JOURNAL OF VIROLOGY, May 1995, p. 3229–3232 Vol. 69, No. 5
0022-538X/95/$04.00+0

Myeloid Depression Follows Infection of Susceptible Newborn Mice with the Parvovirus Minute Virus of Mice (Strain i)

JOSÉ C. SEGOVIA,[1] JUAN A. BUEREN,[1] AND JOSÉ M. ALMENDRAL[2]*

Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT), 28040 Madrid,[1] and Centro de Biología Molecular "Severo Ochoa" (Universidad Autónoma de Madrid-Consejo Superior de Investigaciones Científicas), 28049 Madrid,[2] Spain

Received 28 November 1994/Accepted 14 February 1995

The in vivo myelosuppressive capacity of strain i of the parvovirus minute virus of mice (MVMi) was investigated in newborn BALB/c mice inoculated with a lethal intranasal dose. MVMi infection reached maximum levels of DNA synthesis and infectious titers in lymphohemopoietic organs at 4 to 6 days postinoculation and was restricted by an early neutralizing humoral immune response. After viral control (by 10 days postinoculation), a significant decrease in femoral and splenic cellularity, as well as in granulocyte-macrophage colony-forming unit and erythroid burst-forming unit hemopoietic progenitors, was observed in most inoculated animals. This delayed myeloid depression, although it may be not a major cause of the lethality of the infection, implies indirect pathogenic mechanisms induced by MVMi infection in a susceptible host.

The family *Parvoviridae* includes a group of small (25-nm diameter), nonenveloped, icosahedral viruses containing a linear single-stranded (ss) DNA genome (28). Parvoviruses multiplication relies largely on cell physiology. Factors that are transiently induced during the S phase of the cell cycle (3, 30, 32), as well as other factors expressed at certain differentiation stages (29, 31), are required for these viruses to grow. Both requirements support the characteristic pathogenicity of these viruses towards certain tissues undergoing active proliferation (27).

The hemopoietic system displays a wide repertoire of proliferating cells at diverse differentiation and commitment stages (18) that are potentially susceptible to these viruses. Not surprisingly, parvovirus infections are commonly associated with hematological diseases whose severity depends on host immune competence. The Aleutian mink disease parvovirus causes a persistent infection with severe disorders of the mink immune system (20, 23). Experimental and natural infections with the feline panleukopenia virus develop neutropenia in cats (15, 16). In humans, the B19 parvovirus, with a marked tropism for hemopoietic precursors of the erythroid lineage (6, 21), is the etiological agent of erythema infectiosum in children (2), transient aplastic crisis in patients with hemoglobinopathies (22), or pure erythrocyte aplasia in immunodeficiency syndromes (14). Studies with volunteers intranasally inoculated with the B19 parvovirus showed transient changes in the reticulocyte count and hemoglobin (1) and a severe reduction in peripheral and marrow erythroid precursors 10 days postinoculation at the time of viremia (24).

Two natural strains of the parvovirus minute virus of mice (MVM), termed allotropic variants, show characteristic tropism for mouse cell lines and hemopoietic primary cells in vitro. The prototype strain, MVMp, infects fibroblastic cells (9). The MVMi strain suppresses both a number of T-lymphocyte functions (4, 10) and the clonogenic capacity of different hemopoietic precursors, and it is able to multiply efficiently in primary myeloid cultures (26). In vivo, the pathogenicity of both strains has been studied in intranasally inoculated newborn mice. MVMp infection was asymptomatic, and the virus remained confined to the oropharynx (13), while MVMi caused a generalized infection in which lethal and pathological manifestations were strictly dependent on the host genotype (7). Three cell types were the main targets of MVMi: the endothelium, lymphocytes, and hepatic erythropoietic precursors. The virus host range could be broadened by introducing changes in the allotropic determinant of the capsid (8).

In the present study, we attempted to infer the course of events occurring in the hemopoietic organs of newborn mice during a lethal MVMi infection started by the natural nasopharyngeal route. We determined the kinetics of viral multiplication in lymphoid and myeloid organs, the capacity of susceptible mice to mount a humoral response against the virus, and the extent of hematopoietic damage in the compartment of committed precursors of the spleen and bone marrow. This knowledge may improve our understanding of similar parvovirus-induced hematological diseases in other animals and humans.

Time course of MVMi multiplication in hemopoietic organs. BALB/c mice (originally purchased from the Jackson Laboratory, Bar Harbor, Maine, and bred in the CIEMAT animal facilities) were intranasally inoculated within the first 20 h postbirth with 10^6 PFU of gradient-purified MVMi plaque assayed on human transformed NB324K cells. With this viral dosage, the percentage of mortality for this inbred strain, determined in eight litters of five to seven animals increased from 40% ± 20% at 8 days postinfection (dpi) to a maximum of 85% ± 10% at 12 dpi (data not shown), in good agreement with previous reports (7). Before newborn infections, the dams were routinely monitored for anti-MVM antibodies in serum by enzyme-linked immunosorbent assay (ELISA) and by the neutralization tests described below. To study viral multiplication in the inoculated mice, mice from several litters were sacrificed daily for excision and processing of the main lymphohemopoietic organs (spleen, bone marrow, and thymus) and the lungs. These organs were weighed and homogenized at 10% (wt/vol) in phosphate-buffered saline (PBS). Bone marrow cells were recovered by flushing PBS through the femoral shafts and counted in a hemocytometer. To quantify the infectious virus in the organs, the suspensions were frozen and

* Corresponding author. Mailing address: Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain. Phone: 34-1-3978048. Fax: 34-1-3974799. Electronic mail address: JMAlmendral@mvax.CBM.UAM.es.

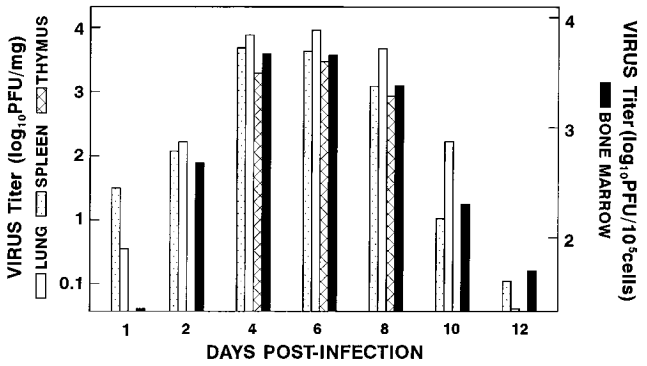

FIG. 1. Kinetics of infectious MVMi appearance in various organs of inoculated newborn BALB/c mice. Tissue homogenates were screened for infectious virus by plaque assay on NB324K cells. The bars represent mean titers from at least three animals from different litters. The virus in the thymus at days 1, 2, 10, and 12 was not determined. The bottom line corresponds to the assay detection limit.

thawed three times and clarified by low-speed centrifugation and serial dilutions were plaque assayed on NB324K cells as previously described (26). Significant titers of infectious virus were detected as early as 1 dpi in the lungs, as well as in the spleen (Fig. 1); this is an indication of the easy dissemination of the virus from the point of entry to internal organs. The kinetics of infectious virus presence were similar in all of the organs, reaching values comparable to those previously reported (7) at 4 to 6 dpi and declining sharply from 8 dpi.

To analyze whether the production of infectious virus in the lymphohemopoietic organs corresponded to viral replication at these sites, we studied the presence of MVM DNA intermediate replicative forms (RFs) and genomic ss forms in these samples. Tissue homogenates at 1, 2, 4, 6, 8, and 10 dpi were digested with proteinase K (100 μg/ml) in the presence of 0.5% sodium dodecyl sulfate and processed for low-molecular-weight DNA isolation by a modified Hirt procedure (17). DNA from equivalents of 1.25 (spleen and thymus) or 0.60 (lung) mg of tissue and 8×10^4 bone marrow cells was run on a 1% agarose gel, transferred to nylon membranes, and hybridized with an [α-^{32}p]dCTP-labeled total MVM probe to a specific activity of 5×10^8 cpm/μg. The results are shown in Fig. 2. RF and ss DNAs were detected in all cases from 2 dpi and reached maximum accumulation by 4 to 6 dpi, in parallel with the

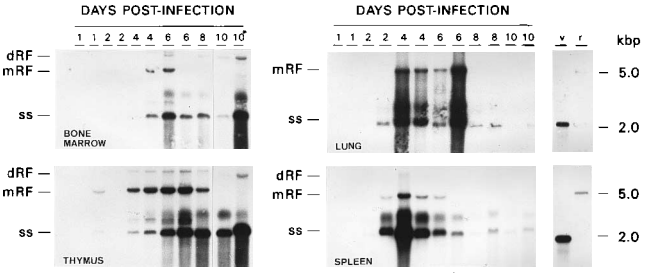

FIG. 2. MVMi DNA replication and maturation in lymphohematopoietic organs and the lungs. Low-molecular-weight DNA was isolated from the organs and analyzed by blot hybridization. Duplicate samples were from two different litters. Bone marrow and thymus samples and lung and spleen samples were from two separate sets of mice. *, samples (bone marrow and thymus) from a moribund mouse with reduced size and marked pathological signs; mRF and dRF, MVM RFs; ss, genomic ss forms; v, viral genomes extracted from purified virions; r, replicative intermediates isolated from EL-4 lymphoma cells infected with MVMi. Exposures were for 48 h with an intensifying screen.

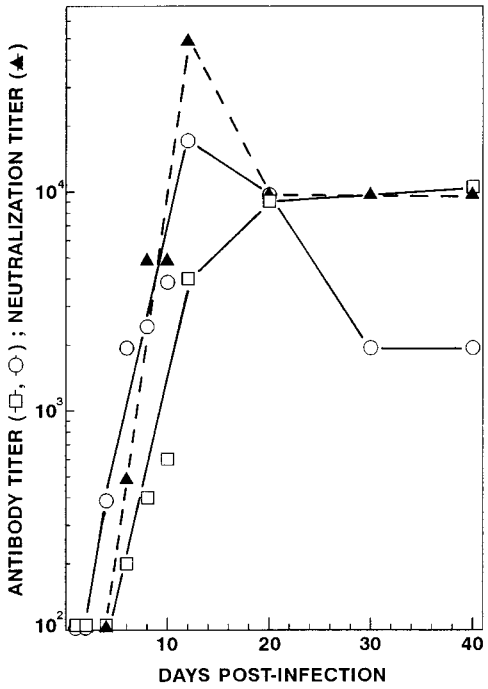

FIG. 3. Anti-MVM antibody production in infected newborn mice. Heat-inactivated sera were analyzed by an indirect ELISA for detection of anti-MVM IgM (○) and IgG (□) and for the ability to inhibit virus plaques on NB324K cells to quantify neutralizing activity (▲). Antiviral titers are mean values of three serum samples. Control sera from uninfected mice gave background values by both assays (data not shown).

infectious virus peaks shown above. These data indicate that the lungs and all of the lymphohemopoietic organs studied are sites of active virus multiplication and maturation. Viral DNA synthesis was apparently halted by 8 dpi since, with the exception of a single thymus sample (Fig. 2), the MVM RFs (mRF and dRF) were barely detectable from that day onward, while the accumulation of genomic ss forms declined progressively.

Humoral immune response to MVMi in newborn BALB/c mice. The fact that the infectious titers and viral DNA in the tissues decreased drastically from 8 dpi could be due to efficient control of the virus by the immune systems of the mice. To explore this possibility, we quantified the anti-MVM antibodies developed in the sera of infected animals, as well as their neutralization capacity for several weeks postinfection. The levels of immunoglobulin M (IgM) and IgG raised against the MVM structural proteins were determined by an ELISA. Flat-bottom wells of polystyrene plates (Nunc) were coated with 100 μl of a solution of 1-μg/ml gradient-purified viral capsids in 50 mM Na carbonate (pH 9.6) and incubated overnight at 4°C. Antibodies were screened by twofold dilution with affinity-purified goat anti-mouse IgG and IgM heavy-chain-specific antibodies linked to horseradish peroxidase (Southern Biotechnology ASS, Inc.), and the color was developed with *O*-phenylenediamine (1 mg/ml) for 15 min. Titers are given in Fig. 3 as the reciprocal of the highest dilution giving an A_{492} of three times the background. Newborn mice had no detectable specific antibodies, but MVMi did activate the immune systems of all of the infected animals at a very early stage of neonatal infection. The antiviral IgM titer rose from 400 at 4 dpi to as high as 20,000 at 12 dpi. Serum anti-MVM IgG developed 2 days later, and the titer rose from 200 at 6 dpi to a plateau of 10,000 at 20 dpi. Animals that survived the infection showed

typically stabilized immunoglobulin titers in serum, resembling the persisting humoral response described for other respiratory virus infections of mice (12). The kinetics of IgM synthesis demonstrate that this immune response is generated by infected newborn mice instead of being accomplished by passive transfer of antibodies from the dams. Similar criteria have been used to discriminate recent from past B19 infections in humans (11).

The capacity of the immune response to inhibit MVMi infectivity was monitored by a neutralization test in vitro. Serial dilutions of sera were incubated in 50 μl of PBS with 10^3 PFU of MVMi at 37°C for 30 min, and the mixture was diluted in PBS and used to determine the remaining infectious virus numbers by plaque assay on NB324K cells. The titer of neutralizing antibodies was estimated as the reciprocal of the serum dilution that gave a half-maximal reduction in the number of plaques. As shown in Fig. 3, a neutralization titer of 600 was detected at 6 dpi and it peaked at 50,000 at 12 dpi, coinciding with the maximum IgM titer, and remained stable in the sera of surviving animals up to 40 dpi. The trend of this curve suggests that both IgM and IgG contribute to the neutralizing activity of the sera. The onset of this humoral immune response correlates with an abrupt interruption of viral DNA synthesis and may presumably contribute to the clearance of MVMi from the organs of newborn mice. However, it protected the animals from neither pathological manifestations (see below; 7) nor generalized mortality.

Analysis of spleen and bone marrow hemopoiesis in MVMi-infected newborn mice. MVMi replication in the spleen and bone marrow may result in hemopoiesis dysfunction in newborns. To gain an overall picture of the cellular events in these organs, we analyzed for 1 to 12 dpi, and in survivors until 40 dpi, femoral and splenic cellularity and the numbers of erythroid burst-forming units (BFU-E) and granulocyte-macrophage colony-forming units (CFU-GM), two committed precursors of different hemopoietic lineages that were highly susceptible to MVMi in vitro (26). The femoral bone marrow and the spleens from 3 to 10 infected and noninfected mice were individually and aseptically removed on each day of analysis. Cell suspensions were carefully prepared in a volume of PBS proportional to the age of the animals, and after triplicate counting in a hemocytometer, precursors were assessed in enriched methylcellulose semisolid cultures as previously described (26). Splenic and marrow nucleated cells at a density of 10^5/ml were dispensed at 0.3 ml per well into 24-well culture dishes (Nunc), and the numbers of colonies were scored under an inverted microscope after 7 days of incubation.

The results of these experiments are summarized in Fig. 4. In noninfected control mice, besides minor variations between them, an age-proportional increase in the numbers of cells and progenitors was observed in the organs, consistent with their growth and increased hemopoietic competence during the postbirth development of the mice (19). Samples from infected animals also showed a certain heterogeneity and a similar rate of increase during the first week postinfection. During the second week, however, reductions in cellularity and the number of CFU-GM and BFU-E progenitors were observed in both the spleen and the bone marrow of infected animals in comparison with the age-matched control group. The loss of committed progenitors was more evident and lasting in the spleen, where all infected animals showed values below the average of the noninfected population from 8 to 12 dpi. Nevertheless, significant reductions in cellularity ($P < 0.05$ according to the Student t test) and CFU-GM and BFU-E progenitors ($P < 0.01$) were also observed in bone marrow at 10 dpi. Given that the reduction in the number of precursors paralleled that found in the bulk population of hemopoietic cells (Fig. 4, upper panels), the committed progenitor compartment, which was highly susceptible to MVMi in vitro (26), does not seem to be a preferential target of MVMi infection in newborn mice. The surviving mice analyzed after 12 dpi showed hemopoietic parameters comparable to those of the uninfected controls.

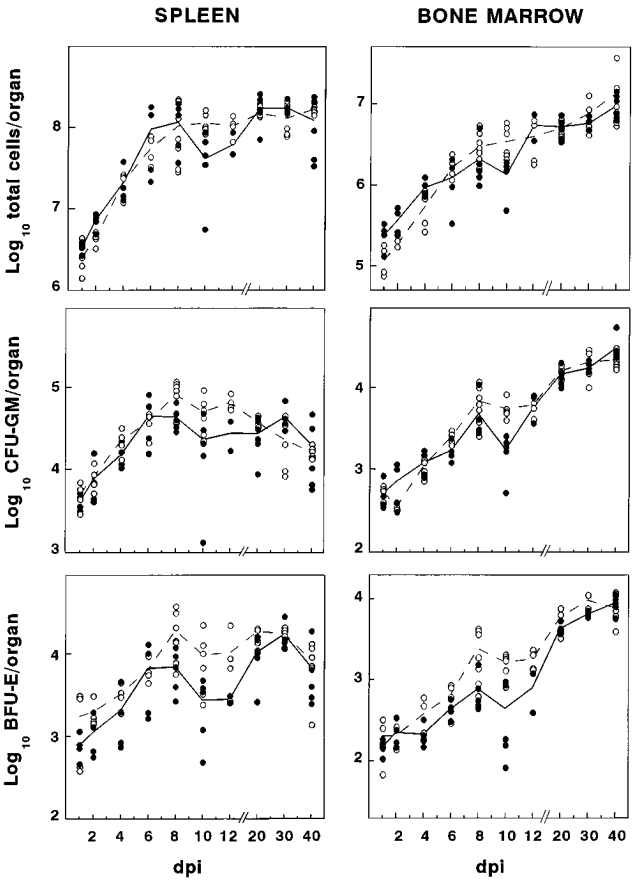

FIG. 4. Hemopoietic progenitor content and total cellularity in the spleens and bone marrow of infected newborn mice. Cell suspensions from the bone marrow and spleen of each mouse were counted and seeded under conditions allowing the growth of CFU-GM and BFU-E precursors. Marrow values are per single femoral shaft. Individual data for control (○) and infected (●) animals are shown. Several points on the graph have been slightly offset to avoid crowding. The lines link the daily mean values determined for control (dashed line) and infected (continuous line) animals. Values beyond 12 dpi are from the fraction (around 10%) of surviving animals.

Conclusions. The parvovirus MVMi causes a systemic infection soon after intranasal inoculation into newborn BALB/c mice. The virus replicates and maturates with similar kinetics in the lungs and the other internal organs studied, suggesting that infectious particles may access the bloodstream at or close to the site of nasopharyngeal inoculation. Virus multiplication peaks in the organs by 4 to 6 dpi and is apparently controlled by a humoral immune response mounted by newborn mice. However, splenic and marrow cellularity and hemopoietic precursors are only decreased between 8 and 12 dpi, a period that correlates with the mortality of the animals and is significantly delayed with respect to the highest titers of infectious virus and viral DNA synthesis in the organs.

Collectively, the global course of cellular and virological events suggests that the hemopoietic impairment in newborn mice is not caused by specific interaction of MVMi with sus-

ceptible myeloid cells, something that could be expected from its in vitro cytotoxicity for committed and primitive mouse hemopoietic precursors (26). Rather, participation of an indirect mechanism(s) in the observed myeloid depression seems likely. Candidates for these effects might be local accumulation of nonstructural MVM proteins with a demonstrated proliferative inhibitory effect on several cell types (5), toxic effects mediated by virus-antibody complexes, or release of inhibitory cytokines. Some of these mechanisms may be shared with the human B19 parvovirus. Although this virus has a narrower hemopoietic host range than MVMi in vitro, since only erythroid precursors are the target of B19 in culture (6, 21), B19 infections result in a reduction of BFU-E and CFU-GM precursors in the marrow of intranasally inoculated human volunteers (24).

In newborn mice with many tissues undergoing proliferation, the myeloid depression may even be connected with the high mortality of the animals via other pathological mechanisms operating at the organism level. In this sense, the histopathologic findings on the kidneys (7) and central nervous systems (25) of analogously MVMi-inoculated newborn mice may be relevant clues worth exploring.

We are grateful to A. Bernard for useful discussions, to R. M. Corot for technical support with the hemopoietic cultures, and to E. López for helping with the mice.

This work was supported by the Comisión Interministerial de Ciencia y Tecnología (grants SAF92-1014-C02-01 and -02), the Commission of the European Communities (contract BMH1-CT92-0629), and an institutional grant from Fundación Ramón Areces to the Centro de Biología Molecular "Severo Ochoa."

REFERENCES

1. **Anderson, M. J., P. G. Higgins, L. R. Davis, J. S. Willman, S. E. Jones, I. M. Kidd, J. R. Pattison, and D. A. J. Tyrrell.** 1985. Experimental parvovirus infection in man. J. Infect. Dis. **152:**257–265.
2. **Anderson, M. J., E. Lewis, I. M. Kidd, S. M. Hall, and B. J. Cohen.** 1984. An outbreak of erythema infectiosum associated with human parvovirus infection. J. Hyg. **93:**85–93.
3. **Berns, K. I.** 1990. Parvovirus replication. Microbiol. Rev. **54:**316–329.
4. **Bonnard, G. D., E. K. Manders, D. A. Campbell, R. B. Herberman, and M. J. Collins.** 1976. Immunosuppressive activity of a subline of the mouse EL-4 lymphoma. J. Exp. Med. **143:**187–205.
5. **Brandenburger, A., D. Legendre, B. Avalosse, and J. Rommelaere.** 1990. NS-1 and NS-2 proteins may act synergistically in the cytopathogenicity of parvovirus MVMp. Virology **174:**576–584.
6. **Brown, K. E., S. M. Anderson, and N. S. Young.** 1993. Erythrocyte *P* antigen: cellular receptor for B19 parvovirus. Science **262:**114–117.
7. **Brownstein, D. G., A. L. Smith, R. O. Jacoby, E. A. Johnson, G. Hansen, and P. Tattersall.** 1991. Pathogenesis of infection with a virulent allotropic variant of minute virus of mice and regulation by host genotype. Lab. Invest. **65:**357–363.
8. **Brownstein, D. G., A. L. Smith, E. A. Johnson, D. J. Pintel, L. K. Naeger, and P. Tattersall.** 1992. The pathogenesis of infection with minute virus of mice depends on expression of the small nonstructural protein NS2 and on the genotype of the allotropic determinants VP1 and VP2. J. Virol. **66:**3118–3124.
9. **Crawford, L. V.** 1966. A minute virus of mice. Virology **29:**605–612.
10. **Engers, H. D., J. A. Louis, R. H. Zubler, and B. Hirt.** 1981. Inhibition of T-cell mediated functions by MVM(i), a parvovirus closely related to minute virus of mice. J. Immunol. **127:**2280–2285.
11. **Harris, J. W.** 1992. Parvovirus B19 for the hematologist. Am. J. Hematol. **39:**119–130.
12. **Hyland, L., M. Sangster, R. Sealy, and C. Coleclough.** 1994. Respiratory virus infection of mice provokes a permanent humoral immune response. J. Virol. **68:**6083–6086.
13. **Kimsey, P. B., H. D. Engers, B. Hirt, and V. Jongeneel.** 1986. Pathogenicity of fibroblast- and lymphocyte-specific variants of minute virus of mice. J. Virol. **59:**8–13.
14. **Kurtzman, G. J., K. Ozawa, B. Cohen, G. Hanson, R. Oseas, and N. S. Young.** 1987. Chronic bone marrow failure due to persistent B19 parvovirus infection. N. Engl. J. Med. **317:**287–294.
15. **Larse, S., A. Flagstad, and B. Aalbaek.** 1976. Experimental feline panleukopenia in the conventional cat. Vet. Pathol. **13:**216–240.
16. **Lawrence, J. S., J. T. Syverton, J. S. Shaw, and F. P. Smith.** 1940. Infectious feline agranulocytosis. Am. J. Pathol. **16:**333–354.
17. **McMaster, G. K., P. Beard, H. D. Engers, and B. Hirt.** 1981. Characterization of an immunosuppressive parvovirus related to the minute virus of mice. J. Virol. **38:**317–326.
18. **Metcalf, D.** 1989. The molecular control of cell division, differentiation, commitment and maturation in haemopoietic cells. Nature (London) **339:** 27–30.
19. **Metcalf, D., and M. A. S. Moore.** 1971. Embryonic aspects of haemopoiesis, p. 172–271. *In* A. Neuberger and E. L. Tatum (ed.), Haemopoietic cells. North-Holland Publishing Co., Amsterdam.
20. **Mori, S., J. B. Wolfinbarger, M. Miyazawa, and M. E. Bloom.** 1991. Replication of Aleutian mink disease parvovirus in lymphoid tissues of adult mink: involvement of follicular dendritic cells and macrophages. J. Virol. **65:**952–956.
21. **Mortimer, P. P., R. K. Humphries, J. G. Moore, R. H. Purcell, and N. S. Young.** 1983. A human parvovirus like virus inhibits haemopoietic colony formation *in vitro*. Nature (London) **302:**426–429.
22. **Pattison, J. R., S. E. Jones, and J. Hodgson.** 1981. Parvovirus infections and hypoplastic crises in sickle cell anaemia. Lancet **i:**664–665.
23. **Porter, D. D., A. E. Larsen, and H. G. Porter.** 1969. The pathogenesis of Aleutian disease of mink. *In vivo* viral replication and the host antibody response to viral antigen. J. Exp. Med. **130:**575–589.
24. **Potter, C. G., A. C. Potter, C. S. R. Hatton, H. M. Chapel, M. J. Anderson, J. R. Pattison, D. A. J. Tyrrell, P. G. Higgins, J. S. Willman, H. F. Parry, and P. M. Cotes.** 1987. Variation of erythroid and myeloid precursors in the marrow and peripheral blood of volunteer subjects infected with human parvovirus (B19). J. Clin. Invest. **79:**1486–1492.
25. **Ramírez, J. C., A. Fairén, and J. M. Almendral.** Unpublished data.
26. **Segovia, J. C., A. Real, J. A. Bueren, and J. M. Almendral.** 1991. *In vitro* myelosuppressive effects of the parvovirus minute virus of mice (MVMi) on hematopoietic stem and committed progenitor cells. Blood **77:**980–988.
27. **Siegl, G.** 1988. Patterns of parvovirus disease in animals, p. 43–68. *In* J. R. Pattison (ed.), Parvoviruses and human disease. CRC Press, Inc., Boca Raton, Fla.
28. **Siegl, G., R. C. Bates, K. I. Berns, B. J. Carter, D. C. Kelly, E. Kurstak, and P. Tattersall.** 1985. Characteristics and taxonomy of Parvoviridae. Intervirology **23:**61–73.
29. **Spalholz, B. A., and P. Tattersall.** 1983. Interaction of minute virus of mice with differentiated cells: strain-dependent cell specificity is mediated by intracellular factors. J. Virol. **46:**937–943.
30. **Tattersall, P.** 1972. Replication of the parvovirus MVM. I. Dependence of virus multiplication and plaque formation on cell growth. J. Virol. **10:**586–590.
31. **Tattersall, P., and E. M. Gardiner.** 1990. Autonomous parvovirus host-cell interactions, p. 111–121. *In* P. Tjissen (ed.), Handbook of parvoviruses, vol. 1. CRC Press, Inc., Boca Raton, Fla.
32. **Tennant, R. W., K. R. Laymant, and R. E. Hand, Jr.** 1969. Effect of cell physiological state on infection by rat virus. J. Virol. **4:**872–878.