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and have found that it is complete and satisfactory in all respects,
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examining committee have been made.

Craig E. Litz

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June 10, 1996

Date

GRADUATE SCHOOL

MORPHOLOGICAL MANIFESTATIONS OF PARVOVIRUS B19 INFECTION IN THE BONE MARROW

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

Lucia Ellen More

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

June 1996

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DEDICATION

I dedicate this work to the memory of my father and mother who provided me with an excellent education and raised me to appreciate the value of hard work, discipline and family.

I am grateful for and blessed by the unconditional love and support of my husband and soulmate, Denny. I thank my children, Daesha, Tasha and Logan, for picking up the slack around the house and letting me have a turn at the computer so I could type my thesis. The love and pride of my family gave me the strength to keep working despite the challenges we encountered.

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My graduate program advisor, Karen Lofsness, Assistant Professor of the Department of Laboratory Medicine and Pathology, has been an inspiration to me since my first day at the University of Minnesota. I consider her my model for the kind of educator and scientist I will strive to be. I will treasure my memories of the many times she listened to my personal and educational concerns with a smile and offered so much encouragement and insight. I look forward to remaining in contact with her as a colleague and a friend.

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Statement of Purpose

The purpose of this study was two-fold: to devise a sensitive assay for the detection of DNA from Parvovirus B19, a virus associated with marrow dysfunction and cytopenias, and to characterize the morphological spectrum of findings in bone marrow specimens from infected patients. A more comprehensive picture of typical morphological findings would aid in the accurate diagnosis, treatment, and prediction of outcome in cases of unexplained cytopenias.

ABSTRACT

Morphological Manifestations of Parvovirus B19 (PV B19) Infection in Bone Marrow.

Parvovirus B19 (PV B19) preferentially infects erythroid progenitor cells in the bone marrow, frequently causing anemia along with transient aplastic crisis and pure red cell aplasia. The giant normoblast, previously described as the classic marrow finding, is not a highly sensitive indicator of infection. We devised a highly sensitive two-round, nested PCR procedure to detect PV B19. Eight of 78 clinical specimens from individuals with unexplained cytopenias which tested positive by this method, were studied to define the effects of this virus. Examination of the bone marrow of these patients revealed a spectrum of morphological manifestations including: the giant normoblast, hypocellular marrow, hypercellular marrow, interstitial depletion, red cell aplasia, erythroid hyperplasia and evidence of erythroid dysplasia. The heterogeneity of these findings may reflect atypical immune responses leading to a prolonged course in some patients.

INTRODUCTION

Parvovirus B19, the only parvovirus known to be infectious in humans, was first discovered by Yvonne Cossart in 1974, when she detected a false positive result on a blood donor serum sample in hepatitis B assay (Cossart et al, 1975). The unusual name, PV B19, originated from the coding of that donor sample as number 19 on plate B. Further assays using this viremic serum as a source of antigen revealed that antibodies to the virus were found in a high proportion of normal adults, suggesting infection in childhood.

The first description of clinically significant disease associated with PV B19 infection was hypoplastic crisis in patients with sickle-cell anemia (Pattison et al, 1981). In 1983 infection with PV B19 was linked to fifth disease in otherwise normal children (Anderson et al, 1983). Since 1985, other syndromes have been clearly linked to PV B19, including post-infectious arthralgia/arthritis in adults and fetal loss in the mid-trimester of pregnancy due to intrauterine transmission from an infected mother (Young, 1995). Chronic PV B19 infection also causes a hematological syndrome similar to pure red cell aplasia (Frickhofen and Young, 1989).

Epidemiology

An ubiquitous benign virus, PV B19 is the cause of a common infection in humans. Most people are infected during their lifetime; by age 15 approximately 50% of children have detectable IgG antibodies to the virus (Cohen et al,1988). Another

study showed a 36% prevalence by the age of 19 and 49% prevalence by 39 years (Anderson et al, 1986) (Table 1). It is apparent that this proportion increases with age; more than 90% of the elderly have detectable PV B19-specific antibodies (Cohen et al, 1988). Seroprevalence is similar worldwide, except among some isolated tribal populations (Young, 1995). The most common manifestation of acute PV B19 infection is fifth disease, with peak occurrence during summer and fall. In epidemic school outbreaks, between 10% and 60% of susceptible children develop fifth disease, while 20%-30% of adult school and day-care personnel also become infected (Chorba et al, 1986; Koch and Adler, 1989).

Table 1. *PV B19 IgG antibody by age group.*

Age Group (year)	% Positive tests (no. Tested)
<5	2 (51)
5-9	21 (32)
10-19	36 (49)
20-39	49(173)

(Adapted from Anderson et al, 1986)

PV B19 is excreted from the nasopharynx; thus, the major route of transmission is probably through the upper airway (Anderson et al, 1985). PV B19 can also be transmitted by transfused blood products (Mortimer et al, 1983a).

Clinical Syndromes

Erythema Infectiosum (fifth disease) of Childhood:

PV B19 infection in humans causes the childhood exanthem known as erythema infectiosum or fifth disease. This childhood disease was probably first described by Robert Willan in 1799 (van Elsacker-Niele et al, 1987). The infection was first named 'erythema infectiosum' in 1899 by Sticker, a German investigator (Sticker, 1899). Six years later, Cheinisse classified it as the 'fifth rash disease' of the classical exanthema of childhood (Cheinisse, 1905).

Infection in immunocompetent individuals is generally self-limited and children are not usually very ill. An incubation period of five to seven days is followed by a prodromal phase characterized by headache, malaise, pyrexia, and respiratory symptoms associated with viremia (Fig. 1). The characteristic fiery red "slapped cheek" facial erythema appears two to five days later, followed in one to four days by the appearance of the second stage, a lacy, reticular, maculopapular eruption on the trunk and extremities. The rash may be transient, or recur over one to three weeks. It may also be accompanied by pruritus, especially on the soles of the feet.

Viral cultures are rarely positive since the clinical manifestations that prompt a patient to seek medical attention are secondary to immune complex formation; by the time a patient presents to a medical provider, the period of viremia has passed. It is the immune response that produces the clinical manifestations of rash and arthralgia that occur during the period of antibody formation. Bone marrow depression, resulting in a transient anemia, occurs during the early viremic phase and normally resolved with the

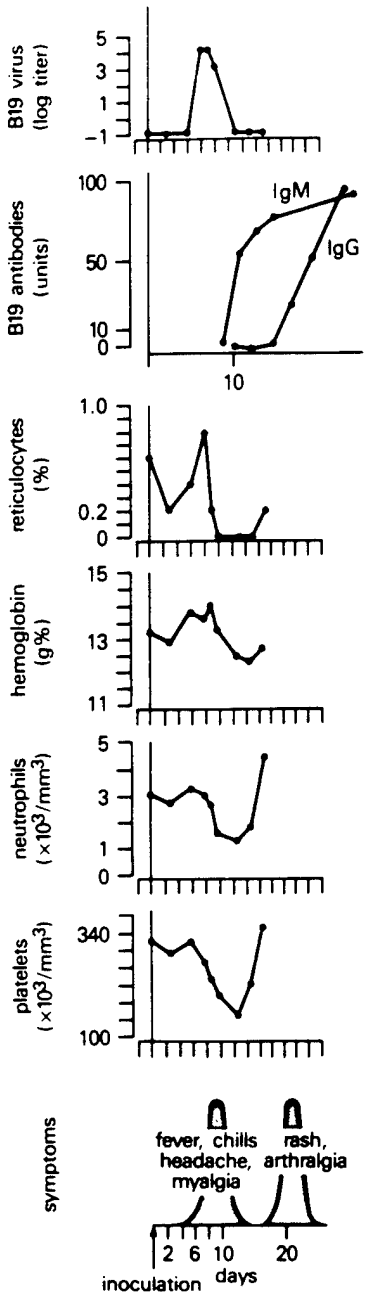

Fig. 1. Virological, immunological and clinical course of PV B19 infection in an immunologically normal individual. (Adapted from Anderson et al, 1985)

appearance of a neutralizing antibody response (Brown et al, 1994).

Parvovirus-related Polyarthropathy Syndrome:

Adults may have an asymptomatic infection or a non-specific flu-like illness. About 50% of adults, especially women, experience a transient arthropathy which lasts one to three weeks (Woolf et al, 1989) with symptoms that mimic rheumatoid arthritis or fibromyalgia (Reid et al, 1985; White et al, 1985). In 20% of affected women the arthralgia may persist from 2 months to 2 years.

A study of patients with classic rheumatoid arthritis (RA) showed a 92.3% prevalence of PV B19 IgG antibodies using RIA , compared to 67.6% of patients with inflammatory arthritis and 60.9% of control patients (Cohen et al, 1986). In studies of synovial fluid, PV B19 has been found in a case of serologically proven infection and in a patient with reactive arthritis (Dijkmans et al, 1988; Kandolf et al, 1989). PV B19 was found in synovial biopsies of 75% of patients with rheumatoid arthritis (RA), but also in 16.7% of those in the non-RA groups (Saal et al, 1992).

PV B19 Infection During Pregnancy:

The most severe consequence of PV B19 infection occurs during the first two trimesters of pregnancy in non-immune women, and can lead to miscarriage in the first trimester or hydrops fetalis during the second (Anderson and Hurwitz, 1988). Assays of PV B19 IgG antibodies in pregnant women showed that only about half have acquired protective immunity (Schwarz et al, 1988). The fetal red cell mass increases

greatly during the second trimester and provides an excellent target for PV B19. Because the developing fetal immune system is unable to mount an adequate immune response to the virus, PV B19 infection is not brought under control, and fetal death results from congestive heart failure due to severe fetal anemia. The estimated risk of spontaneous fetal abortion in infected women is about 5% (Rodis et al, 1990).

Transient Aplastic Crisis (TAC):

TAC is characterized by a sudden cessation of erythropoiesis, absence of erythroid precursors in the bone marrow, and reticulocytopenia followed by anemia (Brown et al, 1994). TAC due to PV B19 infection has been described in patients with underlying hemolytic disorders such as hereditary spherocytosis (Kelleher et al, 1983), thalassemia (Brownell et al, 1986), red cell enzymopathies (Duncan et al, 1983; Rechavi et al, 1989), and autoimmune hemolytic anemia (Smith et al, 1989). TAC can also occur under conditions of erythroid stress such as hemorrhage (Frickhofen et al, 1986), iron deficiency anemia (Lefrere et al, 1986), and following kidney (Neild et al, 1986) or bone marrow transplantation (Niitsu et al, 1990). This ordinarily self-limiting disease can be quite severe in these types of patients. Symptoms are related to the degree of anemia and range from the dyspnea and lassitude of anemia to confusion, congestive heart failure, and severe bone marrow necrosis (Conrad et al, 1988; Godeau et al, 1991). In contrast to normal patients with classic erythema infectiosum, TAC patients are often viremic at the time of presentation (Brown et al, 1994). As serum

virus is cleared from the system, PV B19 specific IgM antibodies become detectable. TAC can be easily treated by blood transfusions and results in lifelong immunity.

Persistent Infection:

Persistent pure red cell aplasia (PRCA) caused by PV B19 infection has been reported in individuals with various immunodeficiency syndromes. These patients fail to mount an adequate immune response to the virus and the subsequent immune-mediated symptoms of fifth disease do not appear. In fact, persistent PV B19 infection may be the dominant manifestation of this infection in some inherited immunodeficiency states (Brown et al, 1994). Severe anemia is the major clinical finding and patients are dependent upon red blood cell transfusions until PV B19 infection is documented and immunoglobulin treatment is administered.

Patients with the acquired immunodeficiency syndrome (AIDS) are predisposed to persistent PV B19 infection and PRCA (Frickhofen et al, 1990). Treatment with immunoglobulin results in a decrease in viral titers and the appearance of specific antibodies in the serum. A few patients have developed symptoms of fifth disease after immunoglobulin treatment, which is consistent with the immune-complex mediated nature of the illness.

Children with lymphoblastic leukemia and other malignancies in remission, during or after chemotherapy, have been known to suffer from chronic PV B19 infection and present with persistent anemia without immune-mediated symptoms (Brown et al, 1994). The megakaryoblasts and lymphoblasts are affected; some

developed pancytopenia. Immunoglobulin treatment can be beneficial, but not necessarily curative in these cases (Kurtzman et al, 1988).

Chronic PV B19 infection has been described following procedures requiring potent immunosuppression such as cardiac, liver, and bone marrow transplantation. Despite the presence of PV B19 antibodies before transplantation, some patients have been shown to be susceptible to the virus due to an undetectable antibody level following immunosuppression (Young, 1995).

Less severely immunosuppressed patients, such as those on steroid therapy for systemic lupus erythematosus, have suffered prolonged anemia following PV B19 infection (Koch et al, 1990). The infection usually resolves spontaneously once an antibody response occurs.

Parvovirus B19

Structure:

Parvovirus B19 is a small, non-enveloped, single-stranded DNA virus of icosahedral symmetry and with a diameter of 15-28 nm (Young, 1995). Each virion contains both a positive and a negative DNA strand, although only a single strand is used for coding genes. Parvoviruses are very stable to physical inactivation due to their lack of an envelope and limited DNA content. Most stages of the PV B19 life cycle have not been studied extensively, but are thought to include attachment to and penetration into the target cell, uncoating, DNA replication, RNA transcription, empty

capsid assembly, insertion of DNA into capsid, virion maturation, and cell lysis and release of virions.

Genome:

The PV B19 genome consists of a single DNA strand of 5,596 nucleotides (nt) (Shade et al, 1986). It is composed of an internal coding sequence of 4,830 nt, flanked by terminal sequences of 383 nt each, which are identical inverted repeats (Fig. 2).

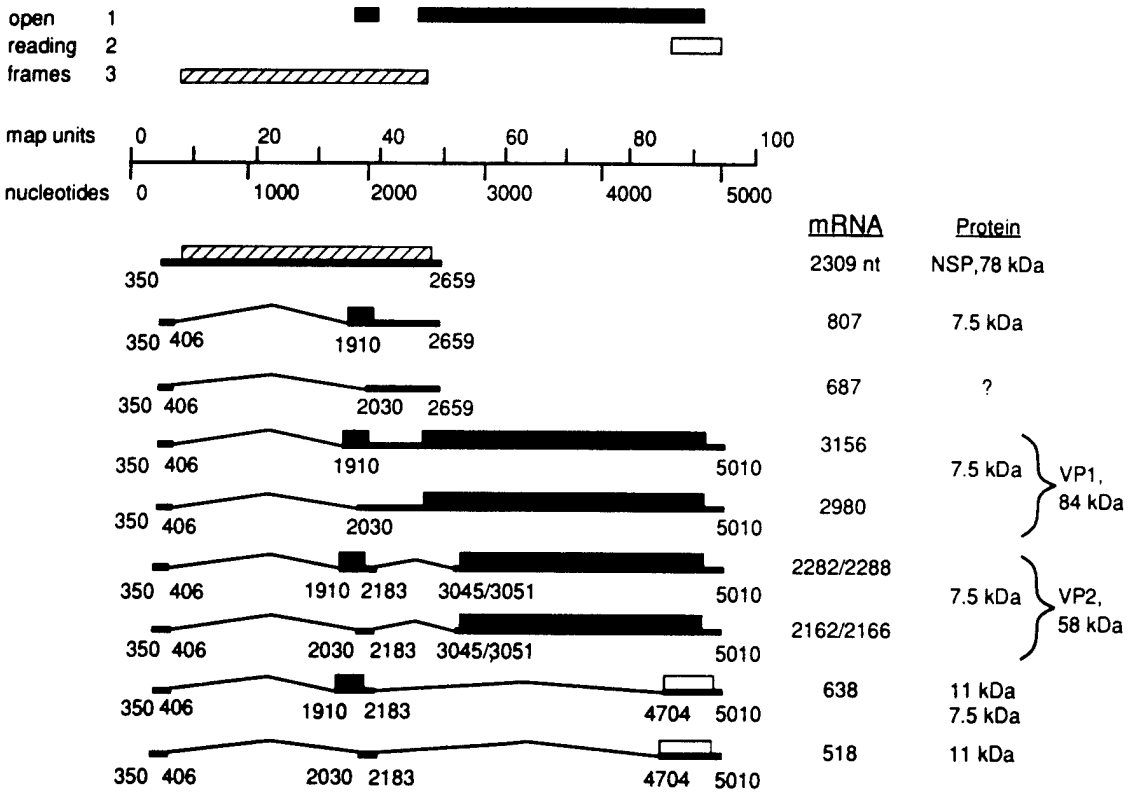

Fig. 2. Transcription map of PV B19. Open bars at top indicate reading frames. (Adapted from Brown et al, 1994)

Replication:

PV B19 is an autonomous virus that replicates in the absence of a helper virus, but requires host cellular functions only expressed during the "S" phase of the cell cycle. Therefore, PV B19 has an absolute requirement for proliferating cells (Brown et al, 1994). Characteristic of PV B19 is that the terminal DNA sequences are palindromic and capable of assuming hairpin duplex configurations at both ends of the DNA strand (Astell, 1990). During viral replication, these double-stranded regions serve as primers that initiate the synthesis of the complementary DNA strand through a rolling hairpin model (Fig. 3).

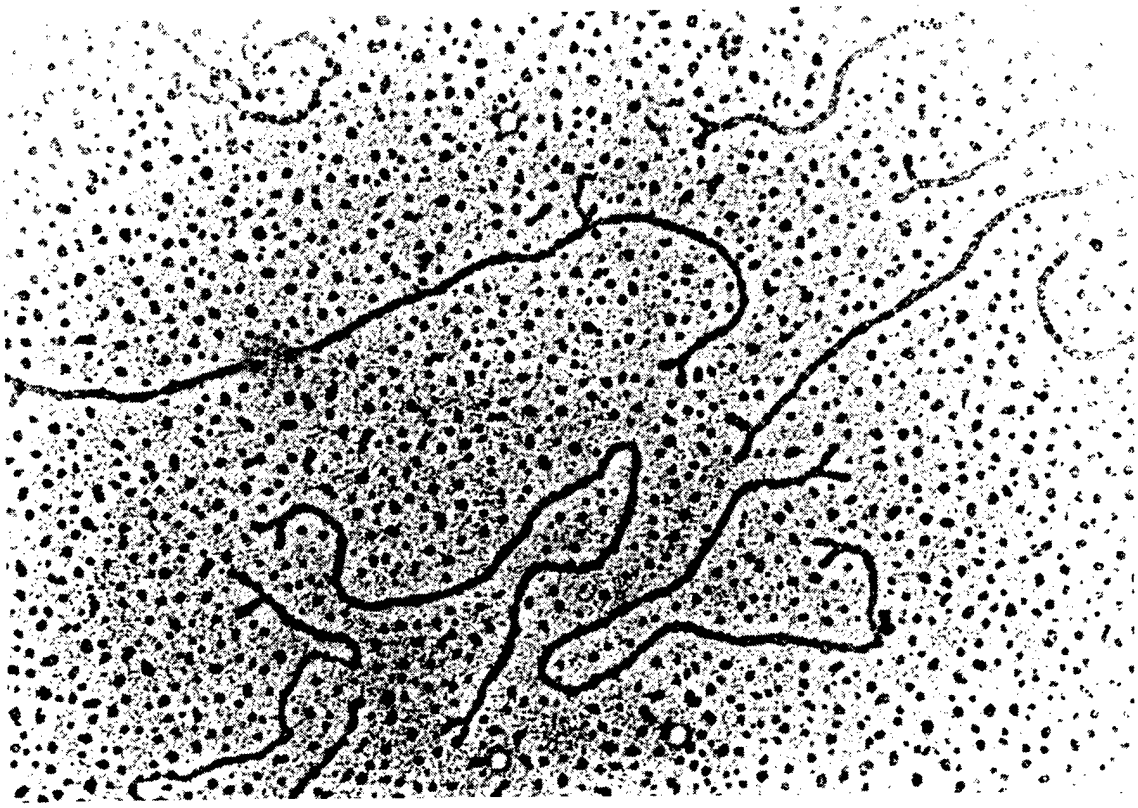

Fig. 3. Electron micrograph of positive and negative PV B19 DNA strands annealed in vitro. (From Brown et al, 1994)

Structural and Nonstructural Proteins:

Two capsid proteins, which are encoded by overlapping transcripts from the 3'end of the genome, make up the structure of PV B19. The major protein, VP2, is a 58 kD protein that constitutes about 90% of the viral capsid. The minor protein, VP1, is a 83 kD protein that includes 227 amino acids at the amino terminus not present in the major capsid protein species. Infected cells can express the PV B19 capsid proteins without any effect on cell proliferation (Kajigaya et al, 1989). The viral DNA constitutes 19-37% of the total mass of the capsid of the complete infectious particle.

The non-structural proteins are required for virus propagation and are encoded by a region of the PV B19 genome sequence encompassing nucleotides 435 to 2,448. PV B19 non-structural proteins are required for replication of the viral DNA and RNA transcription. Expression of the non-structural proteins is responsible for host cell death (Ozawa et al, 1988).

Hematological Manifestations of PV B19 Infection

Studies have demonstrated that PV B19 displays a definite preference for propagation within the erythroid precursors of the bone marrow (Ozawa et al, 1987). The cellular receptor for the virus is the P antigen which is expressed on the surface of red blood cells, megakaryocytes, endothelial cells, trophoblast cells in the placenta, and fetal myocardial and liver cells. Individuals who lack the P antigen are not susceptible to PV B19 infection (Brown, Anderson, and Young, 1993).

The classic finding of PV B19 infection, the giant normoblast, was first described by Owren in 1948 in the bone marrow of patients with chronic acquired pure red cell aplasia of unknown cause (Owren, 1948). This strikingly large cell is an early erythroid precursor with a diameter of about 25-32 um, nuclear inclusions or multiple nucleoli, and cytoplasmic vacuolization (Figs. 4 & 5). Although this cell is a very specific indicator of PV B19 infection, its presence in the marrow is transient. It may be identifiable only during specific stages of the course of the infection. PV B19 is directly toxic to host cells and induces the characteristic morphological change in the erythroid precursors resulting in giant pronormoblasts. The more mature erythroid cells are

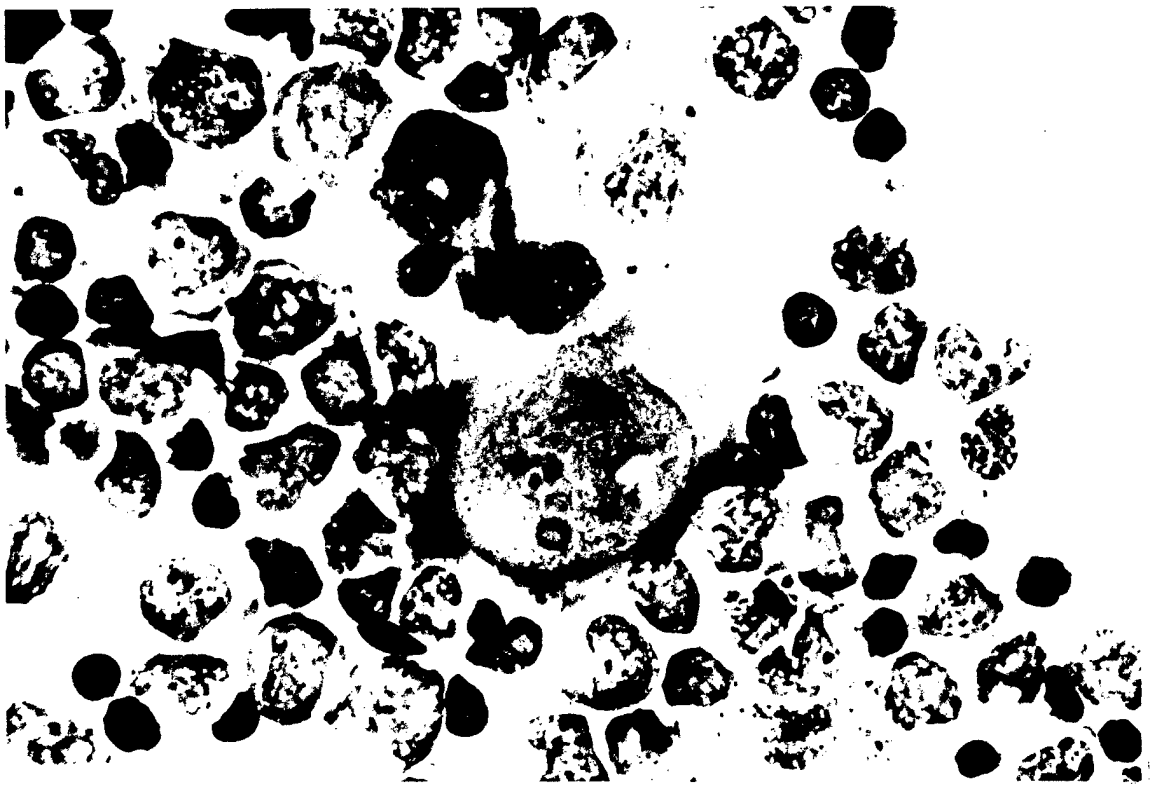

Fig. 4. Giant normoblast in a bone marrow biopsy sample from a patient with PV B19 infection. (Photo courtesy of Craig E. Litz, M.D.)

absent from the bone marrow of individuals with PV B19 induced red cell aplasia. Although the number of giant pronormoblasts in a specimen roughly corresponds to the virus content (Frickhofen et al, 1990), the cells themselves may not contain detectable PV B19 capsid proteins.

In vitro studies, which demonstrated inhibition of late erythroid colony formation by PV B19, confirmed that the cells targeted by the virus were erythroid progenitors (Mortimer et al, 1983b). Southern analysis of suspension cultures of human bone marrow cells infected *in vitro* with PV B19 showed that the virus replicated only in the erythroid fractions of the sample (Ozawa et al, 1987). Susceptibility of erythroid

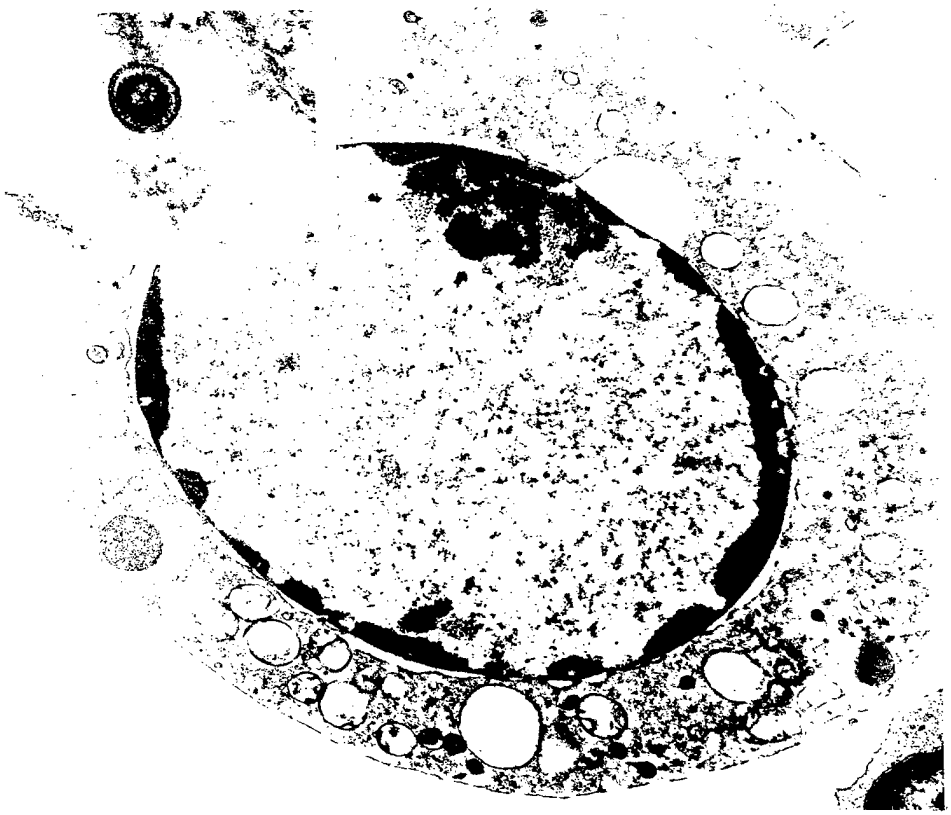

Fig. 5. Electron micrograph (12,000X magnification) of a giant normoblast showing viral inclusions. Inset: Viral particle (62,000X magnification). (EM courtesy of Janet Parkin.)

progenitors to PV B19 increases with differentiation, sparing the pluripotent stem cell and targeting the colony-forming units-erythroid (CFU-E) (Takahashi et al, 1990).

A study of normal volunteers experimentally infected with PV B19 showed a peak in viremia at 9 days. At the onset of viremia the numbers of erythroid burst-forming units (BFU-E) in peripheral blood fell to a very low level. The number rose at the end of viremic phase with a marked overshoot (Potter et al, 1987). Reticulocytopenia occurs in many patients and may reflect the failure of reticulocyte egress from the marrow. It was speculated that PV B19 interferes with the various interactions between cell surface receptors (integrins) and nonhematopoietic components of the marrow such as stromal cells and fibronectin (Murray et al, 1993). Granulocyte-macrophage colony-forming units (CFU-GM) were also markedly decreased at the time of maximum viremia, although the fall in numbers occurred two days later. The lymphocyte count falls sharply during PV B19 viremia, followed a day or so later by decreased platelet and neutrophil counts. Once the viremia clears, the lymphocytes recover, again followed by neutrophil and platelet recovery a day or so later, occasionally reaching values above those at baseline.

Marrow changes were restricted to the erythroid lineage. Both BFU-E and CFU-E are sensitive to infection by PV B19 but, the CFU-E are the prime targets. The bone marrow morphology was normal at 6 days, but at day 10 there was an almost total loss of erythroid precursors (Fig. 6), followed by a 1-3 g/dL drop in hemoglobin and an increase in serum erythropoietin. The mechanism of increased erythropoietin production during PV B19 infection is not clear. A sharp increase in serum

erythropoietin occurs after the peak. This peak coincides with the loss of erythroid precursors in the bone marrow. Once the numbers of BFU-E recover, the erythropoietin levels decline to normal. It is clear, however, that erythropoietin is an absolute requirement for viral propagation, probably to maintain the rapidly dividing erythroid cells viremia (Potter et al, 1987).

Dysplastic alterations in myeloid and megakaryocytic cells may also be present. Thrombocytopenia is thought to be mediated by the expression of the non-structural protein of the virus causing inhibition of megakaryocyte colony formation (Srivastava

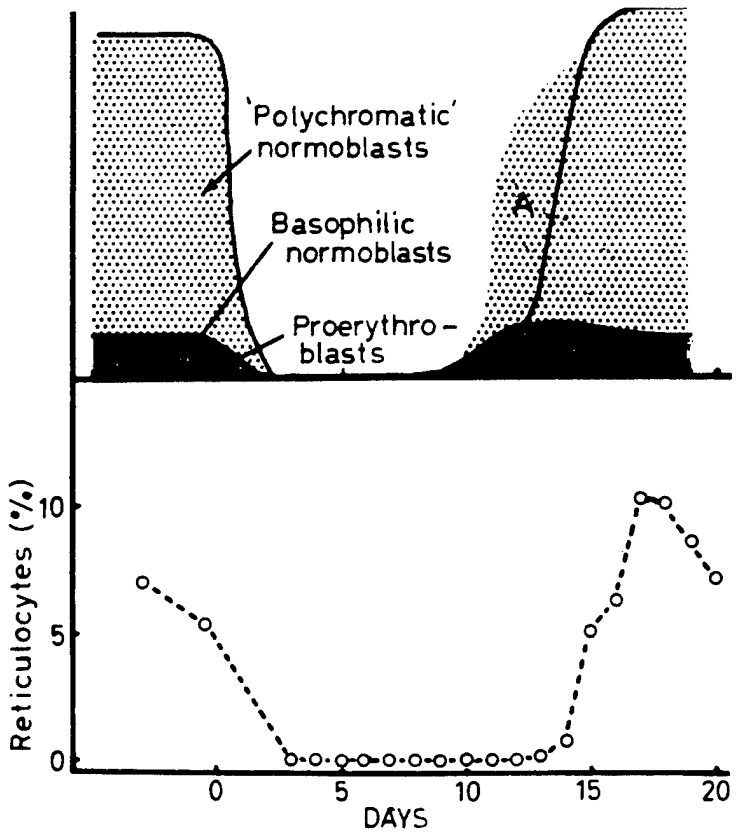

Fig. 6. Loss and recovery of erythroid precursors and reticulocytes in the normal course of infection. (Adapted from Chanarin et al, 1964)

et al, 1990). Srivastava documented complete transcription of the PV B19 genome in mature megakaryocytes, without DNA replication, resulting in impaired megakaryopoiesis. Whether the presence of P antigen on megakaryocytes is involved in this process has not been determined. Thrombocytopenia induced by PV B19 infection may precede the rash due to bone marrow suppression by the virus (Nagai et al, 1992), or occur as the result of immunological mediation (Lefrere and Got, 1987). Since the lifespan of platelets is about 10 days, while that of red cells is 120 days, thrombocytopenia in the absence of anemia may occur as the sole manifestation of PV B19 infection (Yoto et al, 1993).

I has been suggested that neutropenia and thrombocytopenia result from the elimination of granulocytes and thrombocytes which have a PV B19-antibody-complex bound to their Fc receptors (Frickhofen & Young, 1990). One case of persistent PV B19 infection manifested as recurrent agranulocytosis, and bone marrow biopsy showed recurrent granulocytic aplasia. The cause of the persistent infection in this patient was attributed to a subtle immunodeficiency (Pont et al, 1991).

Immune Response

Virus-specific IgM and IgG antibodies appear following PV B19 infection. IgM appears 10 -12 days after exposure and may be detected in serum samples for several months. PV B19 IgG antibodies appear about 2 weeks post infection and persist for life with a rise in levels after re-exposure.

A previous study showed that in serum samples obtained early after infection, antibodies to the major capsid protein VP2 are predominant and often exclusive. Later during convalescence, or in serum samples obtained as part of routine screening of immune individuals, predominant antibodies were those directed to the 83 kD protein. Immunoblot analyses have shown that commercial immunoglobulin preparations contain antibodies to both 58- and 83-kD capsid proteins (Kurtzman et al, 1989).

Persistent infection with PV B19 compromises immune function of both T- and B-cell lymphocytes by direct cytotoxicity or by altered function of persistently infected cells. An attempt to measure T-cell response to viral antigens or lymphocyte proliferation on presentation of cell bound viral antigens in response to PV B19 infection was unsuccessful (Kurtzman et al, 1989). The same study suggested that some immunocompromised patients either do not produce anti-PV B19 antibodies (a qualitative defect) or that they may produce antibodies that fail to recognize the capsid protein epitopes that do not neutralize the virus.

Methods of Detection of PV B19

Currently there are several assays for detection of PV B19 infection. Solid-phase antibody-capture radioimmunoassay is routinely used to measure anti-PV B19 IgM and IgG antibodies (Cohen et al, 1991). Viral capsid proteins can be detected in acetone-fixed cytocentrifuge preparations of bone marrow cells by binding a monoclonal antibody specific to the major capsid protein using alkaline phosphatase-anti-alkaline phosphatase (APAAP) immunocytochemistry (Cordell et al, 1984).

The most sensitive assays for detection of PV B19 are based on nucleic acid hybridization methodologies. The PV B19 genome was first sequenced by Shade et al in 1986, providing the means to manufacture synthetic oligonucleotide probes. Viral DNA and RNA have been demonstrated within specific cells by in-situ hybridization using ^{35}S-labeled parvovirus specific DNA probes (Cotmore et al, 1984). Dot-blot, slot- blot, and Southern-blot analyses, have also been utilized (Frickhofen et al, 1990). Amplification of PV B19 sequences using the polymerase chain reaction (PCR) technique has greatly enhanced the sensitivity level for detection of PV B19 and is now considered the 'gold standard' method for detection of this virus in clinical specimens.

In this work, bone marrow samples from patients with unexplained cytopenias were studied to determine whether there was evidence of PV B19 infection and if these patients displayed common morphological findings, in addition to the classic giant normoblast, which could be used to recognize specimens which should be tested for PV B19 infection. A nested PCR was developed to increase the sensitivity over one-round PCR and maximize our ability to detect samples infected with PV B19 for study.

MATERIALS and METHODS

Specimens

Seventy-eight patients with unexplained cytopenias were retrospectively evaluated for the presence of PV B19 infection. The bone marrow biopsies of these patients revealed no morphological explanation for the presence of cytopenias and no other disturbances were found in their peripheral blood smears. In an attempt to assure that there was no other explanation for the cytopenia, our patient criteria required that none of these patients had myeloblast counts below 4% in the bone marrow and that karyotypic studies, which were performed in 2 patients, were also normal. The bone marrow specimens were collected between 1966 and 1996. DNA from 26 specimens was extracted from paraffin-embedded bone marrow biopsies; DNA from the remaining 52 samples was extracted from fresh bone marrow aspirates.

In addition, bone marrow samples from 144 individuals with known malignancies were selected for PV B19 studies. DNA from these samples was extracted from fresh bone marrow aspirates. The specimens were collected between 1988 and 1996.

DNA Extraction

DNA was extracted from paraffin-embedded bone marrow biopsies according to the methods of Mashal et al (Mashal et al, 1993), as well as from fresh bone marrow aspirates according to standard methods (Sambrook et al, 1989).

Briefly, for the fixed specimen extraction, five 10 um thick sections from a paraffin-embedded bone marrow biopsy block were placed into a 2.0 mL tube. One mL xylene was added to the tube and vortexed for 30 seconds to dissolve the paraffin. After centrifugation for 2 minutes, the xylene was decanted and the wash process repeated. One mL absolute ethanol was added to the tube, vortexed for 30 seconds and centrifuged for 2 minutes. The ethanol wash was repeated. The remaining ethanol was evaporated off the sample in a 45° C heating block. The pellet was suspended in 750 uL of 50mM Tris (pH 8.0), 0.5% sodium dodecyl sulfate (SDS), and 1 mM EDTA. 100 ug/mL proteinase K (Boehringer-Mannheim Biochemicals) was added. After vortexing, the solution was placed in a heating bath overnight at 55° C for digestion. Following digestion, one mL phenol was added to the tube, vortexed for 30 seconds, and centrifuged for 5 minutes. The top layer was transferred to a clean 2 mL tube. 500 uL phenol and 500 uL chloroform were added to the tube, vortexed 30 seconds, and centrifuged for 5 minutes. The top layer of liquid was transferred to a clean 2.0 mL tube. One mL of chloroform was added to the tube, vortexed for 30 seconds, and centrifuged for 5 minutes. The top layer of liquid was transferred to a clean 1.5 mL tube.

A 5% volume of 25% Chelex (Bio-Rad Laboratories, Hercules, CA) in water was added, vortexed for 60 seconds, and allowed to sit at room temperature for one hour to remove remaining heavy metals. After centrifugation for 5 minutes, the supernatant liquid was transferred to a clean 2 mL tube. To 400 uL of the liquid extract 1 mL of ethanol and 133 uL of 10M ammonium acetate were added. The

mixture was vortexed for 20 seconds, and centrifuged 10 minutes to precipitate the DNA. The resulting pellet was washed in 70% ethanol twice and again centrifuged to a pellet. The ethanol was decanted and the pellet dried completely in a heating block at 45° C. The dried pellet was resuspended in 20 uL of TE-4 buffer (0.1mM EDTA, 10 mM Tris-Cl) and used in PCR studies.

PCR Procedure

The PCR technique was modified from the procedure of Frickhofen and coworkers (Frickhofen and Young, 1991). DNA extracted from fixed tissues or 100 ng genomic DNA extracted from fresh marrow was amplified by two-stage PCR.

PCR reactions were performed on each sample. Initially, two separate tubes were set up on each patient; one to determine the presence of genomic DNA sequences (to prove successful extraction), and one for the detection of specific PV B19 DNA sequences in the specimen. The PCR procedures were refined to make it possible to detect the presence of both types of DNA in one tube, thus conserving reagents and decreasing opportunities for carry over contamination.

Nested primers were used to increase the sensitivity of the assay. Nested primers for the major breakpoint cluster (bcr) on chromosome 22 were used for the internal genomic DNA control. For PCR amplification of PV B19, four 20mer oligonucleotide primers were synthesized to flank the non-structural protein gene segment genome (Shade et al, 1986). Primer sequences are shown in Table 2.

For the first PCR reaction, 1 uL of extracted DNA was added to 99 uL reaction mixture containing 1X PCR Taq buffer (Perkin-Elmer Cetus), 0.01% gelatin, 3.5 mM $MgCl_2$ (Perkin-Elmer Cetus), 0.2 uM of each dNTP (Perkin-Elmer Cetus), 2.5 mM units of Taq polymerase (Perkin-Elmer Cetus), and 0.5 uM each of primers PV-1 and PV-2 and 0.1 uM each of primers 22-A and 22-D (Gibco-Life Technologies, Inc., Bethesda, MD). The PCR mixture was overlaid with 45 uL mineral oil. PCR amplification was carried out by using the following parameters: 94° C for 2 minutes (1 cycle); 94° C for 1 minute, 55° C for 1 minute, 72° C for 1.5 minute plus 2 seconds per cycle, (35 cycles); followed by 72° C for 10 minutes (1 cycle), and 15° C soak.

For the second PCR reaction, 1 uL of product from the first PCR reaction was mixed in the same reaction mixture indicated above with different primers, PV-3, PV-4, 22-B, and 22-C. The amplification steps were repeated as described above.

Amplified DNA products were electrophoresed through a 5.0% nondenaturing polyacrylamide gel (Sambrook et al, 1989) in 1X TBE buffer at 160 volts. Gels were stained in 1X TBE buffer containing 0.5 mg/mL ethidium bromide for 5 minutes and examined on a UV light box for the presence of specific bands. A band at the 466 bp position, compared to the standard base pair measuring ladder electrophoresed on each gel , indicated the presence of PV B19 DNA sequence. A band at the 600 bp position indicated the presence of genomic DNA in the specimen.

Table 1. *Primers*

PV B19 Primers

PV-1 5'- TCC TGA ACT GGT CCC GGG GAT GG - 3'
PV-2 5'- AAT GAA AAC TTT CCA TTT AAT GA - 3'
PV-3 5'- GCT GCT TTC ACT GAG TTC TTC AG - 3'
PV-4 5'- AAG CTT GGT GGT CTG GGA TGA AG - 3'

Chromosome 22 Primers

22-A 5'- GTT TCA GAA GCT TCT CCC TG - 3'
22-B 5'- CGC TGA CCA TCA ATA AGG AA - 3'
22-C 5'- AGA AAC CCA TAG AGC CCC GG - 3'
22-D 5'- ACT CTG CTT AAA TCC AGT GG - 3'

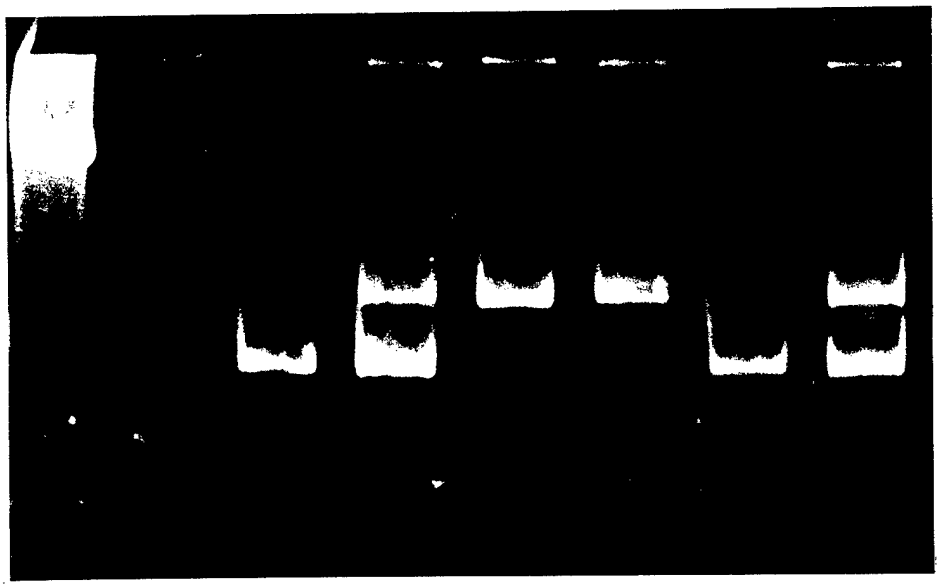

Fig 7. PAGE gel.

PCR Controls

Serial dilutions containing a known concentration of PV B19 DNA PCR amplimers originally derived from a viral capsid template were tested to establish the sensitivity of the PCR procedure. The procedure described was shown to be capable of detecting between 10 and 100 copies of DNA in a specimen.

Each unknown specimen was tested along with the following controls: a negative control to prove reagents were not contaminated, a sensitivity control consisting of a standard solution containing 100 molecules PV B19 DNA, a known positive patient control, and an internal genomic DNA control to demonstrate that DNA template was present in the original samples.

Unknown specimens considered positive showed a 600 bp band (genomic DNA internal control) and a PV B19-specific 466 bp band. To be considered a valid test with a negative result for PV B19, the unknown negative specimens were those showing the 600 bp band of the genomic DNA internal control, and absence of the PV B19-specific 466 bp band.

After initial sensitivity tests were performed, samples of the amplified DNA product were sent for commercial sequencing and were confirmed to be identical to the target area of the PV B19 genome.

Immunohistochemistry

Immunolabeling using a mouse monoclonal antibody to the PV B19 capsid proteins is another method of virus detection in clinical samples (Novocastra

Laboratories, Ltd.,UK). Immunolabeling can be used to demonstrate the cytological distribution of the virus, and whether the virus is located in the nucleus or the cytoplasm of the infected cells.

Two PV B19 PCR positive, formalin-fixed, decalcified, paraffin-embedded specimens from patients with clinically established PV B19 infection were immunolabeled (data not shown) using the alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique with antibody R92F6. Due to the low number of positive cells seen per slide it was felt to be an unacceptably insensitive method of testing.

Southern Blot

Southern blot analyses were performed on 4 of the specimens found infected with PV B19 to confirm the presence of PV B19 by a second method and to determine the approximate number of viral copies present in each specimen.

Introduction:

PV B19 replication is initiated from the short double-stranded regions in the self-annealing, terminal hairpin structures of the virus. Synthesis proceeds from the long palindromes to produce high molecular weight intermediates through a rolling hairpin model. These replicative intermediates correspond to duplexes equivalent to two- or four-fold the original single-stranded template, and can be distinguished from *in vitro* annealed duplexes because the covalent link between their strands allow 'snapback' rather than simple separation of the double-stranded DNA structure after

heating and quench cooling. (Ozawa et al, 1986). The replicative intermediates can be detected directly by Southern blot analysis of DNA extracted under low salt conditions (to prevent annealing) or after restriction enzyme digestion of DNA extracted with normal salt concentration. Asymmetric fragments are obtained after BamHI digestion, resulting in characteristic doublets on electrophoresis and hybridization.

Southern Blot Analysis

Four of the positive PCR tests for PV B19 were confirmed by Southern blot. Briefly, 10 ug of genomic DNA isolated from approximately 10 x 10^6 PCR positive fresh bone marrow cells was digested with 100 units of the restriction endonuclease EcoRI for five hours according to the manufacturer's recommendations (Gibco Life Technology, Inc.). The resulting digests were then electrophoresed on an 0.7% agarose gel and the size separated restriction fragments were transferred to nylon membranes using manufacturer's recommended methods (GeneScreenPlus, Dupont, Inc.). A ^{32}P labeled DNA probe to the non-structural protein gene of PV B19 was hybridized overnight to the resulting nylon filters. This probe was the 466 bp PCR product described above. The filters were then washed under stringent conditions with a final wash of 0.5X SSC/0.1% SDS at 65°C. X-ray film was then exposed to the filters for 2 weeks and the resulting autoradiograms studied densitometrically. Size standards and PV B19 controls were placed on all blots.

Autoradiograms showing hybridization signals at 10.6 kb and/or 5.3 kb and/or 2.7 kb were interpreted as evidence of PV B19 infection. These restriction fragments

correspond to the intact viral dimer and monomer replicative forms and the single stranded non-replicative form, respectively (Shimomura et al, 1992). The PV B19 controls consisted of lanes with varying dilutions of the PCR derived probe. From the signal intensity of these dilutions on the autoradiograms it was possible to derive signal/viral copy standard plots. From these curves data regarding the viral copy/marrow cell were obtainable (See Appendix).

The medical records and laboratory data on the eight patients found to be infected with PV B19 at the time of bone marrow biopsy were reviewed. Data from peripheral blood counts and smears (Table 4) and from bone marrow biopsy examination (Table 5) were compiled in table form to allow for comparison of morphological findings. The results of serological studies for evidence of PV B19 antibodies were available on three patients. The clinical outcome of the infection of ach patient was also indicated in Table 6.

RESULTS

Eight (10.3%) of the 78 bone marrow specimens from patients with unexplained cytopenias were positive for PV B19 DNA by nested PCR (Table 3). Peripheral blood parameters, at or near the time of the PCR-positive bone marrow biopsy, are shown for each subject in Table 4. All but one patient were anemic (<12.0 g/dL) with hemoglobins ranging from 6.0 - 11.5 g/dL. Four patients had macrocytic anemia (>100 fL) with MCV values of 101 - 110 fL (this finding must be tempered by the fact that two of these patients were being treated with AZT, which is known to cause a macrocytic anemia). Four patients had low leukocyte counts ($<4.0 \times 10^9$/L). The platelet counts were normal in four individuals, low in three, and not available in one. Absolute reticulocyte counts were elevated in two patients, low in one, normal in one and not tested in four. Serology for antibodies to PV B19 was performed on four patients; two patients had evidence of IgM antibodies; IgG antibodies were absent from all four.

The bone marrow results for each subject are shown in Table 5. Studies of the bone marrow morphology showed that giant normoblasts were found in four (50%) of the PCR-positive specimens. Red cell aplasia (<5% erythroid precursors in the marrow differential) was seen in four patients. Mild or no erythroid hypoplasia (<15.5% erythroid precursors) was noted in two patients; erythroid hyperplasia (>25% erythroid precursors) was present in two patients. Mild dysplasia of the erythroid precursors was seen in three patients consisting of mild nuclear to cytoplasmic maturational

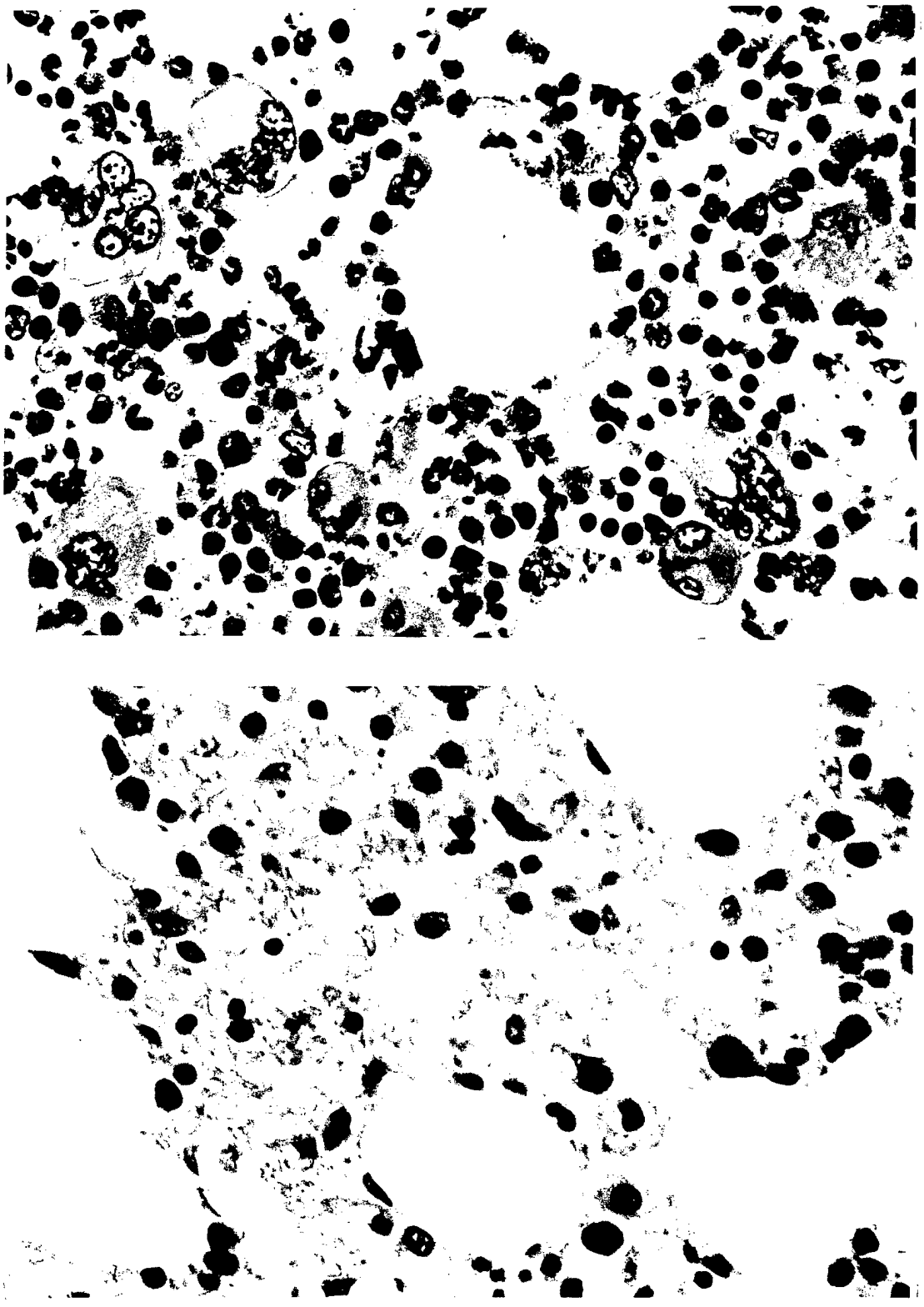

Fig. 8. Bone marrow samples showing normal cellularity (top) and interstitial depletion (bottom). Photos courtesy of Craig E. Litz, M.D.

dysynchrony. These patients had MCV's > 100 fL. When corrected for age, the marrow was hypocellular in six patients, hypercellular in one, and normocellular in one. Interstitial depletion of the marrow architecture was noted in six (75%) of the eight patients (Fig. 8). The megakaryocytes of five patients appeared to be smaller than normal, and, were either normal or slightly increased in number. Granulocyte and lymphocyte percentages varied in proportion to the erythroid aplasia. Mild dysgranulopoiesis consisted primarily of rare bilobated neutrophils and was noted in two patients.

Table 3. *Results of PV B19 PCR studies in patients with unexplained cytopenias*

	Number of patients	PV B19 PCR +
MDS-NOS/RA*	26	3
Erythroid aplasia	23	3
Aplastic/hypoplastic bone marrow	10	1
Normal bone marrow	5	0
Suspected PV B19 infection	2	0
Aplastic anemia	4	0
Macrocytic anemia	2	1
Thrombocytopenia	2	0
Increased hematogones	2	0
Hypereosinophilia	1	0
Erythroid hyperplasia	1	0
Total	78	8

*MDS-NOS/RA = Myelodysplastic syndrome, not otherwise specified/refractory anemia

Table 4. *Peripheral blood findings at time of PV B19 PCR + marrow*

Patient	Hgb	MCV	WBC	Platelets	Reticulocytes
1.	11.5	108	3.9	191	63
2.	10.2	91	9.5	408	185
3.	10.4	105	5.3	196	148
4.	10.1	101	3.5	134	<10
5.	14.0	91	5.8	306	NA*
6.	9.7	110	1.2	128	NA
7.	6.0	NA	1.9	128	NA
8.	6.5	75	5.9	NA	NA
	(12.0 - 16.0 g/dL female) (14.0 - 18.0 g/dL male)	(80 - 99 fL)	(4.0 - 11.0 x 10^9/L)	(150-400 x 10^9/L)	(25-100 x 10^9/L)

NA* = not available

Table 5. *Morphological findings in PV B19 PCR + bone marrow specimens*

Patient	Cellularity	Interstitial Depletion	Erythroid Precursors	Erythroid Dysplasia	Giant Normoblasts
1.	40%-50%	+	30.0%	+	+
2.	40%-50%	+	15.5%	-	-
3.	50%	+	35.2%	-	-
4.	15%-20%	-	1.5%	+	+
5.	20%	+	13.4%	-	-
6.	30%	+	4.0%	+	+
7.	50%-60%	+	2.5%	-	+
8.	75%-80%	-	2.4%	-	-
Normal ranges: (age dependent)			(18%-24%)		

Patient	Granulocyte Precursors	Granulocyte Dysplasia	Lymphocytes	Megakaryocytes: Number	Small Forms
1.	44.7%	-	19.8%	normal	+
2.	68.3%	-	9.5%	increased	-
3.	48.6%	-	11.0%	normal	+
4.	80.7%	-	5.8%	normal	+
5.	56.8%	+	16.4%	normal	+
6.	64.0%	-	11.0%	increased	+
7.	36.3%	+	53.9%	increased	+
8.	42.4%	-	50.4%	increased	-
Normal ranges:	(58%-65%)		(8%-12%)		

Clinical Features and Course of Illness in patients with PV B19 PCR positive bone marrow:

The mean age of the eight individuals found positive by nested PCR for PV B19 infection is 28 (range 1 - 71 years). Three patients were female, five were male. The clinical diagnsosis and outcome of infection in these patients is detailed below.

Patient 1. S.H. was a 21 year old female, 34 weeks pregnant, with a progressive anemia over the previous year before being seen at the University of Minnesota. A bone marrow biopsy was collected December 13, 1989. This specimen tested positive for PV B19 by nested PCR. Peripheral blood parameters post-transfusion are noted in Table 4. Bone marrow parameters are noted in Table 5. The marrow was moderately hypocellular for her age (40%-50%) with a mild erythroid hyperplasia manifested by 30% erythroid precursors. A slight megaloblastic maturation of the red blood cells was noted. Giant pronormoblasts were seen, but did not have the classic appearance described by Owren (Owren 1948). Megakaryocytes were present in normal proportion to the overall cellularity, but appeared small. Moderate interstitial depletion was noted. This patient continues to have a persistent anemia with no evidence of malignancy. As far as can be determined, no studies were done for PV B19 antibodies, nor was the patient treated with immunoglobulin.

Patient 2. G.N. was a 71 year old male presenting with fever of unknown origin. He had a history of peptic ulcer with three major bleeding episodes. He had steroid-dependent rheumatoid arthritis.

Peripheral blood parameters from the time of the PV B19 PCR positive bone marrow biopsy performed March 4, 1991 are noted in Table 4. The bone marrow biopsy (Table 5) revealed a hypercellular marrow for his age (40%-50% cellularity), a mild erythroid hypoplasia with 15.5% erythroblasts, and interstitial depletion. No giant normoblasts were seen. There was mild a dyserythropoiesis with lobated nuclei in the erythroblasts. Megakaryocytes were slightly increased in number. There was a neutrophilic hyperplasia with mild premature segmentation of the nuclei.

It is not known whether this patient has been tested for serological evidence of PV B19 infection. A persistent anemia continues with no evidence of malignancy.

Patient 3. A.M. was a 10 year old female with bilateral polycystic disease who received a kidney transplant January 26, 1994. She was on prednisone, imuran and FK506. Her brother had had a PV B19 infection in December 1994. She tested positive for the presence of IgM antibodies to PV B19, negative for IgG. She was given immunoglobulin March 13, 1995 and again April 3, 1995. The treatment was given in small doses due to allergic responses to the usual therapeutic dosages.

Her bone marrow biopsy, collected April 24, 1995, tested positive for PV B19 by nested PCR. Peripheral blood parameters are noted in Table 4. Parameters for the bone marrow biopsy are noted in Table 5. The marrow was hypocellular for her age (50%) with an erythroid hyperplasia (35.2% erythroblasts). There were areas of mild interstitial depletion. Megakaryocytes were normal in number, but many were small

with unilobate forms. Cytogenetic studies were normal and there was no evidence of malignancy. A PCR test performed by a commercial laboratory (Viromed) was positive for PV B19. There was partial resolution of anemia with immunoglobulin treatment.

Patient 4. B.W. was a 17 year old male diagnosed with medulloblastoma in 1990. A bone marrow biopsy was collected October 8, 1991. Peripheral blood parameters are noted in Table 4. The bone marrow aspirate (Table 5) showed a marked decrease in erythroid precursors (1.5%). The sections showed a hypocellular marrow (15%-20%). Giant normoblasts were identified.

He was admitted to the hospital December 2, 1991 for a bone marrow transplant and died December 11, 1991 of complications related to the transplant conditioning regimen.

Patient 5. A.S. was a 40 year old female presenting with fever of unknown origin, who had been leukopenic since October 1995. Her peripheral blood parameters at that time were: hemoglobin 13.4 g/dL, MCV 89 fL, WBC 3.6 x 10^9/L, platelets 187 x 10^9/L. Serology for PV B19 IgM and IgG antibodies were negative. Clinical symptoms included weakness, fatigue, and a pruritic rash on the extensor surface. A bone marrow biopsy collected January 3, 1996 tested positive for PV B19 by nested PCR. Peripheral blood and bone marrow parameters are shown in Tables 4 and 5. The marrow was hypocellular (20% overall) with a mild interstitial depletion and a slight

erythroid hypoplasia. The megakaryocytes were normal in number, but were slightly small. Occasional neutrophils showed bilobated nuclei. Cytogenetics were normal. A.S. was not treated with immunoglobulin. Her WBC slowly resolved after a period of several months.

Patient 6. J.B. was a 34 year old HIV positive male. He was diagnosed with AIDS in June 1995 and had a fever and shortness of breath. He began receiving AZT at that time. Peripheral blood parameters June 28, 1995 were; hemoglobin 10.1 g/dL, MCV 91 fL, WBC 5.4 x 10^9/L, platelets 285 x 10^9/L, and reticulocytes 0.6%. In August 1995 his hemoglobin dropped to 8.5 g/dL and WBC to 2.1 x 10^9/L; his AZT was decreased, then stopped altogether. He was started on prednisone in September 1995 due to lack of improvement. In October and November his hemoglobin improved to 12.6 g/dL; AZT was restarted December 1. By December 6, 1995 the hemoglobin again dropped to 11.0 g/dL; the AZT was withheld. Peripheral blood parameters from January 3, 1996 are noted in Table 4; a bone marrow biopsy performed January 5, 1996 (Table 5) tested positive for PV B19 by nested PCR.

Immunoglobulin was begun January 12, 1996. By February 7, 1996 his hemoglobin had risen to 11.7 g/dL and his reticulocyte count was 3.1%. Peripheral blood parameters on February 26, 1996 were: hemoglobin 12..8 g/dL, MCV 110 fL, WBC 2.0 x 10^9/L, and platelets 148 x 10^9/L. His anemia resolved after immunoglobulin treatment.

Patient 7. J.A. was an HIV positive male in his thirties. A bone marrow biopsy collected December 13, 1995 was referred to the University of Minnesota hematopathology department for evaluation of anemia and leukopenia (Table 5). This specimen tested positive for PV B19 by nested PCR. Peripheral blood parameters at the time of the bone marrow biopsy are noted in Table 4. The biopsy showed a normocellular marrow with a virtual red cell aplasia. Giant normoblasts were seen in the marrow smear. He was treated with immunoglobulin and responded well. By January 3, 1996 his hemoglobin had risen to 10.5 g/dL, WBC were 3.1 x 10^9/L, and platelets were 189 x 10^9/L.

Patient 8. S.V. was a 1 year old male being evaluated to rule out leukemia. He had a history of diarrhea for 2 months, lethargy, fever, nausea, vomiting, a rash on his right forearm, and a decrease in hemoglobin. Peripheral blood and bone marrow parameters from December 27, 1994 are noted in Tables 4 and 5. Serology for IgM antibodies to PV B19 was equivocal, IgG negative. The marrow smear showed giant normoblasts. Diagnosed with TAC, his aplasia resolved spontaneously.

Table 6. *Clinical diagnosis and outcome*

Patient	Clinical condition	Outcome	Serology
1.	Progressive anemia	Persistent anemia, NEM*	NA
2.	Peptic ulcers	Persistent anemia, NEM	NA
3.	Kidney transplant	Partial resolution with IgG, NEM	IgM+
4.	Medulloblastoma	Died following chemotherapy for BMT**	Negative
5.	Leukopenia, fever	Resolved slowly without therapy	Negative
6.	HIV+	Resolved with IgG	NA
7.	HIV+	Resolved with IgG	NA
8.	TAC***	Resolved rapidly without therapy	IgM+

* No evidence of malignancy
** Bone marrow transplant
***Transient Aplastic Crisis

Southern Blot Analyses

Sufficient genomic DNA from four of the eight patients with unexplained cytopenias and positive nested PCR for PV B19 was available for Southern Blot studies. In all four cases, bands at 10.6 kb and 5.3 kb were present on the 2-week exposure films. Densitometric analysis comparing diluted control DNA signal on the autoradiograms at 2-week exposure times showed that these were between 17-27 x 10^3 copies of PV B19 genomic equivalents per sample. This represents approximately 1.7 - 2.7 copies of PV B19 per 1,000 marrow cells.

Sufficient genomic DNA from 14 patients with hematologic malignancies who were PCR positive for PV B19 DNA was also available for Southern blot studies. All cases except one showed recognizable bands at 5.3 kb and/or 10.6 kb on the 2-week exposure autoradiogram. The intensity of signal for each of these cases was

comparable to that of the group of patients with unexplained cytopenias indicating a similar degree of infection (1-3 viral copies/1,000 marrow cells). There were no extra bands in completely digested DNA, indicating that DNA integration in the tumor cells was unlikely.

PV B19 in Malignancies

An intriguing group of 144 patients, randomly selected and studied retrospectively, had previously been diagnosed with a variety of malignancies (Table 7). This group consisted mainly of patients diagnosed with acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and myelodysplastic syndrome/refractory anemia with excess blasts. Other diagnoses are listed as well. PV B19 DNA was detected in the bone marrow specimens of 12 of these patients (8.3%).

Table 7. *Diagnoses of patients with malignancies*

Diagnosis	Number of patients	PV B19 PCR +
AML M0	4	1
AML M1	4	0
AML M2	16	3
AML M3	10	1
AML M4	13	2
AML M5a	7	0
AML M6	5	2
AML M7	2	0
AML NOS	9	0
CML	21	0
CLL	2	0
CLL-CMML	2	0
MDS-RAEB	18	1
MDS-CMML	3	1
MDS-Myelofibrosis	3	0
Multiple myeloma	6	0
Thrombocythemia	3	1
MPD-AMM	2	0
MPD-NOS	2	0
ALL L1	1	0
ALL L2	1	0
ALL-NOS	3	0
APL	1	0
Lymphoproliferative	1	0
Lymphoma	2	0
Fanconi's anemia	1	0
Blackfan Diamond anemia	1	0
Mastocytosis	1	0
Total	144	12

AML = acute myeloid leukemia
CML = chronic myeloid leukemia
CLL = chronic lymphocytic leukemia
MDS = myelodysplastic syndrome
RAEB = refractory anemia with excess blasts
CMML = chronic myelomonocytic leukemia
MPD = myeloproliferative disease
ALL = acute lymphoblastic leukemia
APL = acute prolymphocytic leukemia
NOS = not otherwise specified

DISCUSSION

The findings of this study indicate that PV B19 infection is more ubiquitous than generally expected and that the peripheral blood and bone marrow findings during this infection are not as homogeneous as originally thought.

A list of morphological manifestations found in bone marrow samples from patients with PV B19 infection would be useful for the diagnosis, treatment, and prognosis of individuals displaying persistent, unexplained cytopenias. To this end we devised a sensitive and efficient PCR method for direct detection of PV B19 DNA and applied it to study a group of bone marrow specimens.

A group of 78 carefully selected patients with cytopenias without a morphological explanation were studied. They all had normal blast percentages, and those in whom cytogenetics were studied had normal karyotypes, indicating that no malignancies were present. 50% of the patients who tested positive for B19 DNA showed giant normoblasts in the marrow. Four were tested for serological evidence of infection, only two of which had evidence of IgM antibodies. Interstitial depletion of the bone marrow, a historically accepted indicator of bone marrow damage or viral infection, was seen in six patients (75%). The overall cellularity of the marrow ranged from 15% to 80%; however, when corrected for age, six patients were hypocellular, one was slightly hypocellular, and one was slightly hypercellular. Four patients showed erythroid aplasia, two showed erythroid hyperplasia. Dysplasia of the

erythroid cells was seen in three patients (37.5%). Mild granulocytic dysplasia was noted in two patients (25%).

We also studied 144 marrow samples from patients with known hematologic malignancies. Interestingly, 8.3% of these samples tested positive for PV B19 by nested PCR. Of note, Southern Blot detected only a low level of PV B19 DNA in the samples and there was no evidence of viral integration in the infected cells. These findings do not indicate any specific relationship of the malignancies to persistent infection with PV B19, and it is more likely the infection was a secondary complication of a suppressed immune status. The bone marrow morphology of this group was not examined, but further studies to determine the effects of PV B19 infection on patient outcome may be revealing. Latent, chronic PV B19 infection appears to be able to cause treatable complications not typical of any of these malignancies (Pont et al, 1991). Identification of these patients may aid in the overall treatment of their conditions.

It is difficult to compare these findings to controlled studies of volunteer subjects infected with PV B19 (Potter et al, 1986). One reason for the discordance of morphologic findings in the current study, compared to previous studies, is that the exact date of exposure to the virus was not known in any of the patients currently studied, and only one patient indicated a recent exposure. The patients in this study were examined at random stages in the course of their illness. Although all were experiencing persistent, unexplained cytopenias, the exact stage of infection at which the bone marrow biopsies were collected is unknown. In addition, the patient

population in this study is biased when compared to normal volunteers. Immunocompetent individuals who respond normally to PV B19 infection do not suffer a prolonged course of disease requiring treatment; only the small percentage who develop persistent cytopenias are extensively evaluated.

In Potter's study of three deliberately infected healthy subjects, the exact time of exposure was controlled and bone marrow specimens were examined at specific stages in the normal infectious process. Details on cellularity and marrow architectural abnormalities were not reported because section material was not collected in their study. Another reason for morphological differences between the infected volunteers and the current group of patients is that studies of normal individuals likely reflect a very homogeneous immune response to infection. However, there have been studies that indicate that a defect in immune response to PV B19 may occur in immunocompromised individuals. One study postulated that immunocompromised patients either do not produce antibody or produce antibodies that fail to neutralize the virus (Kurtzman et al, 1989).

Perhaps the diversity in morphological findings in our study reflects an atypical immune response to this virus, leading to a prolonged disease course, much like that seen in certain individuals infected with hepatitis B. Supporting this hypothesis, five of the eight patients in the current study were in some way immunocompromised and only two of four who were tested showed evidence of an immune response to PV B19.

Available assays that assess PV B19 infection vary widely in their sensitivities. A previous study by Frickhofen et al (1994) showed that the classic finding of the giant

normoblast in the bone marrow biopsy is approximately 63% sensitive as an indicator of infection. The sensitivity of serology is about 50% and is highly dependent on the immune status of the patient. The same study demonstrated detection of 10^8 genome copies/mL of serum by dot blot analysis. Dot blot and Southern blot detection are less sensitive than PCR. One-round PCR amplification was positive in that study in serum containing 10^4-10^5 viral genome copies/mL. The current study demonstrates a similar sensitivity in a single-tube, 2-round, nested PCR; it detected 10^1-10^2 genome copies/uL (10^4-10^5/mL) of PV B19 DNA in a marrow-derived DNA specimen. PAGE gels examined after electrophoresis of the first amplification of the procedure demonstrated that some specimens, which were subsequently positive after 2 amplifications, appeared negative after 1 amplification. This suggested that two amplifications were better able to detect small numbers of genome copies than one amplification (data not shown). The PCR method used in this study was highly sensitive and specific for detection of small amounts of PV B19 DNA in bone marrow specimens.

Although PV B19 infection is normally a self-limited disease, immunocompromised patients suffer a more prolonged and profound anemia than immunocompetent individuals. It can be postulated that variable morphologic manifestations of infection by PV B19 in the bone marrow may depend entirely upon how the individual's immune systems respond to the virus. This also appears to be the case in patients with congenital or acquired immunodeficiencies since they are less able to mount an adequate immune response against the virus. Thus, recognition of the

variable manifestations of PV B19 infection in the bone marrow would be important for appropriate clinical management.

The findings of this study suggest that individuals with long-term cytopenias of unknown etiology should be tested for the presence of PV B19 DNA in their bone marrow. Positive patients may be candidates for immunoglobulin therapy. In addition, the recognition that PV B19 may infect patients with known hematologic malignancies may help to identify individuals in whom immunoglobulin therapy could be indicated to obtain symptomatic improvement. Patients with unexplained cytopenias without morphological explanation should all be considered candidates for persistent PV B19 infection. It must be recognized, however, that it is not possible to determine whether the virus is actually the cause of these patients' cytopenia. A larger prospective epidemiological study, with and without therapy, will be necessary.

APPENDIX

Southern Densitometric Determination of Parvovirus DNA in Marrow Specimens

Control DNA = pyt104 plasmid (8400 base pair)
Control DNA used in lane = 3.75×10^{10}gm
Copies of Control DNA = 3.75×10^{10}gm/8400 bp x 635 gm/mol bp
7.13×10^{17} mol/lane

Formula for densitometric conversion: counts on autoradiogram are proportional to exposure time and mols

or: K(exposure time x mols) = counts
or: K = counts/time x mols
or: Mols = counts/K x time
or: Molecules (copy number) = (counts/K x time) 6.02×10^{23} molecules/mol

Control on blot #280 - counts for 1 hour exposure = 2495
Control on blot #281- counts for 1 hour exposure = 2027
K280 = 2495/1hr x 7.13×10^{17}mol/lane = 3.5×10^{19} counts/mol hr
K281 = 2027/1hr x 7.13×10^{17} mol/lane = 2.8×10^{19} counts/mol hr

Patient A.S.

Counts on blot 280 = 426 for 336 hr exposure (2 weeks)
Mols = counts/K280 x time
X = (426/336 x 3.5×10^{19} counts/mol hr) x 6.02×10^{23} molecules/mol =
21.8×10^{3} /copies per lane/1 x 10^{6} cells/lane
21.8×10^{3} /copies per lane/10 x 10^{6} cells/lane
2 viral copies/1000 cells

Patient J.B.

Counts on blots 280 = 415 for 336 hr exposure (2 weeks)
Mols = counts/K280 x time
X = (415/336 x 3.5×10^{19} counts/mol hr) x 6.02×10^{23} molecules/mol =
21.3×10^{3}/copies per lane
21.3×10^{3}/copies per lane/10 x 10^{6} cells/lane
2 viral copies/1000 cells

Patient G.N.

Counts on blots 281 = 268 for 336 hr exposure (2 weeks)
Mols = counts/K281 x time
X = (268/336 x 2.8 x 10^{19} counts/mol hr) x 6.02 x 10^{23} molecules/mol =
17.1×10^3/copies per lane
17.1×10^3/copies per lane/10×10^6 cells/lane
1.7 viral copies/1000 cells

Patient S.H.

Counts on blots 281 = 268 for 336 hr exposure (2 weeks)
Mols = counts/K281 x time
X = (435/336 x 2.8 x 10^{19} counts/mol hr) x 6.02 x 10^{23} molecules/mol =
27.9×10^3/copies per lane
2.8×10^3/copies per lane/10×10^6 cells/lane
3 viral copies /1000 cells

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