JCI The Journal of Clinical Investigation

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J Clin Invest. 1984;74(6):2024-2032. https://doi.org/10.1172/JCI111625.

Research Article

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Direct Demonstration of the Human Parvovirus in Erythroid Progenitor Cells Infected In Vitro

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Abstract. The human parvovirus (HPV), the cause of transient aplastic crisis of hereditary hemolytic anemia, has been shown to be cytotoxic for erythroid progenitor cells and its presence in these cells demonstrated by morphologic techniques. A relatively pure population of progenitors, isolated by removal of immature erythroid bursts from primary culture, was the target of the virus infection. Infected cells failed to proliferate in secondary culture. Using a monoclonal antibody to HPV, specific fluorescence was demonstrated in a minority of cells 24–48 h after infection with virus. Infected cells examined by electron microscopy showed marked toxic ultrastructural alterations and parvovirus-like particles in crystalline arrays in the nucleus.

Introduction

Parvoviruses, the smallest DNA-containing animal viruses, are responsible for a wide variety of diseases in mammals and birds (1). In 1975 parovirus-like particles were discovered in the sera of blood donors during an investigation of an anomalous serologic reaction (2). Antibody to the antigen of these particles was found to be common in the adult population (3). Screening of sera of children revealed the presence of the anitgen and IgM-type antibody to it among children with sickle cell disease who suffered from aplastic crises (4, 5), and evidence of acute infection with this agent preceding erythroblastopenic crises has now accumulated for several different hemolytic diseases (6). In normal children, the virus has been implicated as the etiologic agent in the common childhood exanthem called fifth disease (7). There is now strong evidence that the virus is a parvovirus (8, 9), and it is generally referred to as human parvovirus (HPV).[1]

We have investigated the mechanism by which HPV inhibits erythropoiesis. Sera containing HPV are potent inhibitors of erythroid colony formation in vitro, and the inhibitory effect is specific for erythroid compared with myeloid progenitor cells (10). Only the virus-particle containing fraction of sera is inhibitory, and antibody to the virus can neutralize its activity (10, 11). A target of HPV can be demonstrated to be an erythroid progenitor cell by experiments in which maturing erythroid colonies are separated from other cultured bone marrow cells before infection (11, 12). We report the analysis of such isolated cells using a variety of techniques, including serial in situ observation, immunofluorescence with a specific monoclonal antibody to HPV, and electron microscopy. These studies show that HPV is cytotoxic for human erythroid progenitor cells and demonstrate HPV in the nucleus and ctyoplasm of red cell precursors.

Methods

Source of HPV and anti-HPV monoclonal antibody. Sera drawn from two patients (JB and JH) with congenital hemolytic anemias and transient aplastic crisis served as the source of HPV; these sera behave identically in tissue culture experiments (11) and are of similar antigenic potency (10). A murine hybridoma IgG antibody to HPV (from blood donor B19) was prepared as described (3).

Cell culture. Bone marrow aspirates were obtained from normal volunteers who had given informed consent; mononuclear cells were separated by sedimentation through Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). Bone marrow mononuclear cells were grown in methylcellulose medium with growth factors to promote formation of erythroid colonies derived from the primitive progenitor, BFU-E, and more mature progenitor, CFU-E (11, 12). Erythroid bursts, derived from the BFU-E, require 2–3 wk to reach full maturity in culture; however, immature nonhemoglobinized bursts can be recognized by their morphologic features at 1 wk and removed from primary culture with a micropipette. Isolated cells from colonies at this stage of development represent a pure population of erythroid progenitors

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Received for publication 18 May 1984 and in revised form 16 August 1984.

The Journal of Clinical Investigation, Inc.
Volume 74, December 1984, 2024–2032

1. *Abbreviation used in this paper:* HPV, human parvovirus.

similar to CFU-E and on replating in fresh medium will form secondary erythroid colonies with a high plating efficiency (11).

To infect cells, 20–100 immature bursts were removed to 50 μl of Iscove's modification of Dulbecco's medium that contained 2% fetal calf serum in 1.5-ml Eppendorf bullet tubes (Brinkmann Instruments Inc., Westbury, NY). Cells were suspended in either HPV-containing or normal sera to a final concentration of 10^{-3} for 2 h at 4°C. To measure and observe intact erythroid colonies, cells in 1 ml of tissue culture medium were transferred to 35-mm suspension culture dishes (Lab-Tek; Miles Laboratories, Elkhart, IN); for experiments in which cells were to be isolated, 0.5 ml of tissue culture medium was added to each bullet, the tubes covered with perforated Parafilm (American Can, Greenwich, CT), and cultured for 24, 48, or 72 h at 37°C, 95% humidity, and 5% CO_2 before harvesting. To recover cells, the Eppendorf tubes were centrifuged in a Beckman Microfuge 12 (Beckman Instruments Inc., Palo Alto, CA) for 7 min at 1,000 rpm. Supernatants were gently removed and the cells washed twice in phosphate-buffered saline (PBS), pH 7.5. For conventional staining and immunofluorescence cells were pelleted onto slides using a Cytospin 2 (Shandon Southern Instruments, Sewickley, PA) cytocentrifuge, 500 rpm × 10 min.

Immunofluorescene. Methods in general use (13) were adapted for detection of HPV. After, cytocentrifugation slides were air-dried and fixed in acetone at 4°C for 1 min. Slides were precoated with normal goat serum diluted 1:5 in PBS-0.2% bovine serum albumin (BSA) to block nonspecific binding. Washes were performed by dipping slides into PBS-0.2% BSA. Anti-HPV antibodies used in the indirect immunofluorescent procedure included hybridoma supernatant from clone VRL/B19/11 (3) and purified IgG from ascites fluid (maximal concentration 2 mg/ml) of the same clone; control sera included mouse IgG (Miles Laboratories Inc., Elkhart, IN), a monoclonal antibody to hepatitis B surface antigen (Hybritech Inc., LaJolla, CA), and nonimmune hybridoma supernatant. The area of the slide onto which cells had been pelleted was treated with 5 μl of purified IgG or 50 μl of hybridoma supernatants for 30 min at room temperature. After washing, 50 μl of affinity purified, fluorescein-conjugated goat anti-mouse gamma chain (Kirkegaard and Perry, Gaithersburg, Maryland) diluted 1:50 in PBS was added for 30 min at room temperature. After final washing and air-drying, slides were mounted using neutral 5% *n*-propyl gallate in glycerol and examined with a Universal III photo microscope (Carl Zeiss, Oberkochen, Federal Republic of Germany) which employed an XBO 150 xenon lamp and standard Zeiss filters for epifluorescence of fluorescein.

Electron microscopy. (14, 15). In order to recover sufficient cells for examination by electron microscopy, special care was taken in harvesting cells from culture. Immature bursts removed from tissue culture media were washed three times in PBS in 1.5-ml polypropylene micro-tubes by centrifugation at 1,200 rpm in a Sorvall RT 6000 centrifuge (DuPont Instruments, Newtown, CT) at 4°C. Supernatants were removed by gentle aspiration. Cells were fixed by exposure to 2% electron microscope grade glutaraldehyde (Polysciences, Inc., Warrington, PA) in PBS for 25 min at 4°C and postfixed with 1% osmium tetroxide (Polysciences, Inc.) for 1 h at 4°C. After removing excess osmium tetroxide by washing, one drop of 12.5% human serum albumin was added and the cells were immediately centrifuged. The resulting gel allowed the cell pellet to be treated as a small block during dehydration and embedding in Epon 812 (Ladd Research Industries, Inc., Burlington, VT). After polymerization, trimmed blocks were cut into 600A sections, placed onto 300 mesh copper grids (Ted Pella Inc., Irvine, CA), double stained with uranyl acetate (LKB Instruments, Gaithersburg, Maryland), followed by staining with lead citrate (LKB), and examined and photographed using a Zeiss EM-10 electron microscope (Carl Zeiss, Oberkochen, Federal Republic of Germany) at 60 kV acceleration voltage.

Results

In situ observations of HPV-infected progenitor cells. Infection with HPV of the cells of an immature erythroid burst, which represent a population of erythroid progenitor cells similar to CFU-E, results in severe reduction of secondary colony formation (11). Detection of colonies requires both cell proliferation and erythroid differentiation, and apparent decreased colony formation could be the result of interruption of either process. Serial observations of replated erythroid colonies by light microscopy showed that HPV-infected cells failed to proliferate. Progressive doubling of cells and formation of 16–64 cell colonies, accompanied by gradual hemoglobinization, were visible in the control, uninfected cultures (Fig. 1 *B*). In the plates treated with HPV, however, no growth beyond the four-cell stage of colony formation was seen (Fig. 1 *A*). At this point, 24–48 h after plating in fresh medium, the cells within these abortive colonies showed crenated outer surfaces and loss of the normal refractile appearance of healthy cells.

Immunofluorescent detection of HPV using a monoclonal antibody. To detect HPV in infected erythroid progenitor cells, immature bursts were removed from culture and exposed to HPV-containing or control sera for 2 h. Cytocentrifuge preparations were made from aliquots of infected and control cells immediately after the 2 h incubation with sera, and 24, 48, and 72 h after replating in fresh medium. Both hybridoma supernatant and purified antibody from ascites fluid allowed detection of a specific pattern of fluorescence in HPV-infected erythroid cells (Fig. 2). At 24–48 h after exposure, particulate fluorescence was observed mainly in the cytoplasm in 5–10% of cells; nuclear fluorescence was also observed in occasional cells (Fig. 2, *A* and *B*). Cell preparations at 2 and 72 h showed no specific fluorescence. The specificity of staining with the monoclonal antibody was confirmed by examination of HPV-infected cells that were stained with mouse IgG, a nonimmune hybridoma supernatant, anti-hepatitis B surface antigen antibody (Fig. 2 *C*), and uninfected erythroid cells that were stained with anti-HPV monoclonal antibody (Fig. 2 *D*).

Ultrastructural changes in HPV-infected progenitors. Uninfected immature erythroid cells showed ultrastructural features that have been associated with erythroid progenitors in methylcellulose culture (16, 17): single large and slightly irregular nuclei; small numbers of mitochondria; scanty endoplasmic reticulum; abundant polyribosomes; infrequent small cytoplasmic vacuoles; and ferritin particles in small vesicles (Fig. 3, *E* and *F*). Progenitor cells examined immediately after a 2-h incubation with HPV-containing serum appeared normal. Cells that had been incubated with HPV and then cultured for 24–48 h showed major morphologic alterations. The cytoplasm showed marked vacuolization and pseudopod formation (Fig. 3, *A–C*); the mitochondria of infected cells were

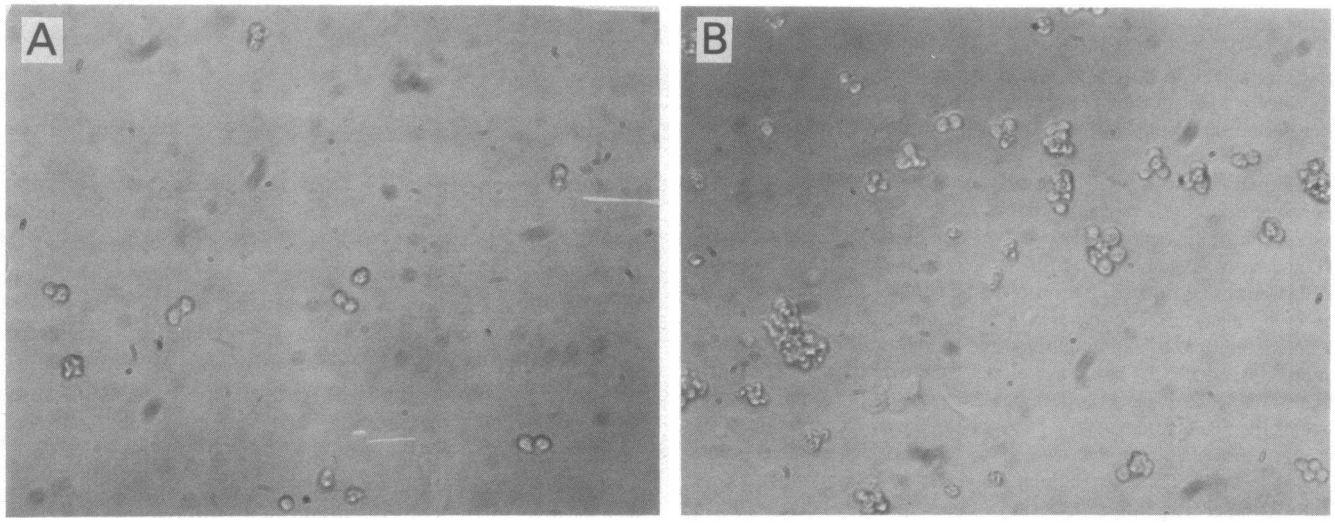

Figure 1. The appearance of developing secondary erythroid colonies in situ after replating. In this experiment, 30 developing bursts were identified by their morphologic characteristics and removed with a micropipette from primary methylcellulose culture. After exposure to HPV-containing (*A*) or normal (*B*) sera for 2 h at 4°C, the cells were replated in fresh culture medium for 48 h. The infected progenitor cells did not proliferate beyond the four-cell stage. × 200 (Polaroid type 665, Zeiss inverted microscope).

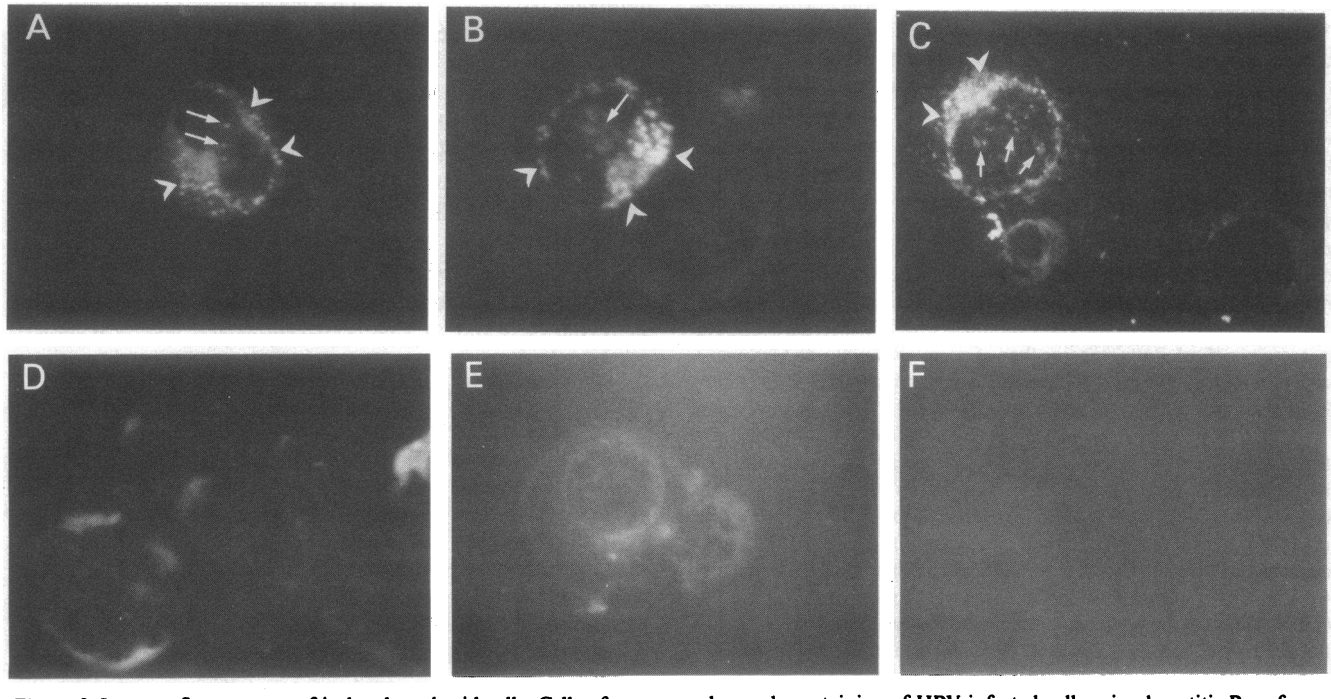

Figure 2. Immunofluorescence of isolated erythroid cells. Cells of immature bursts plucked from primary methylcellulose culture were exposed for 2 h at 4°C to either HPV-containing sera (*A–D*) or control sera (*E, F*) and cultured in fresh methylcellulose for 24–48 h. A specific pattern of particulate green fluorescence was observed mainly in the cytoplasm (large arrowheads) but also in the nucleus (small arrows) of infected cells on staining with anti-HPV hybridoma supernatant 11 (*A–C*). Only a nonspecific, diffuse yellow color was observed on staining of HPV-infected cells using hepatitis B surface antigen monoclonal antibody (*D*) or nonimmune hybridoma supernatant or mouse IgG (not shown), nor was specific fluorescence observed when uninfected cells were stained with hybridoma supernatant 11 (*E, F*). Automatic, full exposure of the film was used to photograph *A–D* and for the control *E;* for control cells in *F*, the exposure time was adjusted to be identical to that of infected cells (4½ min). × 840 (Kodak Ektachrome ASA 400, Zeiss microscope).

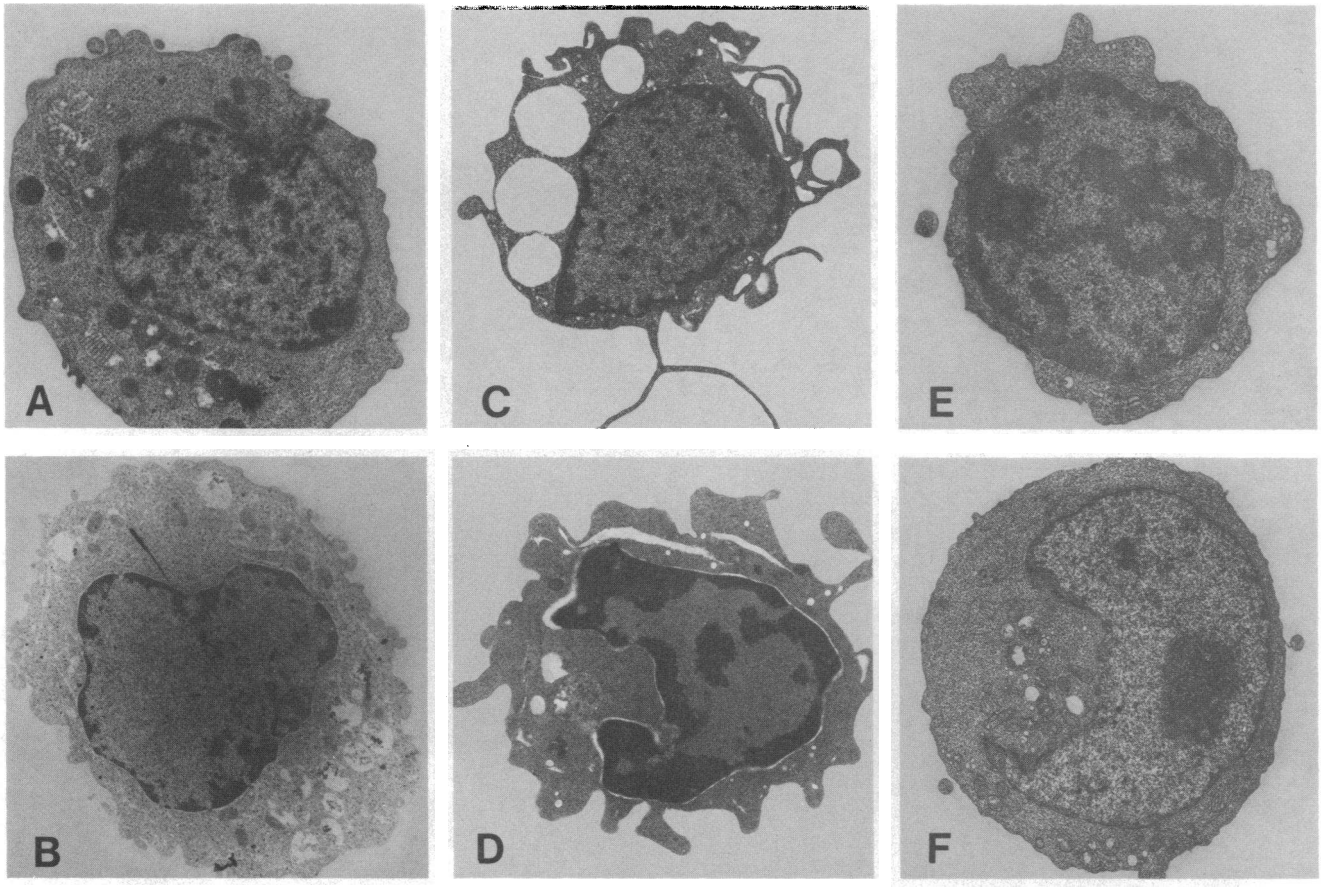

Figure 3. Electron micrographs of HPV-infected erythroid progenitor cells. Immature bursts were isolated as described in the legend to Fig. 1 and incubated with HPV-containing or normal sera. Photomicrographs shown were obtained at 2 h (*A*), 16 h (*B*), and 48 h (*C, D*) after initial infection and subsequent growth in fresh culture medium; uninfected cells at 48 h after treatment with control serum are shown in *E* and *F*. Infected cells at 48 h showed margination of the chromatin, extreme vacuolization, pseudopod formation, and shrunken mitochondria. × ~10,000 (Kodak 4489 film, Zeiss microscope).

smaller than normal and had disturbed lamellar structure. The nuclei of infected cells were characterized by a granular appearance of the nucleoli and margination of the chromatin (Figs. 4 and 5).

Identification of HPV in infected cells. Viruslike particles were identified in the nuclei of HPV-infected cells, especially in the areas of marginated chromatin (Fig. 4), but also within central areas of the nucleus that were not associated with chromatin (Fig. 5). Viruslike particles had an average diameter of 18 nm, and an icosahedral structure could be inferred from their appearance at higher magnification (Fig. 6). Viruslike particles in the nucleus were observed in regular arrays, sometimes within lacunar structures at the nuclear-cytoplasmic border. Particles were more easily observed in the nucleus 48 h after infection than at 24 or 72 h. Because of their similar size, viruslike particles could not be reliably discriminated from ribosomes in the cytoplasm. At 72 h after infection, irregular clumps of viruslike particles were observed in cytoplasmic vacuoles (data not shown).

Discussion

Morphologic studies of normal bone marrow cells infected with HPV and grown in methylcellulose show that this virus is cytotoxic for an erythroid progenitor cell and that parvovirus-like structures can be demonstrated in the nucleus and cytoplasm of these cells by electron microscopy. The technique of reculture of progenitor cells offers the advantage of isolation of erythroid cells at a defined stage of erythroid differentiation. However, the use of sera as a source of virus and isolated bone marrow cells as targets is not directly comparable to infection of synchronized cell populations with purified virus stocks. Because erythroid cells undergo terminal differentiation

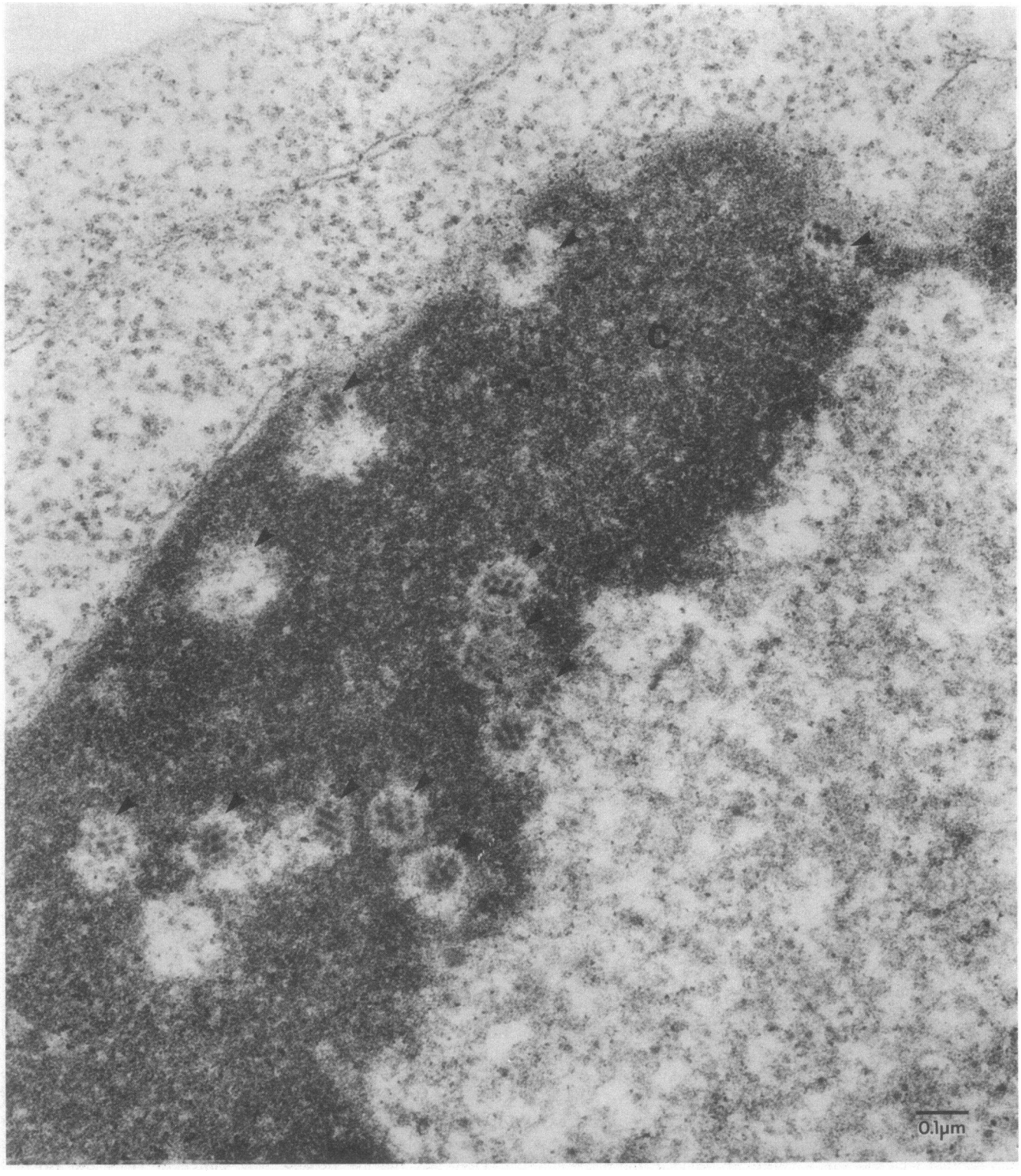

Figure 4. Area of marginated chromatin of HPV-infected erythroid progenitor cell. Bone marrow cells were isolated, infected for 48 h, and cultured as described in the legend to Fig. 1. Within the marginated chromatin (c), there are many lacunae (arrows) containing crystalline arrays of parvovirus-like structures. × 100,000 (Kodak 4489 film, Zeiss microscope).

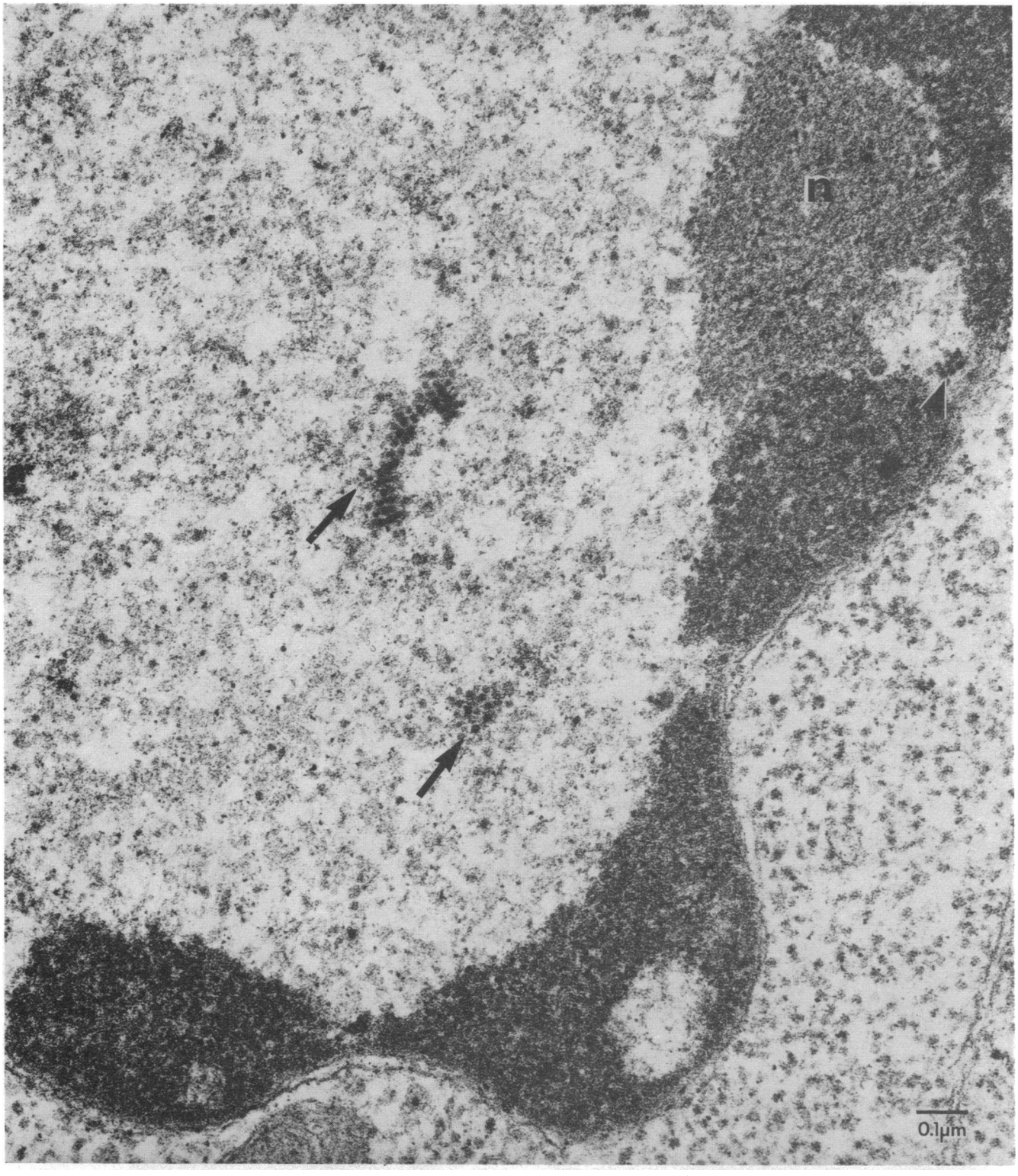

Figure 5. Nuclear interior and nucleolus of HPV-infected erythroid progenitor cell. Cells were isolated, infected, and recultured for 48 h as described in the legend to Fig. 1. The nucleolus (n) is abnormally granular in appearance. Parvovirus-like structures are seen both within lacuane in the marginated chromatin (arrowhead) and in the interior of the nucleus (arrows). × 100,000 (Kodak 4489 film, Zeiss microscope).

Figure 6. Parvovirus-like particles in the nucleus of an HPV-infected cell. Bone marrow erythroid progenitor cells were isolated, infected, and recultured for 48 h as described in the legend to Fig. 1. The virus-like particles (arrow) were icosahedral and measured on average of 18 mm in diameter, which is consistent with the structural features of a parvovirus. × 500,000 (Kodak 4489 film, Zeiss microscope).

to a nonproliferative state, the methylcellulose system would be unlikely to support sustained parvovirus replication, which is very dependent on a substrate of actively dividing cell hosts (18, 19). The usual time course of parvovirus infection in vitro begins with virus absorption within 1–2 h, followed by a latent period of 10–16 h, and expression of complete virus particles at 24–30 h (18, 20–22). In the experiments reported here with HPV, maximal expression was detected by immunofluorescence and electron microscopy at 48 h, but the large sampling errors and small amount of virus detected preclude conclusions regarding real differences in the kinetics of infection by HPV compared to other parvoviruses.

Our observations point to some important aspects of HPV infection of human bone marrow cells. First, like other parvoviruses (18, 19, 21), HPV is cytotoxic for its host cells. In situ observations indicate that infected cells fail to proliferate, and that the effect of HPV is not therefore due to a block in differentiation alone. Infected cells also show cytoplasmic ultrastructural changes associated with toxicity, including vacuolization, mitochondrial deformity, and pseudopod formation.

Second, only a minor proportion of infected cells had evidence of HPV replication by immunofluorescence. In contrast, the functional effect of in vitro HPV infection is to eliminate almost completely secondary erythroid colony formation (11). HPV infection might be expected to be very efficient, as so high a proportion of CFU-E are sensitive to cell-cycle specific agents (23). The viral product detected by the monoclonal antibody may not be expressed in all infected cells. The parvovirus genome also encodes for nonstructural proteins that may alter normal cell regulation (24, 25), and expression of capsid proteins may not be required to cause cell death (Tattersall, P., personal communication).

HPV infection of erythroid progenitors may not lead to production of infectious virions. We have been unable to passage virus from either methylcellulose or suspension cultures of erythroid bone marrow (Young, N., and J. Moore, unpublished data). Nor has HPV been successfully propagated in culture, despite efforts to infect many different cell types including such rapidly proliferating cell lines as African green monkey kidney cells, feline kidney cells, murine myeloma cells, and human fibroblasts, and lymphoid leukemia and erythroleukemia cells (Young, N., and J. Moore; P. Mortimer, unpublished data). Abortive and replication defective production of HPV in progenitor cells in vitro may account for the absence of the pattern of diffuse nuclear fluorescence described in some studies of human adeno-associated viruses (26). The punctate nuclear fluorescence observed with HPV infection was consistent with the relatively small lacunae containing virus particles seen on electron microscopy.

Third, while some parvoviruses, like the human adeno-associated viruses, require co-infection with a second virus in order to successfully replicate, there was no evidence from electron microscopy of a second, morphologically distinct virus proliferating in infected bone marrow cells. Although some biochemical studies have suggested similarities between HPV and the human adeno-associated viruses (8, 9), the molecular features of the cloned virus link it evolutionarily to the helper independent parvoviruses of rodents rather than to human adeno-associated viruses (27). The molecular basis for the fastidious behavior of HPV in tissue culture therefore remains uncertain.

Finally, the appearance of HPV in nuclei is similar to that described for other parvoviruses (21, 22, 28, 29). Viruslike particles were present in the marginated chromatin in clumps and crystalline arrays; by analogy with other parvoviruses, it is likely that virus is manufactured in the interior of the nucleus, employs the nucleolus in its replication, and is transported outwards to the cytoplasm. The 18 nm particle size of HPV in thin tissue section, though smaller than the 23 nm size of HPV in immune precipitated sera (2), is comparable to the size of other animal parvovirus in tissue sections (29, 30). Also, in contrast to the appearance of HPV in sera was the absence in tissue sections of empty capsids.

These morphologic studies complement functional studies in establishing that the pathogenesis of the transient aplastic crises of hereditary hemolytic anemias is cytotoxicity of HPV for bone marrow erythroid progenitor cells. The availability of molecular probes for the HPV genome (27) should facilitate the study of possible HPV involvement in chronic forms of bone marrow failure and in other diseases.

Acknowledgments

We thank Drs. Naomi Luban and Jack Kelleher for providing sera JB and JH. We are grateful for the expert advice in immunofluorescent technique provided by Dr. Stephen Lippman. Drs. David Hoggan, James Rose, and Peter Tattersall were very helpful in the interpretation of our data.

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