Productive Infection by B19 Parvovirus of Human Erythroid
Bone Marrow Cells In Vitro

By Keiya Ozawa, Gary Kurtzman, and Neal Young

B19 parvovirus, the cause of fifth disease and transient aplastic crisis, has been successfully propagated in suspension cultures of human erythroid bone marrow cells obtained from patients with sickle cell disease and stimulated by erythropoietin. B19 inoculation in vitro resulted in a marked decline in identifiable erythroid cells over seven to nine days of incubation. Characteristic giant early erythroid cells were seen on Wright’s-Giemsa stain of infected cultures. By in situ hybridization, 30% to 40% of erythroblasts were infected at 48 hours; a similar proportion of cells showed B19 capsid protein by immunofluorescence. Blasts were infected at 48 hours; a similar proportion of

obtained from patients with sickle cell disease and stimulation cultures of human erythroid bone marrow cells

aplastic crisis, has been successfully propagated in suspension. Previous efforts by ourselves and others to

virus propagation. Previous efforts by ourselves and others to

grow B19 parvovirus in cultured cell lines have failed.

Recently we reported detection of replicative forms of B19 DNA from the nuclei of human erythroid bone marrow cells infected with B19 parvovirus in vitro and cultured in suspension. Replication of B19 in these cultures, as in clonal assays, was highly dependent on the content of erythroid cells and the presence of the hormone erythropoietin. We report here the effect of B19 infection on erythroid cell number and morphology in human bone marrow cultures; the restriction of B19 replication to erythroid cells; localization of viral replication and encapsidation to nuclei; the time course of B19 production in nuclei, cytosol, and supernatant; and the production of large relative amounts of highly infectious B19 virus at low multiplicity of infection.

**METHODS**

**Cell culture, culture conditions, and inoculation procedure.** The source of B19 virus for all experiments was a sample of serum obtained from a patient with sickle cell disease and transient aplastic crisis during a recent B19 epidemic (Minor II, 60 μg B19 DNA/mL, approximately 2 × 10^12 particles/mL). Bone marrow was obtained from patients with sickle cell disease and thalassemia or from normal volunteers after informed consent according to a protocol approved by the institutional review board of the National Heart, Lung, and Blood Institute. Bone marrow mononuclear cells were infected by addition of serum at varying concentrations, diluted in Iscove’s modification of Dulbecco’s medium (IMDM; Flow Laboratories, McLean, VA) at a concentration of 5 × 10^6 cells/mL (total volume 0.4 mL). Virus was allowed to adsorb to cells for two hours at 4°C and was then diluted to 2 × 10^6 cells/mL in IMDM containing 20% fetal calf serum (FCS) and 1 U/mL erythropoietin (epo; step III, Connaught Laboratories, Ontario; or recombinant epo, Amgen Biologics, Thousand Oaks, CA), transferred to 25-cm² flasks (Corning Glass Works, Corning, NY) or 24-well maccrotiter plates (Costar, Cambridge, MA), and cultured at 37°C, 95% humidity, 5% CO₂. Cells were washed free of inoculum virus on day 1. Cells and culture supernatants were harvested from 12 hours to 9 days after inoculation.

**Morphology and immunofluorescence.** Morphology of infected and control cultures was determined by Wright’s-Giemsa staining of cytocentrifuged preparations from periodically sampled cultures. 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 (BSA), or gelatin; blotted dry; and stored frozen until staining with rabbit antiserum to B19 capsid proteins. Optimal results were obtained with anti-B19 antiserum diluted 1:10. 25 μL/slide × 30 minutes, followed by two washes in PBS-BSA and application of affinity purified antirabbit IgG-fluorescein isothiocyanate (FITC) (Kierkegaard Perry, Gaithersburg, MD), 1:50, 50 μL × 30 minutes. Slides were examined using a Zeiss fluorescent microscope.

**Separation of erythroid and nonerythroid cells.** For some experiments erythroid and leukocyte fractions were separated by a panning technique. Cells were harvested from culture and washed twice with IMDM-2%FCS. Two × 10^7 cells were incubated in 500 μL IMDM-10%FCS containing anti-Leu M1 and anti-HLe-1 monoclonal antibodies (MoAbs) (Becton Dickinson, Mountain View, CA), 50 μL each, one hour at 4°C. The cells were washed three times with IMDM-2%FCS and then added to plastic plates coated with
antimouse IgG-IgM for one hour at 37°C. Erythroid cells (>95% by morphology) were recovered in the nonadherent fraction. Leukocytes were recovered by treatment with a solution of 0.05% trypsin-0.02% EDTA (GIBCO Laboratories, Grand Island, NY). For coating of plates, 10 mL of 0.5 mg/mL solution of affinity-purified antimouse IgG-IgM (Cooper Biomedical, West Chester, PA) were added to 100-mm Falcon dishes (Becton Dickinson, Oxnard, CA) and incubated overnight at 4°C. Plates were washed immediately prior to use with IMDM-2% FCS.

Subcellular localization. Cells were washed with Hank’s balanced salt solution (HBSS), suspended in cold reticulocyte standard buffer (RSB: 10 mM Tris-Cl, 15 mM MgCl2, 10 mM Tris-HCl, pH 7.5-0.2% Triton X-100, 100 mM/L phenylmethylsulfonyl fluoride (PMSF), 100 kallikrein inactivator U/mL of aprotinin (Boehringer-Mannheim, Indianapolis), homogenized in a Dounce apparatus (Fisher Scientific, Silver Spring, MD), and separated into cytoplasm and nuclear fractions by centrifugation at 400 x g x ten minutes. The cytoplasm fraction was cleared by centrifugation in a Microfuge apparatus (Beckman, Palo Alto, CA) at 12,000 rpm x five minutes; B19 parvovirus does not sediment under these conditions. Nuclei were rinsed twice in 0.25 mol/L sucrose, 5 mM MgCl2, 0.2% Triton X-100, 0.1 mL/L PMSF. Fractionation was confirmed microscopically.

Reinfection experiments. Infectivity of virus in culture supernatants was compared to the infectivity of B19 present in sera from an acutely infected patient. For these experiments primary cultures of erythroid bone marrow were washed free of residual inoculum at day 1, and supernatants were collected for testing two to three days later. Reinfection was accomplished by adding aliquots of supernatants, 20 to 50 uL, or Minor II serum, 0.004 to 0.13 uL, to a total volume of 1 mL containing 2 x 107 normal bone marrow cells in IMDM-20%FCS, 1 uM/e p. The reinfection cell cultures were incubated for a further two days, the cells were harvested, and input and output B19 DNA was quantitated by dot blot analysis.

Molecular studies. In situ hybridization. In situ hybridization was performed by slight modification of published techniques.14 In brief, cytocoentrifuge preparations were fixed in 4% paraformaldehyde/PBS and stored in 70% ethanol until use. Slides were acetylated in acetic anhydride-triethanolamine and pretreated with a glycine solution to reduce nonspecific binding. The cell preparations were hybridized without prior denaturation using the nick translated 32P-pYT103 probe (derived from a full length cloned B19 DNA15) in 50% formamide, 2 x SSC, 10% dextran sulfate at 42°C for four to six hours. After ten minutes at 37°C, digestion was terminated by addition of equal volumes of 2 x lysis buffer (1% SDS, 20 mL/L Tris-HCl, pH 7.5, 100 mL/L NaCl, 5 mM MgCl2, 0.1 mM PMSF). Cytoplasm prepared from 3 x 106 cells was digested with the same reaction buffer to 100 mL; 10 uL of carrier DNA extracted from uninfected bone marrow cells was added. The particulate fraction of supernatant was concentrated by sedimentation through a cushion of 40% sucrose in PBS at 38,000 rpm for 12 hours at 4°C and suspended in PBS containing 0.02% BSA; the amount equivalent to 1 mL of supernatant with carrier DNA was suspended to a final volume of 100 mL in reaction buffer. DNase I was added to each fraction at final concentrations of 0, 0.03, 0.1, 0.3, 1.0, 3.0, and 10.0 ug/mL. After ten minutes at 37°C, digestion was terminated by addition of double-stranded DNA, purified DNA extracted from infected cultures, obtained from patients with sickle cell disease, greatly reduced the number of erythroid cells, as determined morphologically (Fig 1, Table 1). In control cultures treated with equivalent amounts of normal sera, the total cell number increased modestly after about one week of culture, and the absolute number of erythroid cells remained constant. In contrast, B19-infected cultures showed a marked reduction in erythroid cell content beginning at day 2; by day 9 of culture, erythroid cells were consistently <10% of total cells and occasionally as low as 1%. The content of
Effect of B19 parvovirus infection on total and erythroid cell number. Cultures were inoculated with serum containing B19 (A) or normal serum (B), 10 µL/5 x 10⁶ cells, incubated in IMDM-20% FCS, 1 U/mL epo for times shown on the abscissa. Cell number was determined by counting in a hematocytometer and the proportion of erythroblasts by differential counting of Wright’s-Giemsa-stained cytocentrifuge preparations.

The morphological appearance of isolated erythroblasts in B19 infected cultures was frequently very abnormal (Fig 2). Infected cultures were characterized by the presence of cells of very large size, immature chromatin, and absence of hemoglobinization. Some cells showed large eosinophilic, nucleoli-like nuclear inclusion bodies.

Estimate of number of B19 target cells and B19 genome copy number. The proportion of cells infected with B19 parvovirus was estimated by in situ hybridization under conditions of high viral input (see below) 48 hours after inoculation of cultures. Under the conditions of hybridization used, both single-stranded DNA and RNA were detected. Approximately 15% of total cells from infected cultures showed dense accumulation of grains after hybridization with the B19-specific probe (data not shown). In control, uninfected cultures, no cells or very rare cells showed grains (data not shown). When infected bone marrow cultures were separated into erythroid and leukocyte fractions by a panning method utilizing antileukocyte MoAbs, only erythroid cells demonstrated B19-specific signals by in situ hybridization (Figs 3A, 3B). Between 30% and 40% of erythroid cells were infected. Grains were localized to large and intermediate cells and not to small cells of normoblast size.

To more accurately determine the amount of B19 produced in each target cell, dot blot analysis was performed on each cell fraction (Fig 4). No B19 DNA was detected in uninfected cultures. The erythroid fraction from infected cultures showed a strong B19 DNA signal, which by titration was equivalent to 10,000 genome copies/erythroid cell. Based on the estimated proportion of erythroid cells containing B19 sequences by in situ hybridization, the genome copy number in each infected target cell was approximately 25,000 to 30,000. In fractions enriched by panning for granulocytes, monocytes, and lymphocytes (contaminated <1% with recognizable erythroid cells by morphological

### Table 1. Effect of B19 Parvovirus Inoculation on Total and Erythroid Cells in Suspension Bone Marrow Cultures

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<tr>
<th>Experiment</th>
<th>Days in Culture</th>
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Experiments were performed as described in the legend to Fig 1 (the graphic display of experiment 1). Results did not vary with the patient source of bone marrow except for the initial proportion of erythroblasts.

Fig 1. Effect of B19 parvovirus infection on total and erythroid cell number. Cultures were inoculated with serum containing B19 (A) or normal serum (B), 10 µL/5 x 10⁶ cells, incubated in IMDM-20% FCS, 1 U/mL epo for times shown on the abscissa. Cell number was determined by counting in a hematocytometer and the proportion of erythroblasts by differential counting of Wright’s-Giemsa-stained cytocentrifuge preparations.

Fig 2. Morphology of B19 infected erythroid cells. Cytocentrifuge preparations from infected (A) and control (B) cultures at 48 hours were stained with Wright’s-Giemsa after separation of erythroid cells by panning as described. Some of the large eosinophilic nuclear inclusions are noted by arrowheads. Original magnification ×650; current magnification ×455.
Fig 3. In situ hybridization of B19 infected cells. Cytocentrifuge preparations of separated erythroid (A) and leukocyte (B) fractions from infected cultures obtained at 48 hours were hybridized with 35S-labeled pYT103, a B19 specific molecular probe as described. Erythroid and leukocyte fractions were separated by panning. Original magnification ×860; current magnification ×568.

Fig 4. Quantitation of B19 DNA in erythroid and leukocyte bone marrow cells by dot blot analysis. Mononuclear cells from the bone marrow of a patient with sickle cell disease were infected with Minor II serum as described in the legend to Fig 1. Forty-eight hours after inoculation cells were harvested and separated by panning into erythroid and leukocyte fractions. B19 DNA was quantitated by serial dilutions in a dot blot assay of 10^6 cells: (a) uninfected erythroblast fraction; (b) uninfected leukocyte fraction; (c) B19 infected erythroblast fraction; (d) B19 infected leukocyte fraction; (e) pYT103, 10^3 pg and serial dilutions.

Fig 5. Immunofluorescence of B19 protein in infected cells. Cytocentrifuge preparations obtained 48 hours after B19 inoculation were fixed in acetone and stained with rabbit anti-B19 capsid protein antiserum followed by antirabbit IgG-FITC: (A) infected, (B) control. Original magnification ×840; current magnification ×664.
infected cultures (Fig 7). In this method, nuclei, cytoplasm, and supernatants were digested with DNase I prior to DNA extraction (intact virions are resistant to DNases\(^6\)). The double-stranded monomer replicative form in nuclei was DNase I sensitive, but single-stranded virus DNA in nuclei was completely resistant to enzyme, similar to virus DNA in cytoplasm and supernatant. Purified single-stranded DNA was equally well digested as double-stranded DNA by DNase I, whether extracted from virus present in serum or from infected cell nuclei (Fig 8). This combination of results implied that viral encapsidation occurred within the nucleus immediately after the cleavage of double-stranded replicative forms to single-stranded forms.

**Time course and subcellular localization of B19 production.** B19 production was estimated using dot blot analysis of DNA in infected cells and supernatants (Fig 9A). Ten microliters of serum containing virus was added to a total of \(5 \times 10^5\) erythroid bone marrow cells. The total amount of B19 DNA in cultures (cells and supernatants) was initially reduced at 12 hours, suggesting partial degradation of the input virus. The first strong signal of B19 DNA within cells appeared at 18 hours, and B19 DNA content in cells thereafter increased dramatically, peaking at 48 hours. The decline in B19 DNA within cells from days 2 to 9 paralleled the fall in erythroid cell number. For measurements of B19 DNA in supernatant, residual viral inoculum was removed by washing at day 1. B19 DNA appeared in the supernatants of infected cultures at 32 hours and reached a plateau at day 4.

The subcellular localization of B19 DNA was determined by dot blot analysis after fractionation of infected cells into cytoplasmic and nuclear compartments (Fig 9B). About 95% of B19 DNA was localized to the nucleus, and this ratio remained stable throughout the culture period.

**Effect of multiplicity of infection on B19 virus production.** Efficiency of virus production was highly dependent on the ratio of input virus to bone marrow cells (Fig 10). When measured at day 2, relatively high concentrations of serum-containing virus were required for maximal output. However, when measured at days 5 or 9, tenfold less virus resulted in maximal B19 production, measured in total cultures, cells, and supernatants. At low amounts of input virus, about 50× higher amounts of B19 were produced in supernatant and >200× output virus was measured in total cultures (Fig 10B). These results suggest amplification of the virus signal at low input concentrations by infection of target cells with newly synthesized virus.

**Reinfection with culture supernatants.** To determine if infectious virus was produced in erythroid bone marrow cultures, aliquots of supernatants from days 3 to 4 were added directly to fresh bone marrow cultures (Table 2). Production of B19 in the secondarily infected cultures was assayed at day 2 and compared with B19 production by simultaneous cultures infected with equivalent amounts of B19-containing serum. In supernatants from two cultures, relative infectivity was superior to serum, and in another the supernatant was equally potent to serum.

**DISCUSSION**

The suspension culture system for B19 parvovirus has several important features. First, virus replication is dependent on the erythroid cell content of the cultures and the
Fig 8. Comparison of DNase I sensitivity of single and double-stranded B19 DNA. Purified DNA extracted from Minor II serum (virions: [A]) and from infected nuclei (B) were digested as described in the legend to Fig 7.

presence of erythropoietin; virus replication does not occur in bone marrow cultures lacking detectable erythroid progenitors\(^1\) or in the leukocyte fraction of bone marrow. Second, B19 virus is released from cells into culture supernatants, as demonstrated by the appropriate banding on CsCl gradients of \(^{3}H\)-thymidine-labeled parvovirus particles,\(^2\) the 50-fold output of virus into supernatant at low multiplicity of infection, and the potency of supernatants in reinfecting bone marrow. Because the highest ratio of virus output to input occurred at low multiplicity of infection (moi) characteristic of cytolytic infection, the usefulness of this culture system for production of virus is limited by the number of target cells that can be obtained in a bone marrow aspirate and the difficulty of maintaining erythropoiesis in vitro for extended periods of time.

Except for the use of terminally differentiating cells as targets, B19 propagation in marrow cells appears quite biologically similar to the propagation of other autonomous paroviruses in cell culture lines. Animal paroviruses have generally been cultivated in monolayer cell cultures and quantitated with plaque forming assays,\(^6\) complicating comparison with the bone marrow culture system for B19. All autonomous paroviruses require S phase cells for replication.\(^7\) The kinetics of infection of B19 parovirus appear similar to that of other paroviruses, with appearance of
delayed compared with cell-associated event of release into the supernatant. In contrast to B19 viruses, most virus produced remains cell associated for B19 in bone marrow and for other paroviruses, and the appearance of virus in culture supernatants is delayed compared with cell-associated virus. Virus replicates and is encapsidated only within the nucleus, consistent with electron microscopic data. As shown in the cell fractionation experiments, most of the B19 DNA produced remained in nuclei rather than entering the cytosol for eventual release into the supernatant. In contrast to B19 DNA, most B19 protein was localized to the cytoplasm.

Estimates of true multiplicity of infection are complicated by the absence of a convenient assay for infectious B19 particles, such as a plaque titration, and uncertainty concerning the nature and number of parovirus target cells in erythroid cell culture systems. Most virus produced remains cell associated for B19 in bone marrow and for other paroviruses, and the appearance of virus in culture supernatants is delayed compared with cell-associated virus. Virus replicates and is encapsidated only within the nucleus, consistent with electron microscopic data. As shown in the cell fractionation experiments, most of the B19 DNA produced remained in nuclei rather than entering the cytosol for eventual release into the supernatant. In contrast to B19 DNA, most B19 protein was localized to the cytoplasm.

Estimates of true multiplicity of infection are complicated by the absence of a convenient assay for infectious B19 particles, such as a plaque titration, and uncertainty concerning the nature and number of parovirus target cells in erythroid bone marrow. Assuming roughly 90% of virus input is infectious (by subtraction of the percentage of empty capsids usually seen in immune electron microscopy), a viral titer of $2 \times 10^3$ particles/mL serum, and 15% of the bone marrow cells serving as targets, the optimal multiplicity of infection at 48 hours was about 20,000 viral particles/cell and at 120 hours, 2,000 viral particles/cell. However, the true optimal moi of B19 may be considerably lower if the ratio of infective particles to particles is as low as directly measured for other paroviruses (minute virus of mouse, 1:300 to 400; adenovirus, 1:10 to 100).

B19 parovirus infection greatly reduces the number of recognizable erythroid cells in suspension cultures and alters the morphology of residual erythroid precursors; this culture system thus reproduces two of the cardinal features of the bone marrow of patients with transient aplastic crisis, characterized by a paucity of erythroid precursors and the presence of giant pronormoblasts, the latter possibly related to arrest of mitosis.

Similar bone marrow morphology has been seen in a case of persistent B19 parovirus infection resulting in chronic bone marrow failure. To date human bone marrow cells are the only cells identified to support B19 parovirus propagation in vitro. In normal volunteer infections, viremia and reticulocytopenia were coincident early in the clinical illness. The symptoms of fifth disease—rash and polyarthritis—are late manifestations at the time of appearance of IgM and IgG antibodies specific for B19; these symptoms do not appear with great frequency in transient aplastic crisis patients. These clinical differences may result from different quantities of B19 virus and antibody to virus produced in hematologically normal individuals compared with patients with chronic hemolysis who have greatly expanded erythroid cell compartments.

Because of the much larger numbers of cells that can be studied from suspension cultures compared with clonal assays, a fuller description of the interaction of B19 and hematopoietic progenitors at the cellular and molecular levels is now possible. This cell culture system has allowed construction of a transcriptional map and definition of the structure and function of capsid and noncapsid B19 proteins. Perhaps most important, the availability of a suitable culture system with readily assayed variables of infection—cell number, morphological changes, and B19 gene sequences by dot blotting—in conjunction with nonpermissive cells in culture should allow characterization of the basis of B19 parovirus' extraordinary specificity for erythroid cells.

ACKNOWLEDGMENT

We are very grateful to Dr Peter Tattersall of Yale University for his generous gift of the pYT103 clone and anti-B19 capsid protein antiserum.

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