Epo exposure indicate that Epo's effect is unlikely to be due to any immediate effects of the hormone on membrane protein expression, intracellular calcium flux, or other metabolic actions. Epo dependence of parvovirus propagation is probably mediated indirectly through alteration of the intrinsic genetic program of the cell. In its fastidious behavior, B19 resembles adenovassociated virus, also a human (but nonpathogenic) virus, that usually requires coinfection with helper virus to replicate; under some circumstances, low-level replication can be induced in the absence of helper virus by manipulation of the cell cycle. Under these special circumstances, cellular factors that accumulate during mitosis are presumed to subserve the functions ordinarily provided by genes of the coinciding virus. Similarly, permissivity of minute virus of mice strains that replicate in either fibroblasts or lymphocytes is not determined by surface receptors but at the intracellular level. Recent molecular studies in our laboratory have suggested that full-length transcription of the virus genome is blocked in nonpermissive cell lines into which the B19 parvovirus genome has been transfected (J. Liu, N.S. Young, unpublished data, June 1991). Closely related UT-7 strains that are permissive or nonpermissive for B19 parvovirus propagation may prove useful in determining the precise intracellular events required for virus replication. Manipulation of the cell line may allow increased virus production, which would be practically useful, as well as helpful in defining cellular and extracellular regulation of virus propagation. UT-7/Epo cells may also be more convenient than human bone marrow cells to assay for biologically active B19 parvovirus.

In contrast to the dramatic cytotoxic effects of parvovirus infection on erythroid progenitor cells, B19 was cultivated in UT-7/Epo cells for months with steady production of virus and normal cell growth. B19 parvovirus cell killing is mediated by the single nonstructural protein, and, in general, expression of this same protein is also required for parvovirus replication. UT-7/Epo cells may be resistant to the toxic effect of nonstructural protein while the protein retains its functional role on virus replication; alternatively, lower amounts of nonstructural protein may be produced in UT-7/Epo cell culture than in bone marrow cells. This hypothesis is being investigated by examining the effects of transfection of nonstructural protein gene into UT-7/Epo cells.

There is no ready explanation for the susceptibility of a megakaryocytic cell line to B19 parvovirus when erythroleukemia lines have consistently failed to support virus propagation. Platelet counts decrease during natural and experimental parvovirus infection. In vitro, B19 parvovirus reduces CFU,E-derived colony formation in the absence of virus replication. Predominant expression of the left side of the genome, due to a block in transcription as described above, would result in expression of the cytotoxic nonstructural protein gene and cell death in the absence of virus replication. UT-7/Epo cells have characteristics of both megakaryocytes (cell surface phenotype) and erythroid progenitors (dependence on erythropoietin). B19 parvovirus replication in these cells must require the proper intracellular combination of erythroid transcription permis-

**Table 1. B19 Parvovirus Persistence UT-7/Epo Culture**

| A. Persistence of Infectious Parvovirus in UT-7/Epo Long-term Culture |
|---|---|---|
| **Time** | **Expt 1** | **Expt 2** |
| **Genome copies/cell** | | |
| 0 | 2 wk | 3 mo |
| Infectious virus | + | + |
| **Experiment 2** | | |
| **Genome copies/cell** | | |
| 0 | 2 wk | 1 mo |
| Infectious virus | + | + |

**B. Inhibition of Erythroid Colony Formation by Culture Supernatant**

<table>
<thead>
<tr>
<th>B19-infected</th>
<th>Control</th>
<th>B19</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-7/Epo</td>
<td>UT-7/Epo</td>
<td>Serum</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td>69 ± 5</td>
<td>169 ± 8</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td>40 ± 1</td>
<td>86 ± 1</td>
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UT-7/Epo cell culture supernatants from chronically infected cultures were assayed for active virus by inoculation in hematopoietic progenitor assays using human bone marrow as a source of CFU-E. Genome copy number was detected by DNA dot-blot titration.
sive factors as well as resistance to the cytotoxic effects of viral nonstructural protein.

B19 parvovirus can persist in the immunocompromised host, including the fetus. Adenovassociated virus persists in Detroit 6 cells, and in this cell line the virus is integrated into a specific site of chromosome 19. However, we were unable to detect evidence of integration by B19 parvovirus into UT-7/Epo cells (or in the bone marrow of chronically infected patients; J. Antunoz and N.S. Young, unpublished data, 1991). These data deserve a cautious interpretation because of problems performing gene amplification across the long terminal repeat segments, regions of high second
ary structure due to their content of guanidine and cytosine nucleotides. Nonetheless, evidence accumulated to date suggests that this virus apparently may persist in vitro and in vivo as an episomal structure.

REFERENCES

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First continuous propagation of B19 parvovirus in a cell line

S Shimomura, N Komatsu, N Frickhofen, S Anderson, S Kajigaya and NS Young