Experimental Infection of Cynomolgus Monkeys with Simian Parvovirus

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Simian parvovirus is a recently discovered parvovirus that was first isolated from cynomolgus monkeys. It is similar to human B19 parvovirus in terms of virus genome, tropism for erythroid cells, and characteristic pathology in natural infections. Cynomolgus monkeys were infected with simian parvovirus to investigate their potential usefulness as an animal model of human B19 parvovirus. Six adult female cynomolgus monkeys were inoculated with purified simian parvovirus by the intravenous or intranasal route and monitored for evidence of clinical abnormalities; this included the preparation of complete hematological profiles. Viremia and simian parvovirus-specific antibody were determined in infected monkeys by dot blot and Western blot assays, respectively. Bone marrow was examined at necropsy 6, 10, or 15 days postinfection. All of the monkeys developed a smoldering, low-grade viremia that peaked approximately 10 to 12 days after inoculation. Peak viremia coincided with the appearance of specific antibody and was followed by sudden clearance of the virus and complete, but transient, absence of reticulocytes from the peripheral blood. Clinical signs were mild and involved mainly anorexia and slight weight loss. Infection was associated with a mild decrease in hemoglobin, hematocrit, and erythrocyte numbers. Bone marrow showed marked destruction of erythroid cells coincident with peak viremia. Our findings indicate that infection of healthy monkeys by simian parvovirus is self-limited and mild, with transient cessation of erythropoiesis. Our study has reproduced Koch's postulates and further shown that simian parvovirus infection of monkeys is almost identical to human B19 parvovirus infection of humans. Accordingly, this animal model may prove valuable in the study of the pathogenesis of B19 virus infection.

The human B19 parvovirus was discovered in 1975 but was not associated with any disease entity at that time (8). Although infection is often inapparent or leads to only mild febrile illness, B19 parvovirus is known to be responsible for several human disorders (reviewed in reference 7), including fifth disease in children (3); an acute rheumatoid arthritis-like syndrome, particularly in women (33); transient aplastic crisis in patients with chronic hemolytic anemia, e.g., associated with sickle cell anemia (29, 30); pure erythrocyte aplasia in individuals persistently infected with B19 virus, e.g., associated with congenital (17) or acquired (11) immunodeficiency; and fetal loss and hydrops fetalis in pregnant women (1, 19, 27). It has also been implicated in several other conditions, including congenital anemia (5), congenital malformations (12, 16, 32), myocarditis (25, 28), meningitis (21, 31), and various forms of vasculitis (10, 20).

B19 parvovirus is only distantly related to the other common mammalian parvoviruses and differs also in its predilection for the erythroid cells of bone marrow that express the P antigen, which is known to be the cellular receptor for B19 parvovirus (4). These differences have led to the recent creation of a separate genus within the *Parvoviridae* family, i.e., *Erythrovirus* (14). B19 parvovirus is the sole member of this genus, in contrast to most mammalian parvoviruses, which are members of the *Parvovirus* genus. Accordingly, our understanding of

human parvovirus infections and their consequences has been hampered by the lack of a suitable animal model of infection.

We recently identified a simian parvovirus (SPV) in cynomolgus monkeys (*Macaca fascicularis*) with severe anemia (22, 23). SPV exhibits a high degree of homology with B19 parvovirus (6) and has an identical predilection for erythroid cells (22). These similarities have led to the proposal that SPV be classified as a member of the *Erythrovirus* genus along with B19 parvovirus (6) and raise the possibility that SPV-infected monkeys can be a useful and unique animal model of B19 virus infection in humans. We report here the results of experimental infection of cynomolgus monkeys with SPV that support the former proposal and the latter possibility.

MATERIALS AND METHODS

Experimental protocol. Six seronegative adult female cynomolgus monkeys were maintained in isolation before and after inoculation with SPV. The animals were observed daily for evidence of clinical abnormalities, and blood samples were collected from sedated animals in accordance with a predetermined schedule. In phase 1, two animals (no. 1 and 2) were inoculated intravenously with SPV and blood was collected on postinfection (p.i.) days 0, 1, 2, 4, 5, 6, 7, 10, 12, 14, 17, 19, 27, 34, and 47. Based on initial virological findings and the stress to the monkey associated with such frequent sampling, this frequency of collection was reduced in the second phase of inoculations to p.i. days 0, 2, 6, 10, 12, 14, 16, 18, 20, 22, and 27. In phase 2, two monkeys (no. 3 and 4) received virus by the intranasal route and two received it by the intravenous route (no. 5 and 6). Of the monkeys inoculated in phase 2, three were euthanized and bone marrow was collected at necropsy. This included one monkey (no. 4) that was inoculated by the intranasal route and euthanized on day 15 p.i. and two monkeys (no. 5 and 6) that were inoculated by the intravenous route (euthanasia on days 10 and 6 p.i., respectively). In phase 3, two animals (no. 1 and 3) were sham inoculated with SPV by the intravenous and intranasal routes, respectively, and blood samples were collected in accordance with the same schedule as in phase 2.

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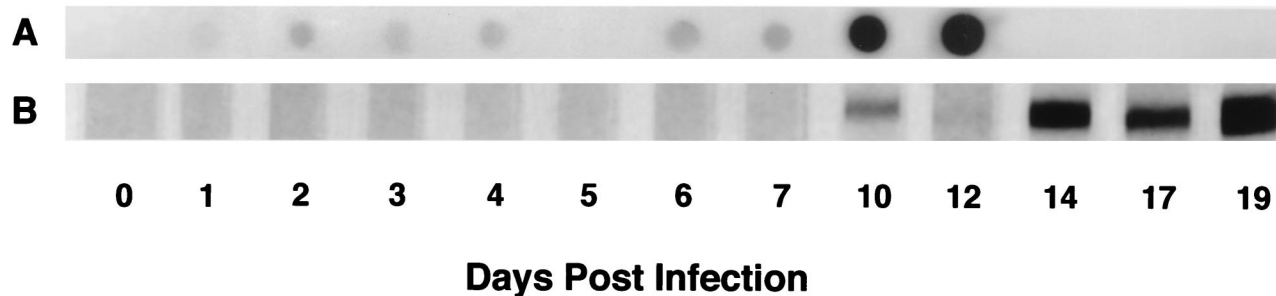
FIG. 1. (A) Viremia detected by dot blot hybridization with the ^{32}P-labeled, cloned P4 SPV probe. (B) Detection of antibody (IgG) to VP2 capsid antigen by Western blotting. Note the rapid virus clearance after appearance of the specific antibody.

Complete hematological evaluations (total leukocyte and erythrocyte counts, hemoglobin, hematocrit, and erythrocyte indices) of blood samples were performed electronically (Coulter Counter M430; Coulter Counter Electronics, Hialeah, Fla.). Reticulocyte numbers were determined on blood smears stained with new methylene blue (Becton Dickinson, Rutherford, N.J.), and differential leukocyte counts were performed on blood smears stained with a modified Wright stain (Sigma, St. Louis, Mo.). Body temperatures and weights of sedated animals were measured at the time of blood collection. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee.

Virus inoculation. SPV was purified by sedimentation of serum from an SPV-infected animal in the original outbreak through 20% sucrose and centrifugation at $150,000 \times g$ for 5 h. The pellet was resuspended in Tris-EDTA buffer (pH 7.5) (22). Aliquots were stored at $-70°C$, and for inoculation they were thawed and resuspended in 1 to 2 ml of sterile normal saline. Monkeys, appropriately sedated with ketamine (0.1 to 0.15 mg/kg), were inoculated ($\sim 6.6 \times 10^{11}$ SPV gene copies per inoculum) by intravenous injection into the saphenous vein or by slow instillation from a syringe directly into the nasal passages.

Identification of virus and antibodies. Viremia was determined by dot blot assay with a 723-bp fragment (P4) of SPV or a cloned full-length SPV probe (22). To detect SPV-specific antibodies, SPV VP2 capsid protein was expressed in a recombinant baculovirus system and purified as described for B19 virus VP2 (15). Purified protein was used to detect SPV-specific immunoglobulin G (IgG) by Western blotting with a goat anti-human IgG horseradish peroxidase conjugate (Tago Inc., Burlingame, Calif.) and diaminobenzidene as the substrate.

Experimental animals were monitored for the presence and potential consequences of intercurrent infection with type D simian retrovirus because of its role in immunosuppression and monkeys' predisposition to anemia in the original outbreak of SPV-associated disease (22). Tissue samples (spleen, mesenteric lymph nodes) collected at necropsy and mononuclear cells obtained from heparinized blood were tested for type D simian retrovirus by formation of characteristic syncytial cells in coculture with the lymphoblastoid Raji cell line (9). Antibodies against type D simian retrovirus were detected in serum samples by using a dot immunobinding assay (13). Both isolation and serologic testing of type D simian retrovirus were done at Virus Reference Laboratory, San Antonio, Tex.

Bone marrow evaluation. Routine light microscopic examination was performed on bone marrow obtained from the femur. After fixation in 10% formalin or 4% paraformaldehyde for 24 h, bone marrow was transferred to 70% alcohol before routine processing through alcohols, embedding in paraffin, and cutting of 6-μm sections that were stained with hematoxylin and eosin. In addition, fresh smears of marrow were fixed and stained with a modified Wright stain (Sigma). SPV was localized in bone marrow by an in situ hybridization technique using a digoxigenin-labeled, cloned 1.3-kb fragment of SPV (6) with detection by an alkaline phosphatase-linked sheep antidigoxigenin antibody and addition of an appropriate substrate (Genius nucleic acid detection kit; Boehringer Mannheim, Indianapolis, Ind.).

RESULTS

Virological and antibody findings. A smoldering, low-level SPV viremia was detectable as early as 1 to 2 days p.i. and persisted until the sudden onset of high-level viremia on day 5 (monkey 1), 6 (monkey 5), or 10 (monkeys 2, 3, and 4) p.i. (Fig. 1 and 2). Viremia had disappeared completely by days 12 and 14 p.i. in monkeys (no. 1 and 2, respectively) inoculated by the intravenous route but persisted until days 15 to 18 p.i. in animals inoculated by the intranasal route (monkeys 3 and 4). Peak viremia preceded a transient but absolute reticulocytopenia. The abrupt cessation of high-level viremia in monkeys 1

and 2 was preceded by the appearance of detectable circulating antibody (Fig. 1). Antibody titers were transiently reduced after their initial detection, and this decrease immediately preceded the complete disappearance of high-level viremia (Fig. 1).

Antibody to type D simian retrovirus was present in five of the six monkeys (no. 1 to 5), but type D simian retrovirus was not isolated from blood samples (monkeys 1 to 3) or from spleen or mesenteric lymph node samples obtained at necropsy (monkeys 4 to 6). No clinical evidence of immunodeficiency (unexplained lymphadenopathy or wasting with chronic diarrhea) was observed in any animal.

Clinical findings. In general, minimal adverse effects of virus inoculation were observed in the experimental animals. Changes in the key parameters monitored for monkey 3 are shown in Fig. 3, and these findings were representative of all of the monkeys studied. Most showed evidence of a slight (less than 10%), temporary weight loss during the first 3 weeks p.i.,

A

DAYS POST INFECTION

#	0	1	2	3	4	5	6	7	10	12	14	17	19	27	34
1 i/v	-	+	+	+	+	-	+	++	+++	+++ R	-	-	-	-	-
2 i/v	-	-	+	+	+	+++	++	+++	+++	+	R	-	-	-	-

B

#	0	2	6	10	12	14	15	16	18	20	22
3 i/n	-	-	+	++++	+ R	++ R	nd	++	+/-	-	-
4 i/v	-	-	+/-	++++	+	+ R	+ *				
5 i/v	-	++	+++	++++ *							
6 i/v	-	+	+ *								

FIG. 2. SPV dot blot assay results. Viremia was detected by dot blot hybridization with a ^{32}P-labeled, cloned P4 SPV probe or a full-length SPV probe. Results are shown for monkeys 1 to 6 inoculated by the intravenous (i/v) or intranasal (i/n) route in phase 1 (A) and 2 (B) studies. Panel A: +, 2×10^8 SPV gene copies/ml; ++, 2×10^9 gene copies/ml; +++, $\geq 2 \times 10^{10}$ gene copies/ml. Panel B: +/-, 3×10^7 SPV gene copies/ml; +, 3×10^8 SPV gene copies/ml; ++, 3×10^9 gene copies/ml; +++, 3×10^{10} SPV gene copies/ml; ++++, $\geq 3 \times 10^{11}$ SPV gene copies/ml. R, absence of reticulocytes; *, euthanized; nd, not determined.
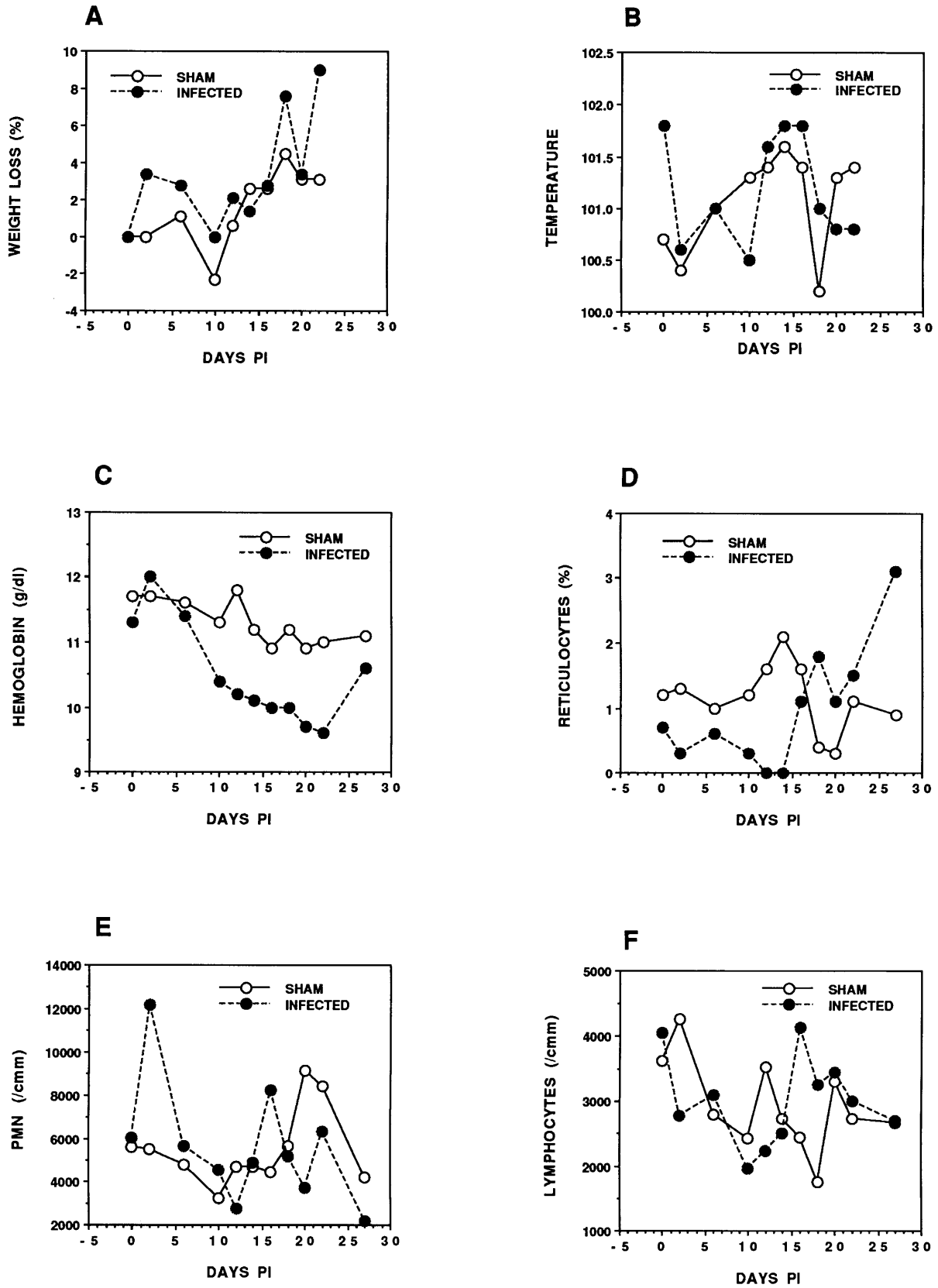
FIG. 3. Alterations in selected parameters in monkey 3 after infection with SPV or sham infection. A, body weight; B, temperature; C, hemoglobin; D, reticulocytes; E, neutrophils (PMN); F, lymphocytes. cmm, cubic millimeter.

which was attributable, in part, to the frequent sedation and blood collection with associated anorexia. One monkey (no. 2) which was obese at the outset continued to lose weight and ultimately was euthanized on day 66 p.i. Necropsy revealed that the ill health was attributable to severe chronic pancreatitis and not to SPV.

The most consistent consequence of infection was transient loss of reticulocytes from the peripheral circulation. Reticulocytopenia was preceded by the peak of viremia and followed by a rebound reticulocytosis. Reticulocytes were undetectable on days 12 (monkeys 1, 2, and 3) and/or 14 (monkeys 3 and 4) p.i. in all of the monkeys monitored. Another consistent finding was a mild anemia reflected in a progressive decline in erythrocyte numbers, hemoglobin, and hematocrit during the first 15 to 17 days p.i. that gradually recovered to normal levels and was not observed in the sham-inoculated monkeys. A slight rise in body temperature (of approximately 1°F) and lower numbers of circulating neutrophils and/or lymphocytes were observed at or around the time of peak viremia.

Bone marrow findings. Bone marrow was obtained and examined before (monkey 6, day 6 p.i.), at (monkey 5, day 10 p.i.), or after (monkey 4, day 15 p.i.) peak viremia. In contrast to the normal appearance of marrow on day 6 p.i., many intranuclear inclusion bodies within maturing cells of the erythroid series were observed on day 10 p.i. in paraffin-embedded sections of marrow and in Wright-stained aspirates (Fig. 4). Inclusions were most prominent in normoblasts but also were seen in pronormoblasts. Marrow examined on day 15 p.i. had relatively fewer inclusion bodies (although these were still common), and there were many proliferating cells of the erythroid series, consistent with a regenerative response. In situ hybridization with a digoxigenin-labeled, cloned SPV probe confirmed the presence of SPV in cells on days 10 and 15 p.i. (Fig. 4). An occasional cell was positive by in situ hybridization on day 6 p.i.

DISCUSSION

Our findings indicate that uncomplicated SPV infection of healthy adult cynomolgus monkeys is characterized by a transient arrest in erythropoiesis that is clinically asymptomatic. In this respect, SPV infection of cynomolgus monkeys resembles uncomplicated, natural B19 parvovirus infection of adult humans (4) or experimental infection of volunteers (2, 26), which is largely asymptomatic or associated with a mild, influenza-like illness.

Infection of monkeys with SPV was followed by a low-grade viremia detectable as early as 1 or 2 days p.i. Peak viremia was present variably (from days 5 to 12 p.i. in animals inoculated intravenously) and was followed by a precipitous decline in levels of the virus in blood that was associated with the appearance of antibody. A similar pattern was observed in animals inoculated intranasally, but the virus persisted in the circulation until day 15 or 16 p.i. These findings resemble those obtained with human volunteers inoculated with B19 parvovirus (2, 26) and suggest that virus replication must occur initially in cells other than those of the bone marrow, perhaps in the upper respiratory tract, with virus shed by initially infected cells appearing as a low-grade viremia. It is also possible that initial bouts of replication in bone marrow or circulating blood cells may contribute to this low-grade viremia (note the few SPV-positive cells in bone marrow on day 6 p.i.), but other, hitherto unidentified, cells and tissues also may contribute to the virus load.

Peak viremia was associated with extensive replication of virus in (and widespread destruction of) erythroid cells, par-
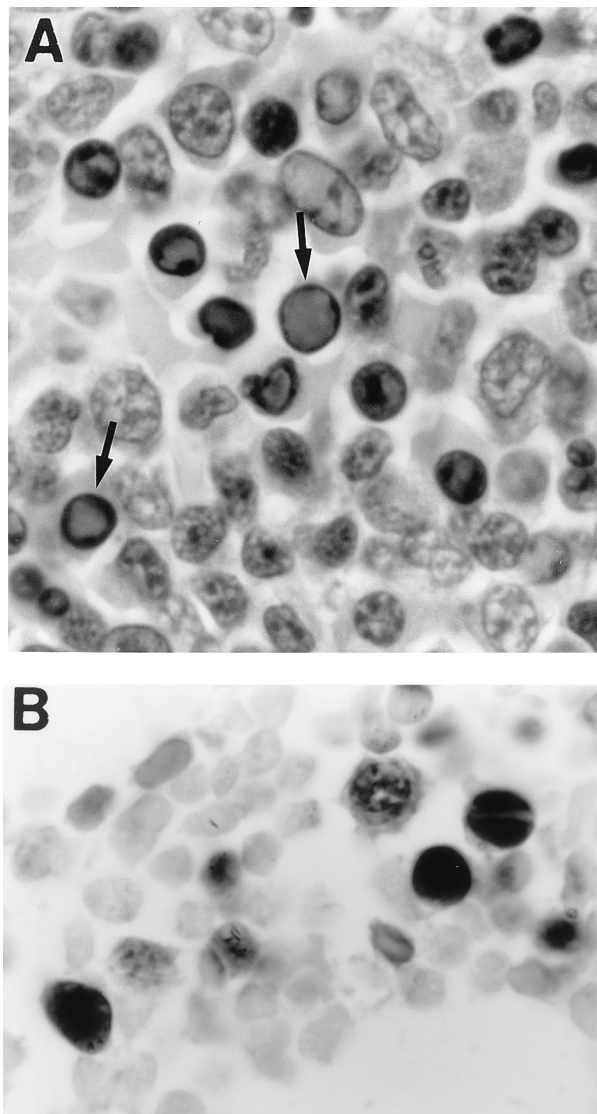

FIG. 4. Bone marrow. (A) Numerous intranuclear inclusions (examples are indicated by arrows) in erythroid cells (X1280, day 10 p.i.). (B) SPV detected in cells (dark blue-black staining) by in situ hybridization with a digoxigenin-labeled, cloned SPV P2 probe (X1024, day 15 p.i.).

ticularly normoblasts, which was reflected in the numerous characteristic intranuclear inclusion bodies. Increased proliferation of erythroid cells occurred as viral titers fell, manifested most obviously as a rebound reticulocytosis. These findings are consistent with rapid virus clearance associated with an effective immune response (reflected in the appearance of SPV-specific antibody as early as 10 days p.i.), are typical for a primary systemic viral infection (18), and are similar to observations for uncomplicated natural infection with SPV (23).

Our findings obtained with SPV-infected monkeys are very similar to those obtained with human volunteers infected with B19 parvovirus (2, 26), particularly with respect to the time of onset of viremia, early reticulocytopenia, the rapid appearance of antibody coincident with clearance of the virus, destruction of erythroid cells during peak viremia, mild anemia, and the presence of mild symptoms. However, some differences in clinical manifestations were noted. Infection of human volunteers with B19 parvovirus was associated with a mild pyrexia (up to

3°F), and some subjects had transient symptoms (observed in three of five seronegative people [2] but not in three B19 virus-inoculated volunteers [26]) of erythematous rash, arthralgia, and mild arthritis, commencing about 17 or 18 days p.i. and lasting approximately 3 to 5 days. Pyrexia was less prominent in the monkeys but may be more difficult to detect because of the need to capture and anesthetize the animals. These procedures themselves can raise body temperature, thereby masking virus-induced rises. Similarly, a mild rash is difficult to detect in haired animals. The apparent absence of arthralgia or arthritis may be because monkeys are adept at masking any evidence of pain or dysfunction, even when it is severe. In humans, lower numbers of circulating neutrophils, lymphocytes, and platelets occurred in parvovirus-infected human volunteers, findings that were less apparent in our monkeys.

This study meets the requirements of Koch's postulates for SPV. SPV was isolated from anemic monkeys that were immunosuppressed by a concurrent type D simian retrovirus infection. Analysis of the original and subsequent outbreaks of anemia in cynomolgus monkeys indicates that infection with SPV is usually asymptomatic in monkeys in the absence of complicating factors such as underlying immunodeficiency (22, 23), and the present study reproduced this essentially asymptomatic infection. As for humans, it appears that asymptomatic or benign infection with transient reticulocytopenia is the usual consequence of parvovirus infection in cynomolgus monkeys, because clinical disease in monkeys is uncommon with SPV, although at least 50% are seropositive (unpublished observations).

Our findings validate SPV-infected monkeys as a useful model in which to study unanswered questions concerning parvovirus infection of humans, because no other animal model of B19 parvovirus infection exists (24). These unanswered questions include the route of B19 parvovirus transmission and virus spread from the port of entry to the site of replication in bone marrow; the role of B19 parvovirus in chronic arthritis, myocarditis, vasculitis, neurological disease, and hydrops fetalis; the pathogenesis of persistent infection; and the possibility of integration of viral DNA with the host's genome. The mechanism whereby infection in utero leads to persistent anemia (5), even when the virus has been cleared by immunoglobulin therapy, also requires elucidation. In addition, studies of SPV in monkeys may be of value in developing and refining effective vaccination regimens for B19 parvovirus and thus may reduce the need for human volunteers for vaccine trials.

ACKNOWLEDGMENTS

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