

Identification of a Novel Simian Parvovirus in Cynomolgus Monkeys with Severe Anemia

A Paradigm of Human B19 Parvovirus Infection

M. Gerard O'Sullivan,* Deborah C. Anderson,* James D. Fikes,* Fairfield T. Bain,* Cathy S. Carlson,* Spencer W. Green,† Neal S. Young,‡ and Kevin E. Brown‡

*Comparative Medicine Clinical Research Center, Clinical Research Center, The Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27157-1040; and †Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, Maryland 20892

Abstract

Although human B19 parvovirus infection has been clearly associated with a number of distinct syndromes (including severe anemia, abortion, and arthritis), detailed knowledge of its pathogenesis has been hindered by the lack of a suitable animal model. We have identified a novel simian parvovirus in cynomolgus monkeys with severe anemia. Sequencing of a 723-bp fragment of cloned viral DNA extracted from serum revealed that the simian parvovirus has 65% homology at the DNA level with the human B19 parvovirus but little homology with other known parvoviruses. Light microscopic examination of bone marrow from infected animals showed intranuclear inclusion bodies, and ultrastructural studies showed viral arrays characteristic of parvoviruses. Another striking feature was the presence of marked dyserythropoiesis in cells of the erythroid lineage, raising the possibility that B19 parvovirus infection may underlie related dyserythropoietic syndromes in human beings. Affected animals had concurrent infection with the immunosuppressive type D simian retrovirus, analogous to HIV patients who develop severe anemia because of infection with B19 parvovirus. The remarkable similarities between the simian and B19 parvoviruses suggest that experimentally infected cynomolgus monkeys may serve as a useful animal model of human B19 infection. (*J. Clin. Invest.* 1994. 93:1571-1576.) **Key words:** parvovirus infections • human B19 parvovirus • haplorhinae • anemia • congenital dyserythropoietic anemia

Introduction

The *Parvoviridae* family comprises a group of single-stranded DNA viruses that are the smallest known viruses that infect mammalian cells (1). Some are important pathogens of human beings and animals, particularly human B19 parvovirus and the canine, feline, and porcine parvoviruses. A key feature

of the *Parvoviridae* is their requirement for actively dividing cells in order to replicate. As such, the characteristic pathogenesis of diseases caused by parvoviruses involves cytotoxicity of tissues with high cellular turnover, particularly the intestine (canine and feline viruses), developing fetus (human B19, feline, and porcine parvoviruses), and bone marrow (human B19, canine, and feline viruses). Nonetheless, the spectrum of clinical disease (skin rash, anemia, fetal loss, or arthritis) associated with B19 parvovirus infections differs from that of other mammalian parvoviruses (2). Also, in contrast to other mammalian parvoviruses B19 is difficult to culture. It will not replicate in established cell lines but requires explant cultures of bone marrow or human fetal liver cells for in vitro studies. A low degree of homology further reflects major differences between B19 and other mammalian parvoviruses. An animal model of human B19 parvovirus infection may contribute to our understanding of the pathogenesis of infection in human beings, especially fetal transmission and congenital infection.

We report here the isolation of a new simian parvovirus (SPV)¹ that shares a high homology with B19 parvovirus at the DNA level and is remarkably similar to the human B19 parvovirus in its predilection for bone marrow and ability to cause severe anemia.

Methods

Animal population. The Comparative Medicine Clinical Research Center (CMCRC) of the Bowman Gray School of Medicine has ~1,500 monkeys that are used in comparative clinical trials of atherosclerosis and osteoporosis. This population consists predominantly of cynomolgus monkeys (~1,200), with smaller numbers of rhesus (~200) and some stump-tailed macaques.

Light and electron microscopy. For light microscopy, tissues were fixed in 10% neutral buffered formalin, dehydrated through alcohols, embedded in paraffin, and 6- μ m sections were stained with hematoxylin and eosin. For electron microscopy, tissues were fixed in 0.1 M cacodylate buffer containing 1% glutaraldehyde, postfixed in 1% OsO₄, dehydrated, and embedded in Epon before sectioning. Tissues were examined with an electron microscope (EM 400; Philips Technologies, Cheshire, CT).

Virological and molecular studies. Serum and tissue samples (spleen, lymph nodes, bone marrow) collected at necropsy were submitted for virus isolation by standard methods, including isolation of simian type D simian retrovirus, as determined by formation of characteristic syncytial cells in coculture with the lymphoblastoid Raji cell line (3, 4).

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Address correspondence to Dr. M. Gerard O'Sullivan, Department of Comparative Medicine, The Bowman Gray School of Medicine of Wake Forest University, Medical Center Boulevard, Winston-Salem, NC 27157-1040.

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1. *Abbreviations used in this paper:* CDA, congenital dyserythropoietic anemias; CMCRC, Comparative Medicine Clinical Research Center; SPV, simian parvovirus.

Parvoviral DNA was detected by dot-blot hybridization of DNA from 10 μ l of serum prepared as previously described for human serum (5) using a 32 P nick-translated full-length B19 parvovirus probe (pYT103) (6).

To further characterize a putative SPV, the virus was concentrated by layering 2 ml of serum over 3 ml of 20% sucrose and centrifuging at 150,000 g for 5 h. The pellet was resuspended in 50 μ l of Tris-EDTA buffer (pH 7.5). Single-stranded DNA was extracted using silica (7) and allowed to self-anneal by incubating the DNA in 50 mM saline at 50°C for 16 h. Southern analysis on the single-stranded and annealed DNA was performed using the pYT103 probe (8).

Sequence data were obtained by cloning a fragment of the DNA into a pUC19 vector. The annealed DNA was digested with the restriction enzyme PstI, and the products were analyzed on a 0.9% agarose gel. A 723-bp fragment was cut from the gel, the DNA purified by GeneClean (Bio 101, La Jolla, CA), and ligated into the Pst I site of pUC19. The inserted DNA was sequenced by the dideoxy method using Sequenase II (U. S. Biochem. Corp., Cleveland, OH); initially "universal" M13 forward and reverse primers were used, and then primers were designed from the previously obtained sequence. The insert was fully sequenced in both directions and the sequence obtained was analyzed using DNASTar (DNASTar Inc., Madison, WI).

To confirm that the sequence was present in the affected animals, a PCR assay was designed using primers and an internal probe from the sequence of the cloned insert. These primers (nucleotides 60–79, 676–657) and this probe (nucleotides 497–515) detect a 616-nucleotide fragment from the insert that is absent in B19 parvovirus. Serum samples were analyzed using a modification of the PCR method for B19 parvovirus (9); product detection was by Southern transfer of agarose gel electrophoresis onto a nylon membrane and probing with the 32 P end-labeled internal probe.

Results

Clinical observations. A cluster of five adult male cynomolgus monkeys with anemia was identified in 1992 at the CMCRC. All had been healthy when living in single cages during the year before their transfer into group housing. After 6 wk, the group of five was disbanded and mixed with other monkeys ("in-contacts," 13 animals in total) to form new social groups. At this time, one monkey (monkey no. 2) was observed to be ill, with clinical signs consisting of diarrhea, dehydration, and moderate anemia (hemoglobin 7.4, hematocrit 29) that progressed over the course of a week (hemoglobin 3.8, hematocrit 19). Despite a blood transfusion, this animal became moribund and was euthanized because of the grave prognosis.

Over the subsequent 4 wk, three of the remaining four monkeys (nos. 1, 3, and 4) constituting the original group became ill with severe normocytic normochromic anemia (Table I). Monkey no. 5 was apparently healthy, with hematological findings that were close to normal (Table I), and this monkey remains healthy to date. Monitoring of hematological parameters in the 13 in-contacts resulted in the recognition of anemia in two additional monkeys (nos. 6 and 7) that were euthanized to limit possible spread of disease. The anemia was classified as predominantly normocytic, normochromic, and nonregenerative, although the presence of reticulocytes indicated erythroid regeneration in two monkeys. Whereas anemia was the major finding (6/6 animals) prompting a decision of euthanasia, a high percentage of monkeys also had diarrhea, dehydration, and positive fecal cultures for *Campylobacter* spp (3/4 monkeys).

Pathological findings. Gross necropsy findings revealed moderate to marked splenomegaly (6/6 monkeys) and moder-

Table I. Hematological Parameters for Anemic Cynomolgus Monkeys

Monkey	WBC	RBC	hGB	Hct.	Retic.
	$\times 10^9/\text{liter}$	$\times 10^{12}/\text{liter}$	g/dl		%
1	4.7	2.1	3.9	0.15	2.4
2	4.0	2.8	3.8	0.19	0.0
3	5.3	1.2	2.2	0.08	0.0
4	12.4	2.7	4.5	0.16	0.0
5	10.0	4.9	9.7	0.33	ND
RV	5.1–13.3	5.6–7.2	9.8–13.3	0.326–0.467	0.0

Except for monkey no. 5, which remains healthy, values represent most recent findings before death or euthanasia. WBC, white blood cells; RBC, red blood cells; hGB, hemoglobin; Hct., hematocrit; Retic., reticulocytes; ND, not determined; RV, reference values.

ate generalized lymphadenopathy (mesenteric, inguinal, and axillary nodes) (5/6 monkeys). Although the most pronounced histopathological findings involved the bone marrow (described below), a variety of other lesions were observed, including moderate to severe typhlocolitis (5/6 monkeys), splenic and lymphoid follicular or paracortical hyperplasia (4/6 monkeys), splenitis (2/6 monkeys), prostatitis (1/6 monkeys), bronchitis (1/6 monkeys), and fibrinopurulent peritonitis (1/6 monkeys). Hepatic centrilobular necrosis was observed in two monkeys and was attributed to hypoxia due to the severe anemia.

Microscopic examination of bone marrow from the proximal femur or sternbrae revealed moderate to marked loss of mature cells of both erythroid (4/6 monkeys) and myeloid lineages, the presence of many medium to large undifferentiated cells, and increased numbers of megakaryocytes. A frequent finding in bone marrows with less marked erythroid depletion was the presence of markedly abnormal cells of erythroid lineage characterized by bizarre nuclear forms, including nuclear blebbing, appendages, or multilobulation (Fig. 1). The other striking finding in some marrows was the presence of intranuclear inclusion bodies, with morphologic characteristics consistent with those observed in tissues infected with B19 and other parvoviruses (10–12) (Fig. 1). Intranuclear inclusion bodies were present in medium to large, less differentiated cells as well as more mature elements of the erythroid series such as normoblasts. They were present in large numbers in one marrow (sometimes as many as five or more per high-powered field; $\times 100$ oil immersion objective), and were observed in small numbers or were rare in the other marrows. Ultrastructural examination of inclusions revealed the presence of ~ 24 -nm virus particles and an occasional viral array characteristic of parvoviruses (11–14) (Fig. 1).

Virus culture. Tissue samples (spleen, lymph nodes, and bone marrow) collected at necropsy from five monkeys and submitted for virus isolation were all positive for type D simian retrovirus. The presence of virus in several tissues and at relatively high titer (based on the rapid appearance of cytopathogenic effects in cell cultures), along with necropsy findings of splenomegaly and lymphadenopathy, indicate that affected animals also had concurrent active infections with type D simian retrovirus, a known immunosuppressive virus (3, 4, 15–17). A mononuclear cell culture from a blood sample obtained from

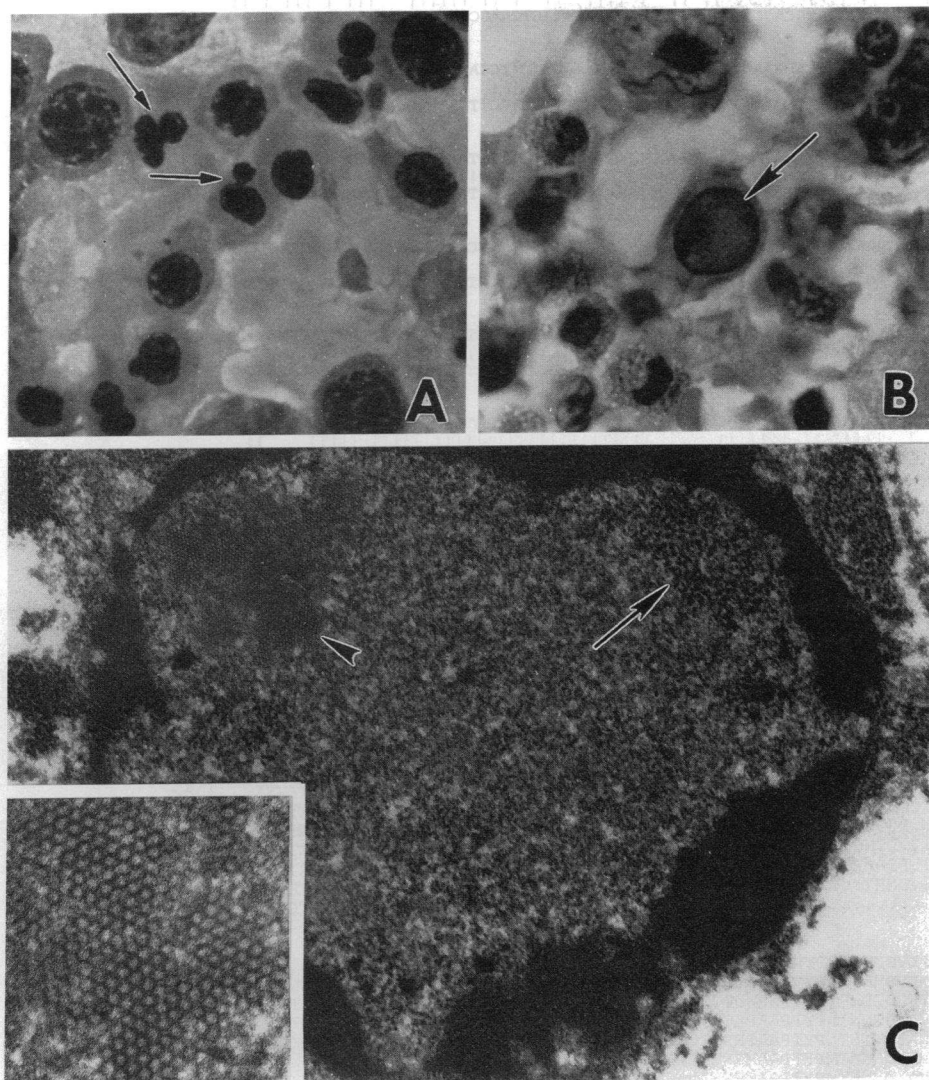


Figure 1. Morphology of bone marrow from anemic monkeys. (A) Dyserythropoiesis (arrows). Impression smear, Wright's stain; $\times 992$. (B) Intranuclear inclusion body (arrow) characteristic of parvoviruses. Hematoxylin and eosin; $\times 992$. (C) Ultrastructure of intranuclear inclusion body showing margination of chromatin, a cluster of parvovirus-like particles (arrow), and viral array characteristic of parvoviruses (arrowhead); $\times 27,000$. (Inset) Viral array; $\times 99,120$.

the healthy monkey (no. 5) also tested positive for type D simian retrovirus. Serum neutralization tests confirmed the presence of serotype 2 in three of three isolates examined.

Parvovirus findings. Serum samples from the four anemic monkeys (nos. 1–4) of the original group of five tested strongly positive by dot blot hybridization for parvoviral DNA (Fig. 2). The sample from the healthy monkey (no. 5) was negative.

DNA from the serum of monkey no. 1 was extracted and examined further. Analysis by agarose gel electrophoresis confirmed that the DNA migrated at the same position as single-stranded B19 DNA and cross-hybridized to a B19 probe (pYT103). Incubation of the DNA in low salt solution at 50°C converted all single-stranded DNA to double-stranded DNA that migrated as a 5.4–5.5-kb band, suggesting that the virus produced equimolar complementary single strands that self-anneal (as for B19 parvovirus) (18).

Analysis of the fragment cloned into a pUC19 vector showed homology (64.7% at the DNA level and 67.8% at the amino acid level) with B19 parvovirus in the VP2 capsid region and almost no homology with other known parvoviruses (Fig. 3). These results indicated that we had identified a previously unrecognized SPV that should be included in the *Parvovirus* family.

Serum samples were tested for the presence of SPV DNA by PCR using the primers and an internal probe designed from the sequence of the 723-bp cloned insert. No DNA was amplified from the pYT103 control, confirming that the PCR is specific for SPV. Samples from the four anemic monkeys (nos. 1–4) from the original group of five were positive for SPV DNA (Fig. 4). Although the samples from the healthy monkey (no.

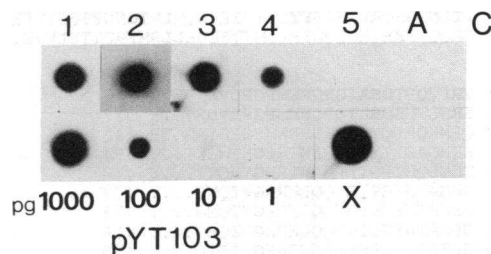


Figure 2. Dot-blot hybridization for parvoviral DNA using the cloned B19 parvovirus probe pYT103 (22). (Top) Serum samples from monkeys nos. 1–5. Samples A and C represent control monkeys not in contact. (Bottom) pYT103 controls (1–1,000 pg). Sample X is serum from a patient with HIV and concurrent B19 infection.

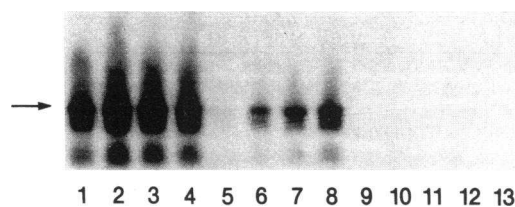


Figure 4. PCR for SPV. Primers designed from the SPV sequence in Fig. 3 (nucleotides 60–79, 676–657) were used to amplify a 616-nucleotide fragment (arrow) of SPV in serum samples; product detection was with a ^{32}P end-labeled internal probe (nucleotides 497–515). (Lanes 1–7) Monkeys nos. 1–7 described in text; (lanes 8–10) monkeys necropsied in 1991; (lanes 8 and 9) two severely anemic monkeys; (lane 10) monkey not in contact; (lane 11) control monkey not in contact (Fig. 2A); (lane 12) pYT103 control; (lane 13) water control.

5) (Fig. 2) and the two in-contact monkeys (nos. 6 and 7) were negative by dot blot analysis (data not shown), samples from monkeys nos. 6 and 7 were positive by PCR (Fig. 4). Of particular interest was our detection of SPV DNA in a serum sample from a cynomolgus monkey that died at the CMCRC in May 1991 (Fig. 4). A total of five animals with a history of severe anemia were necropsied at that time, and sera were tested from two of them (Fig. 4).

Discussion

Considering the clinical, pathological, and virological findings, we conclude that the major cause of the severe anemia in these animals was infection with a previously unrecognized SPV. Moreover, the bone marrow failure caused by SPV is remarkably similar to that caused by the human B19 parvovirus. First, SPV shares a high homology with human B19 parvovirus at both the DNA and amino acid levels, but like B19 parvovirus, the simian virus appears to be distantly related to other parvoviruses. Second, the light microscopic and ultrastructural features of bone marrow infected with SPV are almost identical to that of human bone marrow infected with B19 parvovirus. Third, although B19 parvovirus is widely distributed in the human population (seroprevalence of ~ 60% based on epidemiological studies) (19), anemia is uncommon unless there is an additional predisposing factor, as in sickle cell anemia (resulting in transient aplastic crisis) (20) or immunosuppression (resulting in pure red cell aplasia) (21). Immunosuppression, for example, due to concurrent HIV infection, can allow persistent B19 parvovirus infection that culminates in severe anemia as a consequence of continuing erythroid progenitor destruction (22). In striking parallel to B19 parvovirus-mediated bone marrow failure in human beings, severe anemia in the cynomolgus monkeys consistently was associated with the presence of a known immunosuppressive virus, type D simian retrovirus, along with the SPV. Type D simian retrovirus infection may have predisposed the animals to infection with SPV and may even be a prerequisite for the development of anemia in animals infected with SPV. Most affected monkeys had concurrent illnesses, *Campylobacter*-associated diarrhea in particular, but also including peritonitis and splenitis. We consider these findings to be further manifestations of immunosuppression due to concurrent infection with type D simian retrovirus (3, 4, 15–17).

Our retrospective diagnosis of SPV in an anemic monkey that died in 1991 indicates that the outbreak of anemia described here is not an isolated incident. Rather, it is consistent with the hypothesis that, as for the human B19 parvovirus, SPV may be widespread in the monkey population but only causes illness when the appropriate predisposing factors exist. The observed similarities between SPV and human B19 parvoviruses raise the possibility that SPV may be able to naturally infect human beings, or conversely, that B19 parvovirus may infect monkeys. Indeed, preliminary studies indicate that SPV can infect bone marrow from human beings (Brown, K. E., and N. S. Young, unpublished observations). This may have important implications for the health of personnel working closely with monkeys. Development of a suitable serological test will be necessary to monitor infection of monkeys and human beings and to determine the epidemiologic characteristics of SPV both in nonhuman primate colonies and in the wild.

Finally, experimentally infecting monkeys with SPV or B19 parvovirus could serve as an animal model of human parvovirus infection and should increase understanding of the pathogenesis of disease caused by both the human and simian parvoviruses in their respective natural hosts. In this regard, our finding of dyserythropoiesis in bone marrows from SPV-infected animals is remarkably similar to that of people with congenital dyserythropoietic anemias (CDA), a group of hereditary refractory anemias. This raises the intriguing question of whether B19 parvovirus infection may underlie some of the human CDA, or allied conditions such as Diamond-Blackfan anemia. In support of this possibility is our finding of dyserythropoiesis with prominent binucleated and trinucleated erythroblasts, consistent with a diagnosis of CDA type II, in a patient congenitally infected with B19 parvovirus (22a). Other areas in which experimental infection of monkeys with SPV might increase our understanding of human B19 infections include how the virus is transmitted and in which tissues initial virus replication occurs, the pathogenesis of fetal deaths when infection occurs during pregnancy, the role of B19 parvovirus in arthropathies, and whether latent infection of cells or tissues occurs. Studies with SPV in monkeys also may be of value in developing effective vaccination regimes for B19 parvovirus.

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