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Replication of B19 Parvovirus in Highly Enriched Hematopoietic Progenitor Cells from Normal Human Bone Marrow

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The target cell specificity of the B19 parvovirus infection was examined by isolating highly enriched hematopoietic progenitor and stem cells from normal human bone marrow. The efficiency of the B19 parvovirus replication in enriched erythroid progenitor cells was approximately 100-fold greater than that in unseparated bone marrow cells. The more-primitive progenitor cells identical to or closely related to the human pluripotent hematopoietic stem cells, on the other hand, did not support viral replication. The B19 progeny virus produced by the enriched erythroid progenitor cells was infectious and strongly suppressed erythropoiesis in vitro. The susceptibility of both the more-primitive erythroid progenitors (burst-forming units-erythroid) and the more-mature erythroid progenitors (CFU-erythroid) to the cytolytic response of the virus and the lack of effect on the myeloid progenitors (CFU-granulocyte-macrophage) further give evidence to the remarkable tropism of the B19 parvovirus for human hematopoietic cells of erythroid lineage.

Parvoviruses are among the smallest of the DNA-containing viruses which infect a wide variety of vertebrate animals (4, 12, 27). Whereas parvoviruses of other animal species are frequently pathogenic, a parvovirus of human origin, designated B19 (10), has only recently been shown to be pathogenic and is now known to be the etiologic agent of several clinical disorders in humans, including transient aplastic crisis associated with a variety of hemolytic anemias (23, 26), a common childhood rash called "fifth disease" or erythema infectiosum (2), postinfection arthropathy (24, 29), and possibly hydrops fetalis (1, 5, 13). The B19 parvovirus has been successfully propagated in fresh human bone marrow cells (21, 22) and shown to be cytotoxic to progenitor cells in the erythroid lineage (20, 25, 30, 31). In view of the heterogeneous nature of bone marrow cultures, the low-frequency occurrence of hematopoietic progenitor cells within the bone marrow, and the low-titer B19 progeny virus production in these cultures, we undertook the present studies, in which we enriched hematopoietic progenitor cells from normal human bone marrow, to address these questions directly and to further define the target cell specificity of the parvovirus B19 infection of cells in the erythroid lineage.

Parvovirus B19 replication in unseparated and in highly enriched hematopoietic progenitor cells from normal human bone marrow. Parvovirus B19 replication was measured on quantitative DNA dot blots of culture supernatants of uninfected and B19-infected cell cultures by DNA hybridization to a B19-specific cloned DNA probe, pYT103 (11), essentially as described by Ozawa et al. (21). Briefly, nonadherent, low-density, T-cell-depleted ($NALT^-$) mononuclear cells were isolated by Ficoll-Paque density gradients (specific gravity, 1.077 g/cm^3), adherence, and E rosetting as described previously (17, 18) from fresh human bone marrow aspirates obtained from hematologically normal volunteers with informed consent. $NALT^-$ cells were either used directly or fractionated further to isolate highly enriched progenitor cells which were incubated at 4°C for 2 h with serum containing the B19 parvovirus (Minor II; 60 μg of B19 DNA per ml obtained from a patient with sickle-cell anemia during aplastic crisis [25]). Cells were washed twice with sterile phosphate-buffered saline (pH 7.0) to remove the virus inoculum and suspended in Iscove modified minimal essential medium containing 20% fetal calf serum and 1 U of erythropoietin per ml, as described by Ozawa et al. (21). Cultures were incubated at 37°C (5% CO_2, 95% humidity) for 48 h. Culture supernatants were collected, clarified of cell debris by centrifugation at 13,000 × *g* for 5 min, adjusted to a final concentration of 0.5 M NaOH to disrupt the progeny virions, and incubated at 65°C for 1 h to degrade RNAs and to denature DNAs. Equivalent amounts (200 μl) of culture supernatants from uninfected and B19-infected cells were neutralized with 20× SSC (standard saline-sodium citrate [pH 7.0]), and twofold serial dilutions were filtered through 0.45-μm (pore size) nitrocellulose filters on quantitative DNA dot blots. Baked filters were probed with ^{32}P-labeled B19-specific cloned DNA pYT103 insert (11) (Fig. 1).

Figure 1A shows the extent of B19 replication in 6×10^6 $NALT^-$ cells from bone marrow cells obtained from three different normal donors. All three cultures produced similar degrees of hybridization intensity, whereas the culture supernatants from control, uninfected bone marrow cells showed no B19 hybridization signal. These data are similar to those reported by Ozawa et al. (21).

We next compared the extent of B19 replication in unsorted $NALT^-$ cells with that in highly enriched hematopoietic progenitor cells from normal human bone marrow. The enrichment was carried out as described previously (18; J. Brandt, N. Baird, L. Lu, E. Srour, and R. Hoffman, J. Clin. Invest., in press). Briefly, $NALT^-$ cells were reacted with monoclonal antibodies which recognize antigenic determinants on human hematopoietic progenitor cells. Two monoclonal antibodies, mouse My10 specific for human progenitor cell antigen 1 and mouse anti-major histocompatibility class II, were used at saturating levels and sorted on a Coulter Epics 753 Dye Laser Flow Cytometric System essentially as described previously (18). Two distinct populations of cells were obtained: one population with a high expression of My10 antigen and a low expression of major histocompatibility class II antigen ($My10^{++}DR^+$) and which

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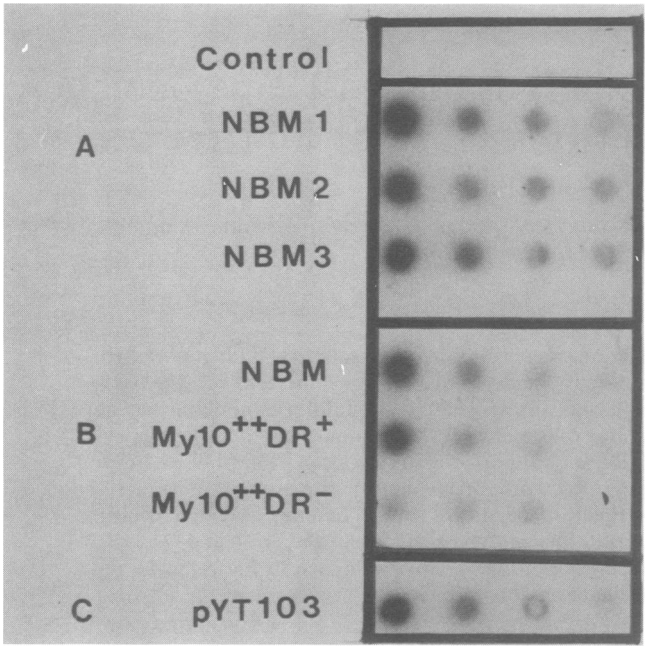

FIG. 1. Dot blot analysis of B19 replication in unsorted and highly enriched hematopoietic progenitor cells from normal human bone marrow. (A) B19 DNA from culture supernatants of unseparated normal bone marrow (NBM) cells from three different volunteer donors. (B) Comparison of B19 DNA replication in 6×10^6 $NALT^-$ cells and 1×10^5 hematopoietic progenitor cells highly enriched with $My10^{++}DR^+$ or $My10^{++}DR^-$ from normal human bone marrow. (C) B19-specific cloned DNA insert. All steps were carried out as described in the text.

contains the majority of the erythroid progenitors (8, 9, 18), and the other population with a high expression of My10 antigen and a nondetectable expression of major histocompatibility class II antigen ($My10^{++}DR^-$) and which contains few erythroid progenitors except for the more-primitive cells similar or identical to the human pluripotent stem cells (Brandt et al., in press). Approximately 6×10^6 $NALT^-$ cells and 1×10^5 cells each from the $My10^{++}DR^+$ and $My10^{++}DR^-$ populations were adsorbed with equivalent amounts of the B19 parvovirus-containing serum at 4°C for 2 h, and the rest of the steps were carried out exactly as described above (Fig. 1B).

It is interesting to note that the B19 hybridization signal from approximately 60-fold fewer $My10^{++}DR^+$-enriched progenitor cells was comparable with that from unsorted $NALT^-$ cells, indicating that the $My10^{++}DR^+$ cells supported a more efficient replication of the B19 parvovirus. The $My10^{++}DR^-$ cell population, on the other hand, was very inefficient in supporting viral replication. In view of the well-known tropism of the B19 parvovirus for hematopoietic cells in the erythroid lineage, it is noteworthy that the $My10^{++}DR^+$ cell population contains the majority of the erythroid progenitor cells (8, 9, 18) and, hence, supports the more efficient replication of the virus. The lack of B19 replication in the $My10^{++}DR^-$ cell population known to contain the more immature blast cells (Brandt et al., in press) further supports the well-known requirement of a precise differentiation state of target cell for parvovirus replication (3, 28). The inclusion of the cloned B19 DNA in these experiments (Fig. 1C) served as a positive control for hybridization and, in subsequent experiments, was useful in determining the B19 DNA copy number per cell.

Biological activity of progeny B19 parvovirus produced by $My10^{++}DR^+$-enriched progenitor cells. Because in the above-described experiments only the extent of B19 virus DNA synthesis was measured, it was necessary to determine whether the progeny virus produced from primary infections of the $My10^{++}DR^+$ cell population was infectious. This verification was carried out by a modified two-cycle infection assay described previously (14; A. Srivastava and P. Nahreini, manuscript in preparation). Culture supernatants from primary infections of the $My10^{++}DR^+$ cells collected at 48 h postinfection and clarified of cell debris by centrifugation at $13,000 \times g$ for 5 min were used directly as a source of the B19 parvovirus and incubated with fresh 6×10^6 $NALT^-$ cells or 1×10^5 each of $My10^{++}DR^+$ or $My10^{++}DR^-$ cells at 4°C for 2 h. The input virus inoculum was removed, and cells were washed twice with phosphate-buffered saline, suspended in fresh Iscove modified minimal essential medium containing 20% fetal calf serum and 1 U of erythropoietin per ml, and incubated at 37°C for 72 h. The rest of the steps were carried out exactly as described above (Fig. 2).

Figure 2A shows the B19-hybridizable signal from culture supernatants of uninfected and B19-infected bone marrow cells. Whereas no hybridization was detected in control, uninfected cells, it is interesting to note once again that in this experiment approximately 60-fold fewer $My10^{++}DR^+$-enriched progenitor cells produced twice as much progeny B19 virus compared with unsorted $NALT^-$ cells. These data further document a more efficient infection and replication of the B19 parvovirus in enriched progenitor cells. The $My10^{++}DR^-$ cell population, however, did not support the productive replication of the B19 parvovirus, as observed before.

Figure 2B shows the hybridization signal produced by cell-associated progeny B19 virus DNA. This signal was

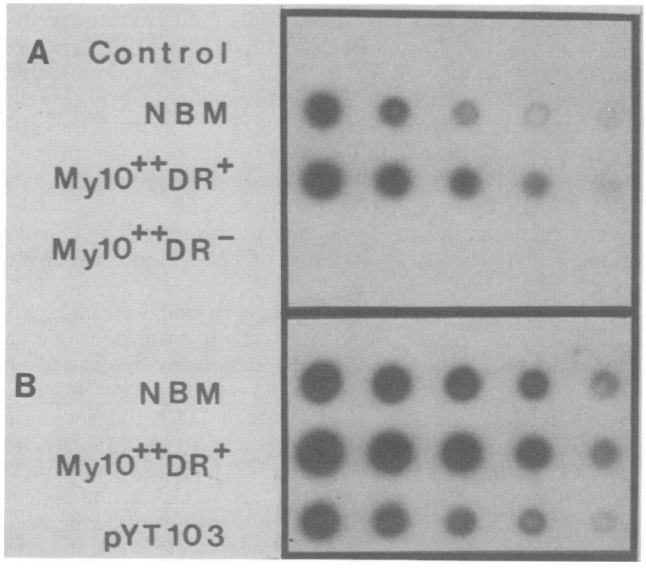

FIG. 2. Dot blot analysis of infectivity of progeny B19 parvovirus produced by $My10^{++}DR^+$-enriched progenitor cells. (A) B19 DNA from culture supernatants of 6×10^6 $NALT^-$ (normal bone marrow [NBM]) cells or 1×10^5 $My10^{++}DR^+$- or $My10^{++}DR^-$-enriched progenitor cells. (B) Cell-associated B19 DNA in 6×10^6 $NALT^-$ and 1×10^5 $My10^{++}DR^+$-enriched progenitor cells. Two-fold serial dilutions of 10^3 pg of the B19-specific cloned DNA insert were also included to determine the B19 DNA copy number per cell, as described by Ozawa et al. (21).

determined as follows. Cells (6×10^6 NALT$^-$ or 1×10^5 My10^{++}DR$^+$) infected with B19 were pelleted at 72 h postinfection by centrifugation at 13,000 × *g* for 5 min, washed twice with phosphate-buffered saline, and suspended in an equal volume of 10 mM Tris hydrochloride (pH 8.6). Cell lysates were prepared by freezing and thawing the cell suspensions three times to disrupt the cells. Cell lysates were treated with proteinase K at a final concentration of 100 μg/ml at 37°C for 16 h, extracted once with phenol, and denatured with 0.5 M NaOH at 65°C for 1 h to degrade RNAs and to denature DNAs. Cell lysates were neutralized with 20× SSC, and twofold serial dilutions of equivalent amounts were filtered through nitrocellulose filters and assayed for B19 DNA on quantitative DNA dot blots as described above. It was estimated that at 72 h postinfection there was ten times as much B19 DNA associated with infected cells as that in culture supernatants (Fig. 2A versus B), and there was twice as much B19 DNA in 60-fold fewer My10^{++}DR$^+$ progenitor cells as that in NALT$^-$ cells (Fig. 2B). It was also possible to determine the B19 DNA copy number per cell in these cultures by comparison of the hybridization intensity obtained with 10^3 pg of the pYT103 DNA on the same blot (Fig. 2B and Table 1).

It was determined that the average B19 DNA copy number in the total NALT$^-$ population ranged from 1,000 to 2,000 copies per cell. While these results are in good agreement with that reported by Ozawa et al. (21) for unsorted normal bone marrow mononuclear cells, it is noteworthy that the B19 DNA copy number per cell in the My10^{++}DR$^+$-enriched progenitor population was approximately 100-fold greater than that in NALT$^-$ cells. However, the B19 DNA copy numbers per erythroid progenitor cell of the two cell populations were not significantly different. It remains possible, however, that a more substantial population of erythroid progenitors was infected in My10^{++}DR$^+$ cells in the absence of a large number of nontarget cells than that in NALT$^-$ cells. Indeed, only 30 to 40% of the erythroid cells have been shown to be infected with the B19 parvovirus in unsorted bone marrow cell cultures (21, 22). Enrichment of progenitor cells, nonetheless, eliminated a large number of nontarget cells, and this result thus substantiated the specificity of the B19 parvovirus for human hematopoietic progenitor cells in the erythroid lineage.

TABLE 1. Parvovirus B19 replication in human hematopoietic cells

Cells[a]	No. of cells[b]	% Erythroid progenitors[c]	% Erythroblasts[d]	No. of B19 DNA copies per cell[e]
NALT$^-$	6×10^6	0.01–0.1	15–35	1,000–2,000
My10^{++}DR$^+$	1×10^5	25–30		80,000–120,000
My10^{++}DR$^-$	1×10^5	0–3		0–250

[a] NALT$^-$ cells were isolated from normal bone marrow aspirates as described before (16–19). Highly enriched progenitor cells from NALT$^-$ cells were isolated by cell sorting as described in the text.

[b] The indicated number of cells was determined by a Coulter Counter and used in each experiment in a total volume of 2 ml of Iscove modified minimal essential medium containing 20% fetal calf serum and 1 U of erythropoietin per ml.

[c] The values represent the normal ranges observed and were deduced from the cloning efficiencies of the sorted cells as described before (18; Brandt et al., in press).

[d] The erythroblast content of unsorted normal bone marrow cells represents the normal range. The total blast (myeloid + erythroid) contents of the My10^{++}DR$^+$ and My10^{++}DR$^-$ cell populations were 50 and 64%, respectively.

[e] The values were determined in three separate experiments on quantitative DNA dot blots by comparison to serial dilutions of 1 ng of pYT103 cloned B19 DNA insert (1 ng = 1×10^{12} base pairs = 2×10^8 copies of $\sim 5 \times 10^3$ base pairs) in the B19 genome.

Influence of B19 infection on hematopoiesis in vitro. Previous studies have identified the B19 target as the late erythroid progenitor cell (20–22, 25, 30, 31). The cytotoxic response of the virus to the more-mature erythroid precursors, measured as CFU-erythroid, has been shown to be more pronounced than that to the more-primitive precursors, burst-forming units-erythroid, in in vitro colony assays (20, 25). We wished to reexamine this response further, because these previous studies were carried out with unfractionated bone marrow cells in which the well-known low-frequency occurrence of hematopoietic progenitors correspondingly results in low cloning efficiencies of colony or burst formation (0.01 to 0.1%). In contrast, the cloning efficiencies obtained with highly enriched progenitor cells range from 30 to 47% (18). Equivalent numbers of My10^{++}DR$^+$- and My10^{++}DR$^-$-enriched progenitors were infected with the B19 parvovirus at 4°C for 2 h, washed twice with phosphate-buffered saline to remove the inoculum virus, and either used directly in in vitro colony assays or incubated at 37°C for 72 h prior to the colony assays performed as follows. Uninfected and B19-infected cells were plated in triplicate in 35-mm-diameter tissue culture dishes containing 1.0 ml of a mixture containing Iscove modified minimal essential medium, 30% fetal calf serum, 1.3% methylcellulose, 5% medium conditioned by the human 5637 cell line (19), 5×10^{-5} M 2-mercaptoethanol, and 1.0 U of erythropoietin. The dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in 5% O_2 (6) for 7 to 14 days. Colony formation by myeloid and erythroid progenitor cells was performed as described previously (15, 16). CFU-erythroid colonies were scored on day 7, and burst-forming units-erythroid and CFU-granulocyte-macrophage clusters or colonies were scored from the same dishes on day 14 (7; Fig. 3).

It is interesting to note that B19 infection had no effect on CFU-granulocyte-macrophage colony formation at either 2 or 72 h postinfection, compared with that by uninfected My10^{++}DR$^+$-enriched progenitor cells, which was taken as 100%. Similar results with unsorted bone marrow cells have been reported before (20, 25). CFU-granulocyte-macrophage colony formation by My10^{++}DR$^-$ cells, albeit at a significantly reduced level (18; Brandt et al., in press), was also unaffected by B19 infection. However, virus infection of My10^{++}DR$^+$ progenitors significantly reduced both burst-forming units-erythroid and CFU-erythroid with 2 h of incubation and completely suppressed cluster or colony formation when the virus was allowed to replicate for 72 h. These data clearly show that both the more-mature erythroid progenitors (CFU-erythroid) and the more-primitive erythroid progenitors (burst-forming units-erythroid) are equally susceptible to the cytolytic response of the B19 parvovirus. The apparent differences between our results and those reported previously (20, 25) may be explained on the basis of the extremely low-frequency occurrence and, possibly, an inadequate infection of the erythroid progenitors in unseparated bone marrow cells with the virus caused by the presence of a large number of accessory or nontarget cells or both (18, 31; Brandt et al., in press). This possibility is further illustrated by the effect of B19 infection of My10^{++}DR$^-$ cells on subsequent colony or cluster formation by a few erythroid progenitors present in this population (Fig. 3). The availability of My10^{++}DR$^+$-enriched progenitor cells may now facilitate further studies on the cellular and molecular interactions of the B19 parvovirus with and the

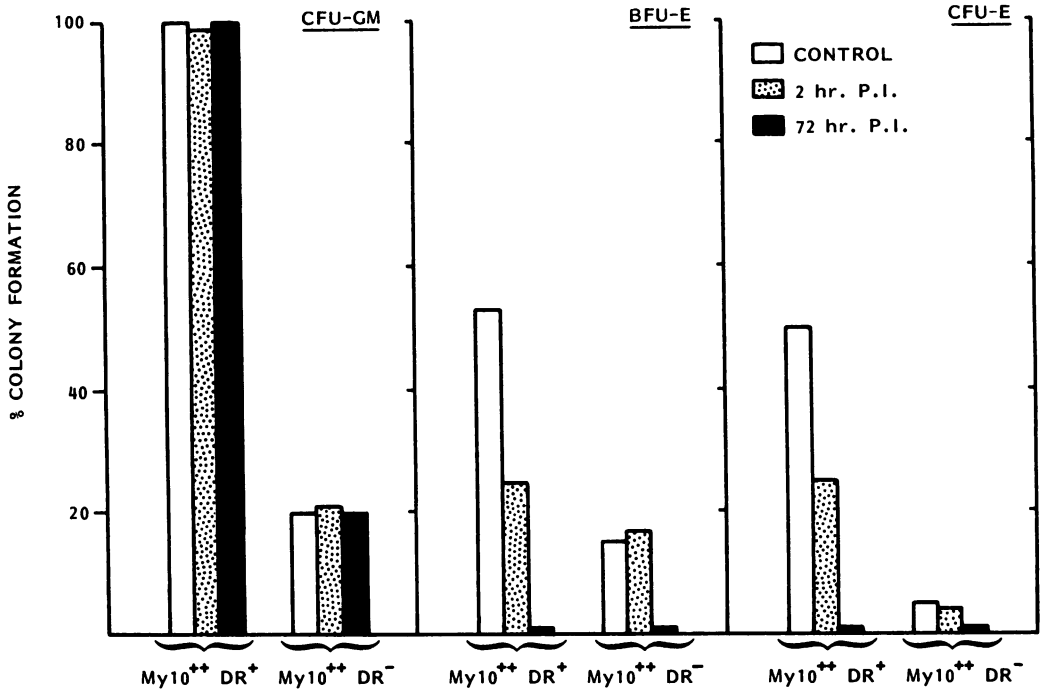

FIG. 3. Effect of B19 infection on normal hematopoiesis in vitro. Colony assays for myeloid and erythroid cell lineages were carried out with enriched progenitor cells as described before (18; Brandt et al., in press). Abbreviations: CFU-GM, CFU-granulocyte-macrophage; BFU-E, burst-forming units-erythroid; CFU-E, CFU-erythroid.

remarkable specificity of B19 for the human hematopoietic cells of erythroid lineage.

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