Infectious Disease Unit
Department of Medicine, Center for Molecular Medicine
Karolinska Institutet, Solna, Sweden

CLINICAL AND LABORATORY FINDINGS IN PATIENTS WITH PERSISTENT PARVOVIRUS B19 INFECTION

Anders Lundqvist, MD

Stockholm 2006

PREFACE – PARVOVIRUS AND FOOTBALL

During the last few years, a lot of my time has been shared between football and parvovirus B19 (B19), and it has been evident that these interests have several similarities. First, the forms of these objects, icosahedral and global, respectively, are rather conserved over time and around the world. Secondly, events surrounding these objects show considerable diversity. If you are interested in football or coaching a football team, you may have great knowledge about individual players, such as body weight, shoe size, running speed and scoring record, but the most important question is: How does he or she play football? In that dimension, every match is a new, unpredictable game. What makes team member Zlatan score more beautifully than ever and why does he miss? These outcomes are matters of coincidence, situation, environment and previous experiences in combination with the same factors in 21 other players in the football field.

In a comparable way, the immune system plays football with microorganisms. The clinical picture of infectious diseases is a synthesis of features belonging to the microbe and to the immune system. The latter has basic characteristics in common among most humans, but the individual patterns of reaction against different enemies and in different situations vary. Consequently, even if a specific infection causes relatively predictable symptoms in most individuals, we have to expect outliers whose symptoms are difficult to recognize. For widespread infections like that from B19, which infects the majority of humans, the total number of individuals with a deviant course may be high, even if the percentage is low.

Our studies of B19 have focused on clinical and immunological aspects of persistent infection, with persistence *per se* of central interest. Hypothetically, an immune system that cannot eliminate an infecting virus is still annoyed by the infection and responds with reactions and symptoms that vary according to the host's individual character. The ball is on the field and one may guess what will happen but cannot know with certainty.

ABSTRACT

Parvovirus B19 (B19) is the etiological agent of the common childhood disease, erythema infectiosum, also named fifth disease or slapped cheek disease. About 50% of humans are infected during childhood, but the virus is also transmitted to adults and about 80 % of the elderly are seropositive. Apart from asymptomatic infection, which is common, the clinical presentations in erythema infectiosum are usually slight catarrhal symptoms and fever followed by rash, typically intense on cheeks and later distributed on the extensor parts of legs and arms. For infected adults, the rash is less common, whereas the frequency of arthropathy is about 50% and may last for months or years. The main replicative sites of the virus are erythropoetic cells, and anaemia is a well-known manifestation of the infection, especially in immunocompromised patients. Pregnant women, when infected, are at risk of spontaneous abortion, hydrops fetalis and, later in pregnancy, intrauterine fetal death. B19 has also been associated with rheumatologic diseases, hepatitis, myocarditis and neurological manifestations.

In 1993, we used PCR-technique and found, for the first time, B19 DNA in the bone marrow (BM) of a patient with chronic fatigue, anaemia and leukopenia. Since then, we have followed her and other patients with chronic symptoms of fatigue, arthralgia and fever and found that some of them had persistent B19 DNA in BM for years after primary infection. Lifelong latency, as a natural course of the disease could account for this persistence, but the prevalence of B19 DNA in BM in the general population was not previously known. To get a hint of the frequency, we tested the BM of 100 patients with haematological disorders from whom BM samples were available and found B19 DNA in four of them. Therefore, we concluded that B19 DNA in BM is not a general finding in seropositive individuals. Arthropathy is a common manifestation of B19 infection in adults and sometimes even meets the official criteria for rheumatoid arthritis. Consequently, the frequency of B19 DNA in BM of rheumatic patients is of interest, which motivated us to study a group of 50 patients with various rheumatological diseases. We found B19 DNA in 13 of 50 patients (26%), a significantly higher level than in our previous study on patients with haematological disorders.

As is well known, immunocompromised patients are susceptible to B19 infection, but our hypothesis is that individuals who are otherwise immunocompetent may have a selective immune deficiency with respect to B19 causing the virus to persist and evoke symptoms of chronic immunological stimulation. To confirm this hypothesis, we have studied patients with persistent B19 DNA in BM with regard to general immunological parameters such as HLA-type, levels of lymphocyte subpopulations and cytokine profiles. In some aspects we have compared with expected values in the general population, in others with reactions in acutely or remotely B19-infected individuals. We also investigated the specific CD8 T-cell response to B19 antigens with an enzyme-linked immunospot assay for detecting interferon gamma (IFNγ) production. Although the patients with persistent B19 DNA in BM had no general immunological aberrations, their B19 specific cellular immune responses diverged from those of controls supporting our hypothesis of a selective immune deficiency.

Key words: Human parvovirus B19, persistent infection, immune defence.

LIST OF PUBLICATIONS

I.   Lundqvist A, Tolfvenstam T, Bostic J, Söderlund M, Broliden K.
Clinical and laboratory findings in immunocompetent patients with persistent parvovirus B19 DNA in bone marrow.
Scand J Infect Dis, 1999. **31** (1): p. 11-6.

II.   Lundqvist A, Tolfvenstam T, Brytting M, Stolt CM, Hedman K, Broliden K.
Prevalence of parvovirus B19 DNA in bone marrow of patients with haematological disorders.
Scand J Infect Dis, 1999. **31** (2): p. 119-22.

III.   Lundqvist A, Isa A, Tolfvenstam T, Kvist G, Broliden K.
High frequency of parvovirus B19 DNA in bone marrow samples from rheumatic patients.
J Clin Virol, 2005. **33** (1): p. 71-4.

IV.   Isa A, Lundqvist A, Lindblom A Tolfvenstam T, Broliden K.
Cytokine responses in acute and persistent human Parvovirus B19 infection
Clin Exp Immunol, 2006. In press

V.   Isa A, Norbeck O, Hirbod T, Lundqvist A, Kasprowicz V, Bowness P, Klenerman P, Broliden K, Tolfvenstam T.
Aberrant cellular immune responses in persistently human parvovirus B19 infected individuals.
J Med Virol. 2006 Jan; **78** (1): p.129-33.

CONTENTS

LIST OF ABBREVIATIONS

Aa	Amino acid
B19	Parvovirus B19
BM	Bone marrow
CD	Cluster of differentiation
CFS	Chronic fatigue syndrome
Con A	Concanavalin A
DNA	Deoxyribonucleic acid
EIA	Enzyme immuno assay
ELISpot	Enzyme linked immunospot assay
ETS	Epitope type specificity
FACS	Fluorescence activated cell sorter
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFN	Interferon
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IUFD	Intrauterine fetal death
IVIG	Intravenous immunoglobulin
kDa	Kilo daltons
mAb	Monoclonal antibodies
MHC	Major histocompatibility complex
ml	Milliliter
ns	Not significant
NS	Non-structural protein
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SFC	Spot forming cells
VP	Virus capsid protein
TAC	Transient aplastic crisis
Th	T helper cell
Tc	T cytotoxic cell

1 BACKGROUND

1.1 BACKGROUND AND TAXONOMY

Parvum is Latin for small, and viruses in the family of Parvoviridae are small non-enveloped particles with a diameter of 18 to 26 nm and single stranded DNA. These pathogens are very common in insects and animals. The virion is stable and resistent to heat, detergents and radiation. The Parvoviridae family is divided into Densovirinae, which infect insects, and Parvovirinae, which infect vertebrates (Heegaard et al. 2002). Three genera are defined in the Parvovirinae group:

A) Dependovirus
   Infects humans and other vertebrates and are dependent on other viruses, e.g., adenovirus or herpesvirus to infect and replicate. In humans they are not associated with any known disease.

B) Parvovirus
   Is a common pathogen among animals and, e.g., canine parvovirus causes gastrointestinal infection in dogs, a disease associated with high mortality. Humans are not susceptible to these viruses.

C) Erythrovirus
   Characteristically they have a tropism for and replicate in erythropoetic cells, thereby causing anaemia, which is a well-known clinical feature of the infection. At least three different strains infect humans (Servant et al. 2002).

   1. Erythrovirus Genotype 1 Parvovirus B19
   2. Erythrovirus Genotype 2 Parvovirus A6/K71
   3. Erythrovirus Genotype 3 Parvovirus V9

   Among these, B19 is the only one known to cause disease. Although B19 was identified 1974, it was not associated with disease until 1981 when the relationship to aplastic crisis in patients with sickle cell anaemia was discovered (Pattison et al. 1981). Genotypes 2 and 3 were subsequently identified (Nguyen et al. 1999; Hokynar et al. 2000; Nguyen et al. 2002) and are proposed as the cause of human disease in case reports, but their importance has yet not been clarified.

1.2   THE VIRUS

1.2.1 History

Parvovirus B19 was first discovered in 1974 in London, when Yvonne Cossart and colleagues were testing assays for hepatitis B in samples of blood donors (Cossart et al. 1975). Specimen 19 in panel B revealed an unexpected antigen in a counter-immunoelectrophoresis, and analysis with electron microscopy as well as further molecular biology studies classified the virus in the Parvoviridae family.

1.2.2 Morphology

The B19 virion is composed of two distinctive proteins that build an icosahedral capsid containing 60 capsomeres and have a molecular weight of 5.6×10^6 and a diameter of about 23 nm (Corcoran et al. 2004).

1.2.3 Genetics

Parvovirus B19 DNA is single stranded and consists of about 5600 nucleotides among which 4830 encode proteins and the rest are palindromic terminal repeats at each end. The nucleotide sequence variability of B19 is known to be low, i.e., no higher than 1% when one compares different isolates.

1.2.4 Proteins

Two proteins provide building blocks in the B19 capsid. VP1 is the larger of these proteins (84 kDa) but constitutes only about 4% of the capsid structure. VP2 is, consequently, the major structural protein, forms the remaining ~96% of this structure and has a molecular weight of 58 kDa. VP1 and VP2 are partially identical except for a 227 amino acid sequence, named the unique region (VP1ur), which ends the N-terminal part of VP1 (Brown et al. 1994).

The major non-structural protein (NS1), which is hidden inside the capsid, is believed to function as a regulator of transcription and translation and also, probably, is important for apoptosis of infected cells (Moffatt et al. 1998). The molecular weight is 77 kDa, and the corresponding genome is well conserved (Erdman et al. 1996). Another two NS proteins whose molecular weights are 7.5 and 11 kDa have been described; although their functions are currently unproven, they may be involved in a signalling pathway (Shade et al. 1986; Deiss et al. 1990).

1.2.5 Culture and animal model

Parvovirus B19 is difficult to culture, and trials to propagate this virus in several erythropoetic cell lines have failed except for short periods of viability with limited expression of viral antigens. Since B19 does not infect species other than humans, the only similar animal model for experimentation is macaque monkeys infected with simian parvovirus, which resembles B19. Immunocompromised monkeys infected with the simian virus develop persistent anaemia, and fetal infection with this virus is associated with hydrops fetalis analogous to that caused by B19 infection in humans (Brown et al. 1997).

1.3 EPIDEMIOLOGY AND TRANSMISSION

During the early viremic phase of infection, B19 is found in respiratory secretions indicating a respiratory route of transmission, but there is no known replication in the mucosal cells. B19 infection is common in that 50% of the population is infected during childhood (Chorba et al. 1986), and more than 80% of elderly humans are seropositive (Heegaard et al. 2002). The extent of its contagiousness is rather high; that is, about 50% of non-immune contacts within a household have been shown to seroconvert (Chorba et al. 1986). Additionally, the attack rate among personnel working in child day care centres or schools is described as 20-30% during outbreaks (Gillespie et al. 1990). B19 infection is distributed worldwide and low seroprevalence has been found only in occasional isolated tribes in Africa and South America. However, viremia is rare considering that the frequency of B19 DNA in sera from blood donors varies from 1:167 to 1:35000 in different studies (Yoto et al. 1995; Tsujimura et al. 1995). When large pools of plasma are collected to produce immunoglobulin or factor VIII concentrates for haemophiliacs, the risk is greater, because B19 is so resistant to heat treatment and detergents that eliminating it from such products is difficult (Wu et al. 2005). Vertical transmission has been registered in about one-third of pregnant women with primary B19 infection, and these patients endure a potential risk of spontaneous abortion and intrauterine fetal death. The annual seroconversion rate among women of childbearing age is estimated to about 1.5% (Koch et al. 1989) but may be higher during an epidemic situation (Valeur-Jensen et al. 1999).

1.4 INFECTION OF HUMAN CELLS

Characteristic for B19 is the tropism for erythropoetic cells and the binding to P-antigen that serves as a receptor mediating B19's internalization into cells and makes replication possible. Individuals lacking P-antigen (1/200 000) are naturally resistant to B19 infection. P-antigen is present on the surfaces of haematopoetic precursor cells but also in several other cell lines, such as endothelial cells, fetal myocytes and placental trophoblasts (Brown et al. 1993). Yet no replication of the complete virus has been shown in these latter cell types. However, erythropoetic cells also express a co-receptor, identified as an alfa-5-betaintegrin, which is involved in viral replication (Weigel-Kelley et al. 2003). Another co-receptor, Ku80, was recently described and is expressed in erythropoetic cells and also in lymphocytes (Munakata et al. 2005).

1.5   VIRAL PERSISTENCE

Two well-known manifestations of B19 infection are its potentially chronic course in immunocompromised individuals and the persistent arthropathy it may cause in adults with primary infection. Many other diseases with a chronic course have been proposed as related to B19 based on findings of B19 DNA with PCR technique in various tissues. Further studies have shown that B19 DNA is often present in, e.g., the synovia and skin of healthy individuals. Consequently, studying the etiological relationship between B19 and disease is a difficult task, and the clinical relevance of finding B19 DNA must be established organ-by-organ. BM is of special interest, since it is the known site of viral replication, and microscopy typically shows erythropoesis with giant pronormoblasts, which may also indicate B19 infection (Brown et al. 1996; Heegaard et al. 2002). The frequency of B19 DNA in BM of healthy individuals or autopsied material has varied from 2.1% (Heegaard et al. 2002) to 17% (Eis-Hubinger et al. 2001).

1.6   IMMUNE RESPONSES

Viral infections, in general, induce activation of both humoral and cellular immune defence. The humoral response is initiated by production of IgM antibodies followed by IgG and IgA. As shown, antibodies *per se* may provide immunity against viral infections, e.g., prophylactic treatment with commercial immunoglobulin to individuals exposed to hepatitis A virus (Mosley et al. 1968). However, the neutralizing effect of antibodies is, in principle, extracellular, and viral infections are by nature intracellular and need the cellular production system for replication. On the other hand, infected cells have viral antigens on their surface and, in cooperation with the complement system, antibody binding to the cell may cause cytolysis and neutralization of viruses that are set free (Lachmann et al. 1997). The cellular immune response, mediated by cytotoxic T-cells (CD8), is important in the defence against intracellular pathogens such as virus and fungi by killing cells that present microbial antigens on their surface (Zinkernagel 1996). In recent years, many new techniques to study cellular immune responses have evolved, e.g., intracellular cytokine staining and phenotyping using new markers for maturation and proliferation detected by FACS. The ELISpot-technique has made it possible to elucidate aspects of the T-cell response in various infections and also the variability of reaction in distinct infections in different individuals (Larsson et al. 1999).

The multiple mechanisms of immune reactions are of special interest in our understanding of persistent infections. Patients who spontaneously recover from hepatitis B infection usually produce high levels of specific CD8 cells, yet others who develop chronic infection have a less intense reaction (Chisari et al. 1995). Individual immunological characteristics including HLA-type of the host determine which viral epitopes become exposed and, thereby, modulate the immune reaction (Penna et al. 1991). Theoretically, the chronic course of an infectious disease may be related to selectively impaired immune responses to specific pathogens rather than a generalized immune deficiency.

In B19 infection the humoral response typically is initiated by the appearance of specific IgM antibodies ten days after infection and their disappearance two to four months later, although the IgM-reaction is sometimes prolonged (Anderson et al. 1985; Musiani et al. 1995). Viremia peaks at about the same time as IgM-antibodies reach a detectable level but declines when IgG-antibodies become evident some days later. Knowledge of the epitope-specificity of the humoral response is important for the development of diagnostic assays. IgM and IgG antibodies are directed against both VP1 and VP2, but different types of antigens vary in certain properties. Reactivity to linear epitopes of VP1 is lost, whereas antibodies against conformational antigens persist (Kerr et al. 1999). NS1 is an important protein of the virus, and NS1-specific antibodies have been described in several but conflicting reports. In some studies, a relationship between NS1-antibodies and persistent infection has been proposed (Modrow et al. 2002; von Poblotzki et al. 1995; Kerr et al. 2000), a theory that has not been confirmed by others (Jones et al. 1999). Also IgA-antibodies are produced and may be important in the mucosal immune reaction (Erdman et al. 1991). IgE-antibodies have been demonstrated, but their clinical relevance is still under evaluation (Bluth et al. 2003).

The cellular immune defence against B19 has been difficult to investigate and is not as well characterized as the humoral response. The cellular immune response is probably important though, since infections with a chronic course have been noted in the presence of circulating and neutralizing antibodies (Cassinotti et al. 1997; Dobec et al. 2006). Tolfvenstam et al. used peptide pools representing the B19 NS1 protein to stimulate CD8 cells and were able to demonstrate epitopes associated with cellular responses (Tolfvenstam et al. 2001). A strong and long-lasting CD8 response against NS1 has also been demonstrated (Norbeck et al. 2005).

Some studies of cytokine responses to acute B19 infection have shown prolonged up-regulation of serum IFNγ and TNFα during symptomatic B19 virus infection (Kerr et al. 2001). Furthermore, patients with acute B19-associated arthritis have been demonstrated with lower levels of IL6, TNFα, and GM-CSF than patients without arthritis, and B19 related rash has been associated with decreased levels of TGFß1 (Kerr et al. 2004).

1.7 CLINICAL MANIFESTATIONS

1.7.1 Asymptomatic infection

Although most of us have antibodies against B19, proving prior exposure to this infection, rarely is its diagnosis recorded, which reflects the commonly mild clinical picture of the disease. In an epidemiological study done in England during 1985, of 54 adults who were serologically positive for recent B19 infection, 14 (25%) were asymptomatic (Woolf et al. 1989). In another study, 32% were without symptoms; however, fewer B19-infected blacks are diagnosed, possibly because the rash is difficult to see on dark skin (Chorba et al. 1986). The frequency may be even higher as reported in an outbreak, including medical students, in which 68% were asymtomatic (Noyola et al. 2004).

1.7.2 Erythema infectiosum

The clinical picture of erythema infectiosum was first described in 1799 by the famous dermatologist Robert Willan and later by Ager (Ager et al. 1966). The manifestations, however, were not attributed to B19 until 1983, when 31 individuals in a London outbreak were found to have B19 IgM antibodies (Anderson et al. 1984). This relationship was also proven experimentally by inducing the disease with inoculation of the virus intranasally in healthy individuals (Anderson et al. 1985). Later, B19 was established as the only cause of this disease, which is also called slapped cheek disease, Stickers disease or, more commonly, fifth disease. Fifth disease is a designation used after a classification of common childhood diseases introduced by Cheinisse in 1905. The typical course is initiated with non-specific symptoms about one week after infection, including fever, often mild, malaise, coryza and myalgia. These prodromal symptoms usually vanish after a few days but are replaced with a rash about 15 to 17 days after exposure (Figure 2). The facial erythema usually starts on cheeks and includes circumoral pallor, the slapped cheek appearance (Figure 1). Some days later, the rash appears on the trunk and typically the extensor sides of extremities, often in a lacy or reticular pattern that may recur for several weeks and vary in intensity depending on environmental factors such as heat or stress. The clinical appearance may vary greatly in different individuals, and the classical eruption is much more common among children than in adults (Woolf et al. 1989).

Figure 1.
My daughter Malin presenting a typical facial rash of fifth disease. She also had exanthema on arms and legs and later on her two sisters showed the same clinical picture while their mother during a period of two weeks suffered from arthralgia.
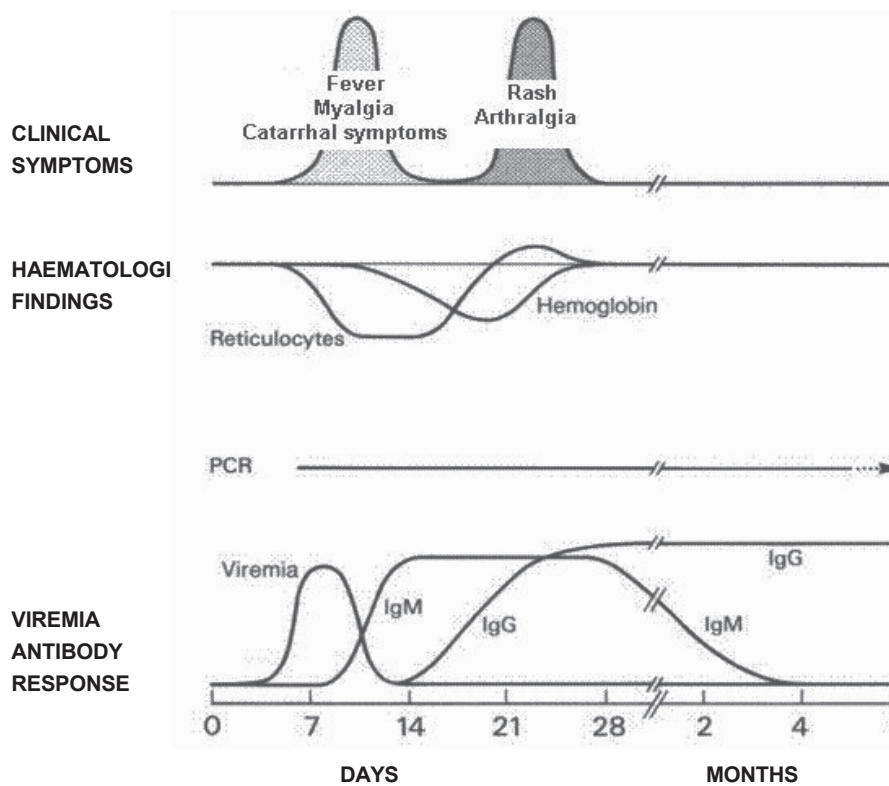
Figure 2.
Virologic, immunologic and clinical course following B19 infection (modified from
Heegaard et al. 2002).

1.7.3 Arthropathy

Of children with B19 infection, about 10% develop arthralgia, but the frequency is higher in adults, reported as 30% in men and 59 % in women (Torok 1992). Arthropathy is the most common manifestation of B19 infection in grown-ups. In one study, five women of 26 patients (six men, 20 women) with B19 arthropathy had persisting symptoms for two months or more (Woolf et al. 1989). Some patients with B19 arthropathy also meet criteria for rheumatoid arthritis (RA) according to the American College of Rheumatology and sometimes test positive for rheumatoid factor or other autoantibodies (Naides et al. 1988; Kerr et al. 1996). White et al. investigated 153 patients with early synovitis and found B19-specific IgM in 17 of them whereas other infections were diagnosed in five (White et al. 1985). Other investigators examined 90 patients with arthritis of unknown origin for the presence of B19 DNA in synovial fluid and synovial tissue (Cassinotti et al. 1998). The results yielded B19 DNA in one of 78 synovial fluid samples and in 15 of 90 synovial tissue samples. The 15 patients with B19-positive synovial tissue were further investigated, and nine (60%) of them had BM aspirates that turned out to be B19 DNA positive. Söderlund et al., on the other hand, reported B19 DNA in synovia from eight of 29 children with arthritis (28%) but also in 13 of 27 individuals (48%) in the control group including young healthy adults with joint trauma. Moreover, when results from these patient and control groups were summarized, 18 of 20 seropositive individuals also had B19 DNA positive synovia (Söderlund et al. 1997; Vuorinen et al. 2002).

The most frequently affected joints in patients with B19 arthropathy are metacarpophalangeal joints, knees, wrists and ankles (Woolf et al. 1989). Therefore, this virus has been postulated as a possible cause of RA (Takahashi et al. 1998), although other reports disagree (Kerr et al. 1995; Nikkari et al. 1995; Söderlund et al. 1997). B19 arthropathy is generally not known to include erosions or destruction of joints (Naides et al. 1990) even though such conditions have been described (Tyndall et al. 1994). Additionally, an increased seroprevalence for B19 in patients with RA has been reported (Cohen et al. 1986) but not confirmed (Nikkari et al. 1994). Therefore, the role of this virus in rheumatic diseases, if any, remains to be clarified. An interesting point, however, is that HLA DR4, which bears a relationship to RA, has also been associated with B19 arthropathy (Gendi et al. 1996).

1.7.4 Other autoimmune diseases

B19 has been suggestively implicated in several chronic autoimmune diseases such as systemic lupus erythematosus (SLE), Wegener`s granulomatosis, Kawasaki's syndrome, systemic sclerosis and others (Moore et al. 1999; Nikkari et al. 1994; Nigro et al. 1994; Pugliese et al. 2006; Ferri et al. 2005). Although the clinical symptoms of B19 in patients with these diseases may be similar, no statistical proof of any general etiologic connection has emerged.

1.7.5 Chronic fatigue syndrome and fibromyalgia

The diagnosis of chronic fatigue syndrome (CFS) is based on internationally accepted criteria, first defined in 1988 and revised in 1994 (Fukuda et al. 1994). That description depicts a disease with debilitating fatigue of more than six months duration associated with at least four of the following symptoms: impaired memory or concentration, tender lymph nodes, myalgia, arthralgia, headache, unrefreshing sleep and postexertional malaise. The etiology is unknown but among the microbial causes proposed are herpesviruses (Kawai et al. 1992) and enteroviruses (Chia 2005). Several case reports have associated CFS with parvovirus B19 (Jacobson et al. 1997; Kerr et al. 2002), and treatment with immunoglobulin has been successful in some cases (Kerr et al. 2003). On the other hand, a study of seven patients with haematological and rheumatological symptoms who also fulfilled the criteria for CFS could not demonstrate any sign of ongoing B19 infection (Ilaria et al. 1995). However, CFS is very complex and probably has a multifactorial etiology including endocrine, psychological, neurological and immunological aspects (Maquet et al. 2006). CFS is connected with various disturbances in the immune system according to analyses of cytokines and cell lines of various kinds. The IFNγ level may be elevated as well as the IL6 response to stimulation of PBMC. NK-cell activity may be decreased, and cytotoxic T-cells activated (Barker et al. 1994). B19-related CFS has been associated with elevated levels of IFNγ and TNFα (Kerr et al. 2003).

The diagnostic criteria of fibromyalgia are based on chronic, general diffuse pain and tenderness in defined points, but a subset of these patients also has symptoms mimicking CFS and sometimes resembling B19 infection (Wolfe et al. 1990). Case reports of B19 infection in patients developing fibromyalgia have been published (Leventhal et al. 1991), but a study of 15 patients with fibromyalgia showed no association with B19 infection (Berg et al. 1993).

1.7.6 Haematological manifestations

Historically, the first clinical manifestation of B19 observed was transient aplastic crisis (TAC) in children with haemolytic anaemia. Six hundred children admitted to a London hospital were examined for B19, and all six patients with laboratory signs of recent infection turned out to be Jamaican immigrants with sickle cell disease on presentation with aplastic crisis (Pattison et al. 1981). Later, B19 was identified as the major cause of aplastic crisis, which is a well-known complication of sickle cell anaemia, occurring just once during a patient's life (Serjeant et al. 2001). The pathogenic process stems from the tropism of B19 for erythropoetic cells, where the virus replicates and causes apoptosis (Hsu et al. 2004). Usually TAC is a self-limiting disease, but these patients may be severely ill with symptoms that include acute chest syndrome, splenic sequestration and BM necrosis, occasionally leading to death (Smith-Whitley et al. 2004; Lowenthal et al. 1996; Eichhorn et al. 1999). Blood transfusion is commonly needed, but these patients usually recover within ten days. B19 may also be the origin of aplastic crisis in other disorders and may be the initial presentation of a previously undiagnosed chronic haemolytic disease (Eriksson et al. 1988).

For patients whose disease involve a compromised immune defence, including HIV or disorders related to haematological malignancies or chemotherapy, B19 infection may be associated with chronic BM failure (Abkowitz et al. 1997; Broliden et al. 1998). The most common manifestation in these cases is pure red cell aplasia (PRCA). Tests of peripheral blood demonstrate anaemia and reticulocytopenia, and in the BM, reduced erythropoesis with giant pronormoblasts is typical (Kurtzman et al. 1988). The patient may recover spontaneously but the anaemia may persist, sometimes for years (Kurtzman et al. 1989). Treatment is possible with intravenous immunoglobulin, (IVIG), usually given in doses of 0,4 g /kg for five consecutive days (Mouthon et al. 2005). Immunocompromised patients infected with B19 may present with leukopenia or thrombocytopenia and occasionally pancytopenia (Smith et al. 1995). In the general population, B19 infection usually decreases hemoglobin level but not to the point of clinical importance and without overt anaemia. Occasionally, though, previously healthy individuals with no known immune deficiency may develop PRCA as well as agranulocytosis and thrombocytopenia in association with B19 infection.

Apart from bone marrow insufficiency, thrombocytopenia may have an immunological source, so-called idiopathic thrombocytopenic purpura (ITP). This disorder has been associated with B19 infection in children (Heegaard et al. 1999; Murray et al. 1994) but not in adults (van Elsacker-Niele et al. 1996).

1.7.7 Complications associated with pregnancy

About 30 to 40% of pregnant women are seronegative for B19 and susceptible to infection. The annual seroconversion rate is about 1.5% in women of childbearing age but may be higher in epidemic situations (Valeur-Jensen et al. 1999; Jensen et al. 2000). Infected pregnant women may have exanthema or arthralgia, but asymptomatic B19 infection is so common that the diagnosis is often overlooked. The risk of an adverse outcome of pregnancy after primary maternal infection with this virus has been estimated at 5 to 10% (Enders et al. 2004; Yaegashi et al. 1998). The fetus is most vulnerable in the second trimester, a period when the erythropoetic activity increases rapidly (Yaegashi et al. 1998); however, fetal loss correlated with B19 infection has occurred throughout pregnancy and been considered responsible for intrauterine fetal death in the third trimester (Tolfvenstam et al. 2001). The best-documented disorder attributed to B19 infection during pregnancy is non-immune hydrops fetalis, with a pathogenetic cause of anaemia in association with heart failure (Yaegashi 2000). Treatment is possible with intrauterine blood transfusion (Enders et al. 2004) or immunoglobulin (Selbing et al. 1995).

1.7.8 Other manifestations

B19 has, in case reports, been associated with a large number of diseases including myocarditis (Lamparter et al. 2003), neurological disorders (Aguilar-Bernier et al. 2006), hepatitis (Sokal et al. 1998) and glomerulonephritis (Mori et al. 2002). This association is sometimes probable when a typical clinical picture of B19 infection is followed by complications in specific organs, by the presence of B19-specific IgM antibodies and by B19-specific IgG seroconversion. However, validating the diagnosis by using PCR to locate B19 DNA in different tissues is more difficult, since B19 DNA can also be found in healthy individuals (Söderlund et al. 1997; Vuorinen 2002).

1.8 TREATMENT AND PROPHYLAXIS

1.8.1 Symptomatic treatment

Non-steroidal anti-inflammatory drugs and paracetamol may be of use in cases of arthralgia (Golstein et al. 1996).

1.8.2 Antiviral drugs

The non-structural protein, NS1, is involved in viral replication, but viral proliferation is more likely catalyzed by host-related DNA-polymerase (Astell et al. 1997). Unlike HIV treatment with nucleoside analogues, no such antiviral treatment is available for B19. However, HAART treatment for HIV infection may be enough to resolve co-existent chronic B19 infection in an indirect way, since HAART increases the number and functions of immune cells (Mylonakis et al. 1999).

1.8.3 Blood transfusion

Patients with sickle cell anaemia and (TAC) often need blood transfusions, which yield a favourable prognosis and commonly resolve the anaemia within ten days. When hydrops fetalis is diagnosed and associated with B19 infection, intrauterine blood transfusion is a possible treatment, as described in several reports, and should be considered (Enders et al. 2004).

1.8.4 Intravenous immunoglobulin (IVIG)

In immunocompromised patients with chronic B19-related anaemia, IVIG is a well-established treatment and usually given in doses of 0.4 g/ kg consecutively for five days, as stated above. Sometimes this therapy is curative, but relapses may occur, in which case repeat doses of IVIG are needed (Broliden 2001). However, one must consider that IVIG is a possible route of B19 transmission, since the virus is stable and difficult to eliminate during the procedure of IVIG production (Erdman et al. 1997; Hayakawa et al. 2002). Nevertheless, for patients with chronic B19 infection but not obviously impaired immune defence, IVIG has been found successful for treating chronic fatigue syndrome, arthritis and intrauterine infection (Kerr et al. 2003; Lehmann et al. 2004; Rugolotto et al. 1999; Selbing et al. 1995).

1.8.5 Cessation of chemotherapy

For patients with B19 infection for whom cessation of immunosuppression is possible or planned, the withdrawal or switch of chemotherapy may be enough to resolve the infection (De Renzo et al. 1994; Wong et al. 1999). Yet, for patients with renal failure treated with erythropoeitin, B19 may cause severe worsening of anaemia, necessitating a withdrawal of and a contradiction in case management (Lim 2005).

1.8.6 Vaccination

B19 vaccine has been tested in clinical trials and induced strong humoral responses. By recombinant technology, empty capsids consisting of 25% VP1 and 75% VP2 have been produced. The immune response to such vaccine can be measured by assessing levels of neutralizing antibodies (Ballou et al. 2003). However, to receive long-term effects from the vaccine, cellular immunity must be considered (Isa et al. 2005). Presumably, certain patients at risk for B19 infection, such as those with sickle cell anaemia, could benefit from vaccine prophylaxis. Moreover, widespread vaccination might be worthwhile if the clinical spectrum of B19 becomes related to a larger number of diseases than is known today.

2 AIMS OF THE STUDY

- To investigate the frequency of B19 DNA in bone marrow of patients with haematological and rheumatological diseases.

- To study clinical and immunological parameters in patients with signs of persistent B19 infection, specifically to define whether persistence is associated with a general immunodeficiency or a selective failure of the immune response.

3 MATERIALS AND METHODS

3.1 STUDY SUBJECTS

The patients included in the five papers and their connections are illustrated in Figure 3.

3.1.1 Paper I

In March 1993, a 36-year-old woman was referred to the Clinic of Infectious Diseases in Borås, Sweden, after presenting with six months of variable symptoms including myalgia, fever and exanthema. Routine laboratory analysis had revealed leukopenia and anaemia. BM aspiration demonstrated megaloblastic erythropoesis of unknown etiology. B19 serology was IgM negative but strongly positive for IgG. When BM aspiration was repeated, a sample for PCR analysis of B19 was sent to the laboratory capriciously and found positive. This result was the beginning of my interest in this subject and resulted initially in acquiring BM samples from other patients with chronic, unexplained symptoms such as fatigue, arthralgia, leukopenia and fever. The index case and nine others are described superficially and retrospectively in Paper I. Results for patients 1-5 were, in contrast to those for patients 6-10, all B19 DNA positive in BM. Subsequently, patients 1-4 were found to be persistently infected and are further studied in Papers IV and V.

3.1.2 Paper II

During the period October 1994 to March 1996, one hundred patients were included in this study. They were all undergoing BM sampling indicated by haematological reasons, at the Clinic of Medicine, Södra Älvsborg Hospital, Borås, Sweden. They were diagnosed with acute lymphatic leukaemia (1), acute myeloid leukaemia (17), chronic lymphoproliferative disease (23), chronic myeloproliferative disease (15), plasma cell disease (13), anaemia (11), other cytopenias (11), adenocarcinoma, (3) and other diagnoses (6). Patients were offered inclusion in the study regardless of symptoms or diagnosis, simply because BM sampling was planned. We do not know how many patients chose not to participate or how many BM samples were taken during the study period.
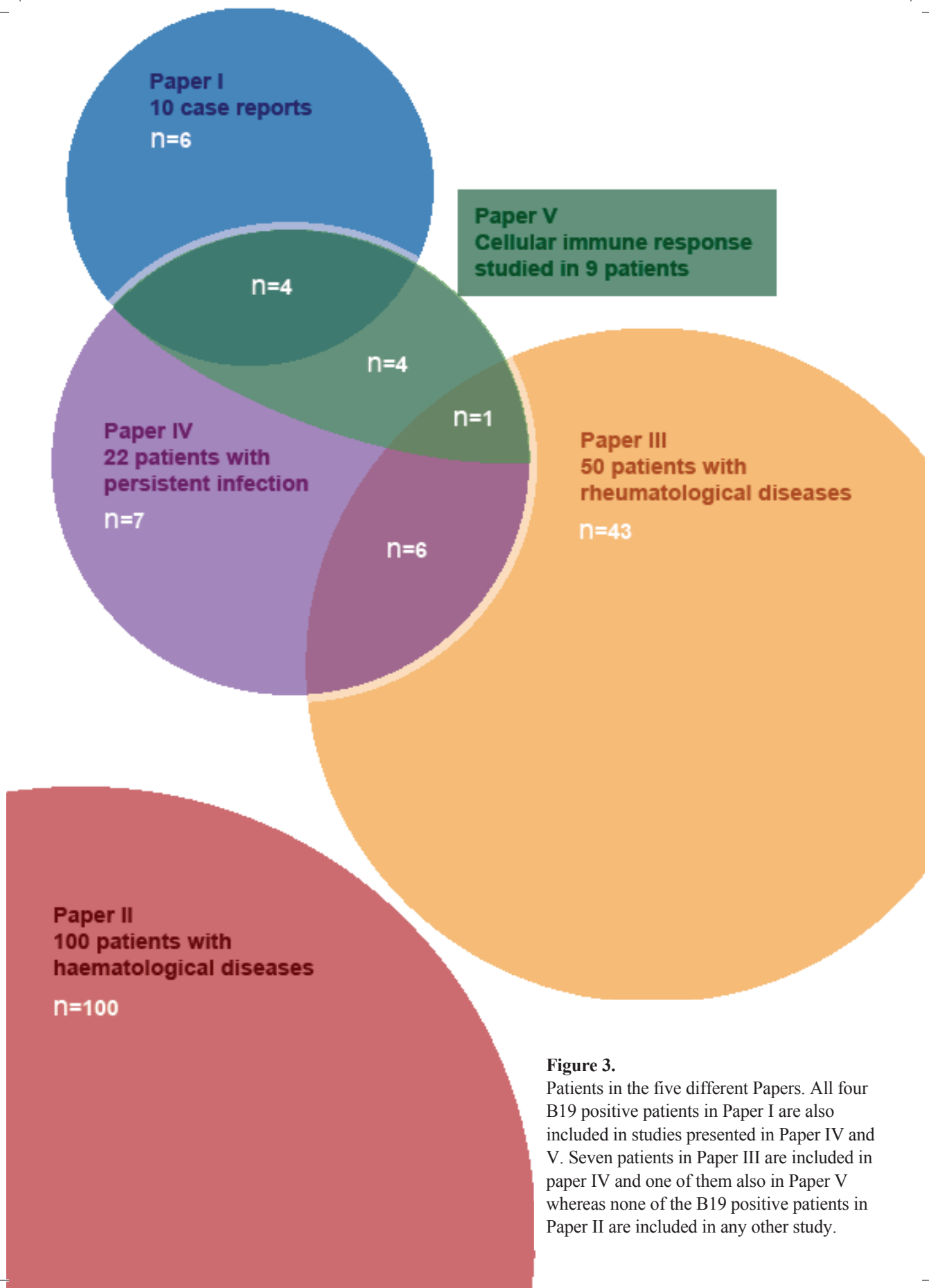
Figure 3.
Patients in the five different Papers. All four B19 positive patients in Paper I are also included in studies presented in Paper IV and V. Seven patients in Paper III are included in paper IV and one of them also in Paper V whereas none of the B19 positive patients in Paper II are included in any other study.

3.1.3 Paper III

During the study period between 1997 and 1999, when 996 patients visited the Department of Rheumatology in the Clinic of Medicine at Södra Älvsborg Hospital Borås, Sweden, 130 of them were randomized and invited to participation in the study, and 50 accepted. The diagnoses of study subjects appear in Table I. Patients with septic arthritis or crystal-induced tenosynovitis were not included. A healthy control group had been desirable but was not available.

Table 1.
Distribution of diagnoses in the study group,presented in Paper III, and compared with all patients attending the clinic during the study period.

Diagnosis/ symptoms	Study group		Population	
	n	%	N	%
RA	22	44	592	59
SLE	8	16	43	4,3
Sjögren's disease	3	6	47	4,7
Wegener's granulomatosis	1	2	4	0,4
Giant cell arteritis	1	2	15	1,4
MCTD	1	2	9	0,9
UCTD	6	12	25	2,5
Arthralgia	7	14	102	10,2
Myalgia	1	2	49	4,9
Total number	50	100	996	100

RA	=	Rheumatoid arthritis
SLE	=	Systemic lupus erythematosus
MCTD	=	Mixed connective tissue disease
UCTD	=	Undefined connective tissue disease.

3.1.4 Paper IV

Acutely B19 infected patients

Eight previously healthy female adults were prospectively identified and included in the study after their anti-B19 IgM serology proved to be positive, and B19 DNA was found by PCR in samples referred to Clinical Virological Laboratory at Karolinska Hospital. All these women had at least three of the four following symptoms: fever, arthralgia, fatigue and rash.

Persistently B19 infected patients

Initially 25 patients were included in the study because of fulfilling criteria for suspected persistent B19 infection based on chronic clinical symptoms and a B19-positive PCR-sample from BM. Some were recruited after being diagnosed in routine clinical practice in Clinic of Infectious Diseases, Södra Älvsborg Hospital, Borås, Sweden (n=16) and some from the study presented in Paper III (n=9) who were included consecutively regardless of diagnosis or other parameters until we reached the number 25. Later, criteria were altered to be more restrictive, and two B19-positive BM samples with at least six months interval were required for our own definition of persistent infection. In this way three patients were excluded, and 22 subjects remained: four males and 18 females.

Healthy seropositive individuals

Eighteen B19 IgG positive healthy laboratory workers were included, all negative for B19 IgM and B19 DNA in serum and without history of recent B19-related symptoms.

Healthy blood donors
Nineteen healthy blood donors served as controls in one the part of the study, investigating cellular immune response to concanavalin A.

3.1.5 Paper V

Persistently infected patients

Seven females and two males also included in the study described in Paper IV participated in the study. They were B19 IgG positive and IgM negative but B19 DNA positive in BM during follow up (two to eight years).

Healthy seropositive individuals

Fourteen healthy individuals were included, seven males and seven females, all were B19 IgG positive but negative for B19 IgM and DNA in serum.

Healthy seronegative individuals

Three individuals negative for B19 IgG were used as methodological controls.

3.2 COLLECTION OF SAMPLES

3.2.1 Bone marrow

BM puncture was performed with patients given local anaesthesia (xylocain) in the posterior superior iliac spine. BM samples were delivered to the laboratory in sterile tubes without additives. BM smears so-collected were examined by light microscopy at the Clinical Chemical Laboratory, Södra Älvsborg Hospital, Borås, Sweden.

3.2.2 Blood

Blood was sampled by venous puncture and delivered to various laboratories for each test specified.

1. Sera were placed in sterile tubes without additives for PCR and serology.
2. Blood samples in tubes with EDTA and ACD, respectively, were used for analysis of HLA-type.
3. Blood in tubes with EDTA were analysed with flow cytometry for subpopulations of lymphocytes.
4. Blood in tubes with heparin were used for analysis of IFNγ production by the ELISpot-technique.

3.3 DETECTION OF B19 DNA

Serum and BM samples were tested in two different nested PCRs in Paper I and II. These assays represented the structural (VP) and the non-structural (NS) proteins of the B19 virus, as described previously (Broliden et al. 1998). Briefly, in each PCR, the result was considered positive if at least two of three separate amplifications gave identical results, and all controls were correctly positive or negative. At least one of the two PCR:s (VP and NS) was required to be positive for each patient. Serum and BM samples were analysed after heat treatment at 94°C for 10 minutes, followed by centrifugation at 12,000 rpm for five minutes. Two μl of the supernatant was used in the PCR assay. The first amplification consisted of 35 cycles and the nested round 25. The following primers were used in Paper I and II.

VP-PCR.
First amplification: nucleotides 2955 to 2974 and reverse primer 3364 to 3349.
The nested round: nucleotides 3002 to 3020 and reverse primer 3291 to 3272.

NS-PCR.
First amplification: nucleotides 1355 to1374 and reverse primer 1723 to 1703.
The nested round: nucleotides 1399 to1422 and reverse primer 1682 to 1659.

In Paper III only NS-PCR was used and with a slightly modified outer forward primer.

3.4 SEROLOGICAL ASSAYS

3.4.1 Specific B19 IgG in serum

Enzyme linked immunosorbent assay	*Papers*
1. ELISA; Eurodiagnostica, Malmö, Sweden	I , II
2. EIA; DAKO, Glostrup, Denmark	I
3. EIA; Biotrin International Ltd, Dublin England	III, IV, V

3.4.2 Specific B19 IgM in serum

Indirect immunofluorescence test
IFA; Biotrin, International Ltd, Dublin, England	I

Enzyme Linked Immunosorbent Assay
1. EIA; DAKO, Glostrup, Denmark	I, II
2. EIA; Biotrin International Ltd, Dublin, England	I , II, III, IV, V

3.4.3 Avidity

These assays were kindly performed by Professor Klaus Hedman at the Department of Virology, Haartman Institute, University of Helsinki, Finland. Avidity of B19 antibodies was measured by protein denaturing EIA:s using recombinant antigens consisting of VP1 alone, VP2 alone and VP1 and VP2 together (VP1/2) in ratio 1/11. Wells with antigen were incubated with serum and then washed with 8 M urea in PBST (PBS containing 0.05% Tween) or only PBST, respectively. All wells were treated for 1 h with alkaline phosphatase-conjugated anti-human IgG and substrate for 30 min. Denaturation of antigens with urea results in decreased bond between antibodies and antigen especially in newly infected individuals who have antibodies with lesser avidity than individuals infected long ago. By using the EIA absorbance quotient between urea-treated and urea-free samples, the time interval since primary infection in each patient could be estimated. Avidity levels >25% indicate past infection of more than six months (Söderlund et al. 1995).

3.4.4 Epitope type specificity

Epitope type specificity (ETS) was, just like the avidity test, used to estimate if antibodies against B19 were signalling past or acute infection. By using denaturated B19 antigens, namely self-assembled VP1/2 capsid and VP2 capsid with 8 M urea, antibody response to VP1/2 antigens have been shown to be more stable than to VP2 in patients with past infection. On the contrary, there is no such difference in acute infection. Consequently, the extent of antibody responses to VP1/2 relatively VP2 rises over time, resulting in low quotient (<2) for acute infection and elevated scores (>5) afte more than six months after the infection.

3.4.5 Neutralizing antibodies

Dr. Jennifer Bostic, MedImmune Inc, Gaithersburg, Maryland, USA helpfully performed analysis of neutralizing antibodies. A megakaryoblastic cell line (UT-7/Epo), adapted to grow with erythropoietin and semipermissive to B19 infection, was used and incubated with serum at various dilutions. The neutralizing titre was defined as the highest dilution that yielded no B19 RNA, which was analysed with reverse transcriptase-PCR (RT-PCR).

3.5 IMMUNOPHENOTYPING OF LYMPHOCYTES

Subpopulations of lymphocytes in blood from persistently infected patients were
determined by fluorescent activated cell sorter (FACS) and presented in Paper IV. The
samples were stained with combinations of murine monoclonal antibodies directly
conjugated with fluorochromes, fluorescein isothiocytanate (FITC), phycoerythrine
(PE) or peridinin chlorophyll protein (PerCP). Blood was incubated at 4° C for 15
minutes with antibodies in the concentrations recommended by the manufacturer. The
following flurorochrome-conjugated antibodies were used: anti-CD3, -CD4, -CD5,
-CD8, -CD16, -CD19, -CD45RA, -CD45RO, -CD56, and anti-HLA DR (all antibodies
were purchased from BD Biosciences, Mountain View, CA, USA). Cell analysis was
performed by flow cytometer (FACSCalibur, BD Biosciences). Dot plots and quadrant
statistics from three-colour analysis were generated by CellQuest software (BD
Biosciences). The absolute number of blood lymphocytes was determined using a
haematological cell counter (Sysmex K-4500; TOA Medical Electronics Co, Japan).
The results for each subpopulation were expressed as the percentage of lymphocytes
and as the number of cells x 10^9/L.

3.6 PEPTIDES

Amino acid sequences published by Shade were used as templates for synthesizing a
total of 210 peptides representing NS1, VP1ur and VP2 (Shade et al. 1986). Nonamers
with an overlap of one amino acid were purchased (Mimotopes, Clayton, Victoria,
Australia) except for the VP2-nonamers, which together with all other peptides were
synthesized in-house by F-moc chemistry. Peptides were first dissolved in
dimethylslufoxide (DMSO) and were then diluted with distilled water to a DMSO
concentration of less then 5%.

3.7 INTERFERON GAMMA (IFNγ) ELISPOT

The cellular immune response in general, described in Paper IV, was investigated by
analyzing responses to concanavalin A, and helpfully performed by Associate Professor
Bengt Andersson at Clinical Immunology Laboratory, Sahlgrenska University Hospital,
Gothenburg, Sweden.
Sterile microtitre plates with nitrocellulose bottoms (Multiscreen-HA plates; Milipore,
Mulsheim, France) were coated overnight at 4° C with 100 μλ of mouse monoclonal
antibodies to human IFNγ in sterile filtered PBS (final concentration of 15 μg/ml;
Mabtech, Stockholm, Sweden). After washing five times with PBS, 200 μL of
peripheral blood mononuclear cells (PBMC) in cell culture medium were added in
different concentrations (5 x 10^4, 2 x 10^4 and 1 x 10^4 cells per well) in duplicates.
Concanavalin A was added at a final concentration of 25 μg/ml and unstimulated cells
were used as controls. The PBMC were incubated for 48 hours at 37° C in a humid
atmosphere with 5% CO_2. Plates were washed and 100 μl of biotinylated mouse
monoclonal anti-IFNγ (final concentration 1 μg/ml; Mabtech) was added and they were
incubated at room temperature for 2 hours. After washing, 100 μl of streptavidin con-
jugated with alkaline phosphatase (final concentration 4 μg/ml) was added. The spots
were developed by BCIP-NBT (Life Technologies) substrate diluted in Tris buffer.

Blue immunospots, considered to represent individual IFNγ secreting cells, were counted in a dissection microscope.

The cellular immune response to B19 virus, described in Paper V was measured *in vitro* by analysing antigen-specific secretion of IFNγ with a previously described ELISpot assay (Larsson et al. 1999), but modified by the use of streptavidin conjugated with alkaline phosphatase and a corresponding substrate (BioRad, Hercules, CA, USA). PBMC were mixed with single or pooled peptides at a concentration of 10μg/ml and plated in triplicates of $2x10^5$ cells/ well. After 18 hours of incubation (37°, 5% CO_2, with 95% humidity), spots were visualized, counted and registered as spot-forming cells (SFC)/10^6 PBMC after subtracting the release from negative controls in wells with PBMC without peptide stimulation. A cut-off level was set, and responses were determined to be positive or negative. IFNγ-release in the ELISpot assay was previously shown to correlate well with the presence of specific CD8 T-cells and cytolytic activity (Lalvani et al. 1997; Horton et al. 2004). To ensure that IFNγ detected in the ELISpot originated from CD8-cells, depletion assays in ELISpot were done, as described previously (Norbeck et al. 2005). The responses in ELISpot were lost by depletion of the CD8 cells using mini-MACS CD8 microbeads and subsequent magnetic separation (Miltenyi Biotec, Bergisch Gladbach, Germany) compared to non-depleted PBMC.

3.8 HLA-TYPING

HLA expression was analysed by Consultant Jan Konar at the Tissue Typing Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden. HLA class I expression was determined by serology. Lymphocytes in peripheral blood were isolated, and HLA was assessed by using a complement dependent cytotoxicity technique (CDC) (Vartdal et al. 1986). HLA-DR genotyping was performed using a PCR-SSP technique as described elsewhere (Olerup et al. 1992).

3.9 QUANTIFICATION OF CYTOKINES

The cytokines' concentrations were longitudinally analysed by multiplex beads assay (Luminex). We quantified twelve different cytokines: IL1β, IL2, IL4, IL5, IL6, IL8, IL10, IL12, IL15, GM-CSF, INFγ and TNFα in sera from all acutely infected individuals (Paper IV). Cryopreserved serum samples from persistently infected and healthy seropositive individuals were analysed on one occasion. A commercially available multiplex beads immunoassay, based on the Luminex platform (Biosource International, Inc, Camarillo, CA, USA) was used according to the manufacturer's procedure. All samples were run in duplicate. Briefly, beads with defined spectral properties were conjugated to the analyte-specific capture antibodies. Beads, samples, standards and controls were pipetted in a filter-bottomed 96-well plate and incubated for 2 hours while shaking (550 rpm). After three washes, the biotinylated detector antibodies were added to the beads and incubated for one hour at room temperature. Streptavidin conjugated to R-phycoerythrin (SA-PE) was added to the wells after several washings and incubated for 30 minutes. By monitoring the spectral properties of the beads and the amount of fluorescence associated with PE, the instrument measures the concentration of the analytes presented in the original specimens. The data (mean fluorescence intensity) were analyzed using a Luminex reader (Luminex, Austin, TX), and the mean concentration was calculated as pg/ml serum.

4 RESULTS AND DISCUSSION

4.1 PAPER I: CLINICAL AND LABORATORY FINDINGS IN IMMUNOCOMPETENT PATIENTS WITH PERSISTENT PARVOVIRUS B19 DNA IN BONE MARROW

This retrospective report describes our initial experiences of PCR analysis of B19 DNA in BM samples and how my interest begun. In March 1993, at the Clinic of Infectious Diseases, Södra Älvsborg Hospital, Borås, Sweden, a 36-year-old woman presented with a six-month-history of variable symptoms including myalgia, fever and exanthema. Previously, routine laboratory analysis had revealed leukopenia and anaemia. BM aspiration demonstrated megaloblastic erythropoesis of unknown etiology. Parvovirus serology was IgM negative but highly positive for IgG. When BM aspiration was repeated, a sample for PCR analysis of B19 was sent, to the Virological Laboratory at thee Swedish Institute for Infectious Disease Control, and found positive. This unexpected result inspired me to analyse B19 DNA in BM samples in other patients with chronic, unexplained symptoms such as fatigue, leukopenia and fever. That original sampling was irregular, intuitive and without systematic order so could be referred to "randomized biased selection." Our first doubts and questions, which still continue, were how to evaluate the relevance of B19 DNA in patients' BM and whether further immunogical investigations were clinically indicated. Knowledge of the natural virological course of B19 was, and remains, limited and several explanations for positive finding of B19 DNA are possible.

1. Chronic active infection
2. Persistence as a part of the natural course of B19 infection analogous to that of herpesvirus infection
3. Fragments of B19 DNA without the ability to replicate viruses

The diagnosis of this first patient (case 1) was relatively obvious, since she had several clinical signs characteristic of B19 infection. She had anaemia associated with megaloblastic erythropoesis (Figure 4), suffered from arthralgia and had exanthema, a combination that made B19 infection probable. Initially, her serum was declared IgM negative, but reanalysis determined that it was positive. Furthermore, when the patient was confronted with the diagnosis, she recalled an outbreak of fifth disease in the day care centre where she worked during the same year as she fell ill. Her persistent symptoms, i.e., anaemia and leukopenia, led to treatment with IVIG that was initiated in 1995, after she had endured more than two years of disease (Figure 5). The levels of haemoglobin and leukocytes were improved after IVIG therapy, but her fatigue and arthralgia persisted, and the virus was not cleared from BM. ETS and avidity were tested to estimate the duration of the disease, and was helpfully performed by Dr. Maria Söderlund at the Department of Virology in Helsinki, Finland. The results indicated past infection of more than six months duration. The patient is further described in Paper IV and V.
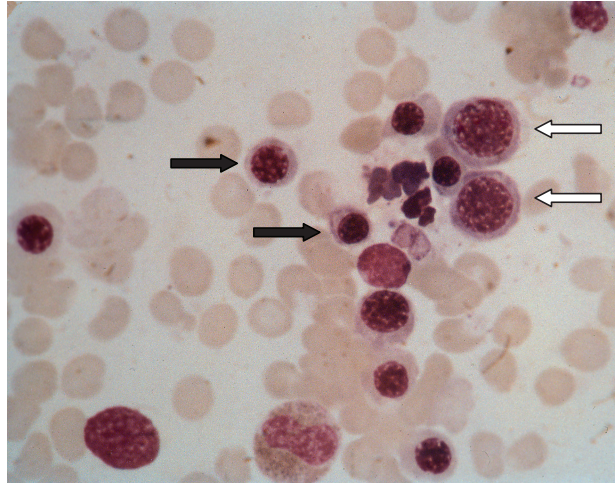
Figure 4.
BM preparation in case 1 presented in Paper I. Megaloblastic erythropoetic
cells are shown with white arrows and normal erythropoetic cells with black
arrows.
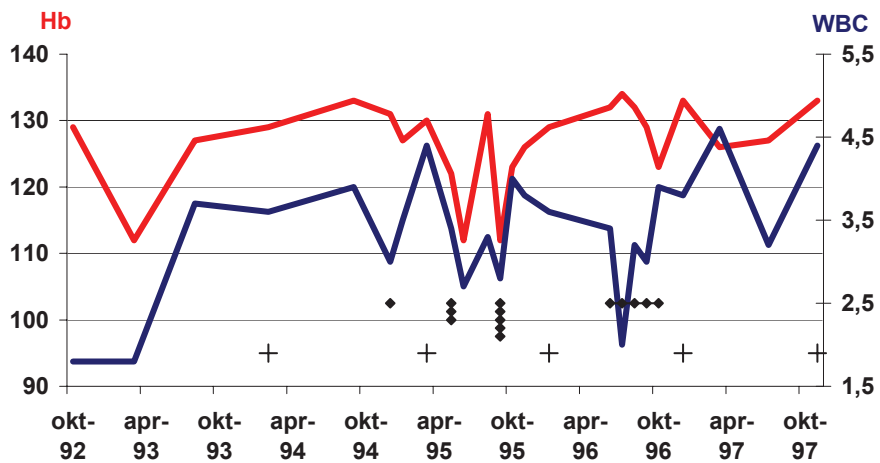
Figure 5.
Laboratory parameters associated to case 1 in the first paper. She is also described in
Paper IV and V. The figure show variations in hemoglobin (Hb, g/l) leukocyte count
in blood (WBC, leucocytes x 10^9/l) and in time corresponding treatment with
immunoglobulin (♦) Initially the treatment was given intravenously (IVIG), one dose
of 30 g in December 1994, three doses during three days in May 1995 and five doses
during five days in September 1995. Subcutaneous treatment with immunoglobulin
(SCIG) was given in doses of 40 ml twice a month from April to October 1996. B19
positive PCR samples in bone marrow are also shown (+).

The patient recorded as case 2 suffered from arthralgia fever and fatigue subsequently to acute respiratory symptoms. She was working in a childrens' day care centre, so stimulated by the results from case 1, we examined her BM searching for B19 DNA and the result from the PCR assay was positive. Her symptoms, including those of chronic fatigue syndrome, persisted for several years, but her haematological status remained normal and, except for B19 DNA in the BM, no other obvious clinical signs of B19 infection appeared. Treatment with immunoglobulin was given and to some extent subjectively connected to improvement. BM samples have been B19-DNA negative several times but periodically positive again. Although the explanation could be reinfection, variations in viral load combined with limited sensitivity in the PCR procedure are more likely. ETS and avidity assay demonstrated a prior immune reaction more than six months before, but the initial respiratory symptoms may or may not have been related to B19 infection.

The patient designated as case 3 also worked in a childrens' day care centre, but her medical history was more complicated than the others'. In childhood she had symptoms of motor and sensory dysfunction, and by the age of twelve, she underwent a muscle biopsy, examined on the suspicion of hereditary muscle dystrophy but proving to be negative. However, her symptoms persisted and propagated slowly. In 1991, neurophysiologic examination demonstrated motor and severe sensory neuropathy of unspecified origin. She was admitted to the Clinic of Infectious Diseases in 1994, because of a long-lasting ulcer on her right foot, probably related to neuropathy. The patient also experienced recurrent episodes of erythema nodosum since 1979, and when it reappeared in 1995, rheumatologic investigation was initiated. Additionally, for many years, she had suffered from periods of facial erythema and non-arthritic arthralgia in knees, wrists and shoulders. Results of testing for rheumatoid factor and antinuclear antibodies were positive. After thorough investigation in the Department of Rheumatology at Sahlgrenska Hospital, Gothenburg, in 1997, including examination of saliva secretion and lip biopsy, she was diagnosed with primary Sjögren's syndrome. In 1996, arthralgia, facial erythema, anaemia and probably the patient's profession as preschool teacher all contributed to the decision to look for B19 DNA in her BM. When that result proved to be positive, the current question about relevance was evident.

Various neurological manifestations associated with B19 infection are well described in several reports (Barah et al. 2003; Aguilar-Bernier et al. 2006; Kerr et al. 2002), and IVIG treatment is considered of potential value (Nigro et al. 1994). Therefore, this patient's slow but serious progressive symptoms motivated tentative treatment with IVIG, which was given in doses of 0.4g / kg five days followed by one dose monthly for six months. No obvious alteration in clinical status was noted initially but her subsequent symptoms of erythema nodosum occurred much less frequently, and the neuropathy, which according to neurophysiologic tests, had worsened between 1991 and 1996 was without evident changes between 1996 and 2000. After therapy ceased, follow-up analysis of B19 DNA in BM performed in 1996, in 1997 and most recently in 2000 all showed positive results, illustrating the failure of viral clearance.

Interestingly, the very first muscle biopsy from 1962 was found, re-tested and declared positive for B19 DNA. With respect to the difficulty of establishing accredited analysis of 40-year-old, formalin-fixed samples, the finding was astonishing. Was the patient already persistently infected in childhood? Was the chronic immune stimulation by this virus the pathogenetic origin of her disease manifestations including erythema nodosum, facial erythema, autoantibodies and progressive neuropathy?

The profession of the patient case 4 represents was, not surprisingly, pre-school teacher. She was admitted to the clinic in October 1996 after four weeks of continuous fever and arthralgia in the knees. Her routine haematology status was normal, and serology concerning cytomegalovirus, Epstein-Barr virus and human immunodeficiency virus was negative. Serological testing for B19 demonstrated highly positive results for B19 IgG and slightly positive B19 IgM indicating primary B19 infection. The symptoms proceeded without relief, and persistent B19 DNA remained in BM samples in November 1996 and March 1997. Treatment with IVIG 0.4 g/ kg was given once in May 1997 and additional three doses three weeks later. In connection with the latter doses, the patient became more acutely ill with high fever but no focal sites of symptoms for some days. Subsequent BM sampling in July was negative, and the arthralgia vanished, but the inconvenience of fatigue and fever has continued over the years fulfilling criteria for CFS. B19 DNA in BM was again positive in September 1997 and in 1998 but negative when analysed in 1999. That year, she was struck down by a miscarriage in week 15 of pregnancy, but another pregnancy ended successfully in 2000 when an acute Caesarean was performed because of pregnancy-related toxaemia. The child was born healthy without signs of infection. Although the patient had another miscarriage in 2002, in 2003 she gave birth to a healthy girl after a pregnancy without complications.

Primary B19 infection during pregnancy is associated with miscarriage but also hydrops fetalis, mainly in the second trimester, and intrauterine fetal death later in pregnancy (Norbeck et al. 2002; Skjoldebrand-Sparre et al. 2000). B19 has also been linked with preeclampsia and eclampsia (Selbing et al. 1995; Yeh et al. 2004). If and how chronic B19 infection might affect the foetus is not known but should be considered, since persistent infection logically might be related to some sort of impaired or deficient immune response to B19 in the mother.

The patient known here as case 5 had fever and fatigue for ten months previous to examination for B19 DNA in BM, which proved to be positive. Sampling 11 months later was negative, and in follow up the patient was asymptomatic.

The patients designated as cases 6 to 10 were examined for similar reasons as those constituting cases 1-5, chronic symptoms of unknown origin, but with components consistent with known manifestations of parvovirus B19. They were all B19 DNA negative in BM as summarized in Table 2.

Table 2.
Clinical and virological findings in five patients with negative B19 DNA in BM presented in paper I.

Case	Clinical presentation	B19 IgG	B19 IgM	B19 PCR
6	Leukopenia, anaemia, fever	+	-	-
7	Chronic leukopenia	+	-	-
8	Anaemia and fever	+	-	-
9	Arthralgia	+	-	-
10	Recurrent arthralgia and fever	+	-	-

The conclusions from these case reports in which B19 DNA was consistently found in BM are and were difficult to draw, but we established that some individuals were B19 DNA positive in BM and others were not. One explanation could be that life-long viral persistence in BM is the natural course after primary infection and that our method was not sensitive enough to detect all instances. If so, viral load could be of interest, and a positive result in our qualitative test could indicate a relatively high extent of viral replication. Actually, a recent study with a sensitive quantitative PCR has demonstrated B19 persistence in serum for more than one year after acute infection (Lindblom et al. 2005). Our qualitative PCR has turned out to have sensitivity about 10^3 copies/ ml.

Another explanation for positive PCR results could be fragments of DNA remaining from primary infection, even over long periods of time. This possibility was not confirmed, since B19 DNA from some of the patients (cases 1 through 4) was later sequenced, and the complete B19 virus genome was present in each patient (unpublished data).

CFS is a diagnosis based on internationally accepted criteria, defined first in 1988 and revised in 1994, describing a disease with debilitating fatigue lasting more than six months and associated with at least four of the following symptoms: impaired memory or concentration, tender lymph nodes, myalgia, arthralgia, headache, unrefreshing sleep and postexertional malaise (Fukuda et al. 1994). That patients with B19 infection frequently develop this syndrome is well known, but when larger populations of patients with CFS were investigated, no sign of B19 was found. Undoubtedly, this disease has a broadly heterogeneous aetiology (Maquet et al. 2006).

Numerous viral infections, not only that from B19, have been discussed as possible causes of a full range of autoimmune inflammatory diseases. One theory regarding pathogenesis has been a "hit and run phenomenon" in which virus acts as a trigger factor that initiates an immunological reaction. Subsequently, this reaction becomes self-generating and, possibly by molecular mimicry, continues to evoke a destructive autoimmune response even after the virus has been eliminated (Holtzman et al. 2005; Rouse et al. 2002). Another hypothesis, illustrated by case 3, is that persistent viral infection causes autoimmune manifestations from chronic immune stimulation. An example is the association between chronic hepatitis B virus infection and periarteritis nodosa, in which the deposition of immune complexes may cause vasculitis in various organs (Trepo et al. 2001). The autoimmune disease Sjögren's syndrome has been linked with such viral infections as that by Epstein-Barr virus (Wen et al. 1996) herpes virus 6 (Newkirk et al. 1994) and HTLV1 (Nakamura et al. 1997). An association with B19 has been investigated in two small studies but with negative results (De Stefano et al. 2003; De Re et al. 2002). The patient in case 3 was diagnosed with Sjögren's syndrome, yet her B19 DNA analysis was negative when a sample from the salivary gland was examined with PCR. However, immunological manifestations secondary to infectious diseases may not necessarily be related to a viral presence in the affected organ.

In conclusion, this retrospective report contains the results of our first use of PCR to search for B19 DNA in BM samples. The positive findings in patients with relevant, chronic symptoms raised questions that were important to answer and became the basis for our further studies.

Paper II:
Prevalence of parvovirus B19 DNA in bone marrow of patients with haematological disorders
How common is B19 DNA in BM samples in a larger population?

Paper III:
High frequency of parvovirus B19 DNA in bone marrow samples from rheumatic patients
How common is B19 DNA in patients with rheumatologic diseases?

Paper IV:
Cytokine responses in acute and persistent human Parvovirus B19 infection
Do patients with persistent B19 infection have any general immunological characteristics in common?

Paper V:
Aberrant cellular immune responses in persistently human parvovirus B19-infected individuals
Do patients with persistent B19 infection have any specific B19-related immunological characteristics in common?

4.2 PAPER II: PREVALENCE OF PARVOVIRUS B19 DNA IN BONE MARROW OF PATIENTS WITH HAEMATOLOGICAL DISORDERS

Paper I presented the histories of patients with chronic symptoms and positive findings of B19 DNA in BM samples. These findings led to questions concerning the frequency of B19 DNA in the general population. If, speculatively, B19 persistence in BM was the natural course of primary B19 infection, the frequency of positive test results ought to be rather high, since the seroprevalence in the adult population is over 50% and more than 80% in the elderly. A large study including healthy individuals would have been desirable, but BM puncture is an invasive method limiting the possibilities. That was the reason for investigating B19 DNA in BM of patients with haematological disorders. Their BM was examined for diagnostic purposes or as follow-up after chemotherapy; that is, sampling BM for B19 DNA analysis was not a motive. A total of 100 patients was enrolled in the study between October 1994 and April 1996 at the Haematology Department, Clinic of Medicine, Södra Älvsborg Hospital, Borås, Sweden. Regrettably, neither the total number of BM punctures performed during the study period nor count of patients denied inclusion in the study was recorded. Moreover the population was heterogeneous including patients with such varied diseases as acute lymphatic leukaemia (1), acute myelocytic leukaemia (17), chronic lymphoproliferative disease (23), chronic myeloproliferative disease (15), plasma cell disease (13), anaemia (11), other cytopenias (11), adenocarcinoma (3) and other (6).

The main purpose of the study was to examine the frequency of B19 DNA in BM, but ETS and avidity were also assessed to establish if the primary infection was more recent than six months. Results indicated borderline values in the patient designated case 1 and signs of acute infection in that of case 2. They were IgM- positive indicating recent infection, but B19 PCR was negative in BM as well as in serum. Both patients had, related to their haematological diseases, elevations of polyclonal IgM in serum; therefore, falsely positive B19 IgM cannot be excluded, or is even probable. In general, results from serological assays are questionable in patients with haematological disorders; additionally, our analysis of avidity in these cases is of limited value. Problems with serological testing are further illustrated by the fact that only one of nine myeloma patients was IgG-positive. Myeloma is characterized by the production of the monoclonal M-component, but the production of polyclonal antibodies against various infectious agents is depressed. Consequently, when studying seroprevalence, the true history of exposure to B19 may be underestimated. Nevertheless, the seroprevalence in these patients was 59%, corresponding to the expected figure in an adult population. However, patients with haematological diseases are at risk for acquiring B19 infection by transfusion and immunoglobulin treatment (Cohen et al. 1997; Hayakawa et al. 2002).

Four patients were positive for B19 DNA in BM, whereas none was positive in serum. Symptoms, possibly related to B19 infection, such as arthralgia, exanthema and anaemia, were a part of the medical history for three of them, but they had no obvious signs of ongoing B19 infection at the time of BM puncture. Obviously, it is difficult to determine if manifestations like anaemia are B19 related or not in patients with haematological diseases.

As mentioned, patients at a haematological department are at risk for acquiring B19 infection, but they might also be prone to developing persistent infection. When one attempts to approximate the prevalence of B19 DNA in BM in the general population, the result may be an underestimation. In a previously published study, B19 was detected in the BM of four patients with chronic B19-related arthropathy but not found in any of six seropositive healthy controls (Foto et al. 1993). The frequency of B19 in BM samples was described in two studies published 1997. Liu detected B19 DNA by in situ hybridization, which was positive in seven of 81 (8.6%) AIDS patients (Liu et al. 1997), and Cassinotti reported positive B19 DNA in four of 45 healthy bone donors (Cassinotti et al. 1997). Later, Heegaard presented data from 153 healthy BM donors of whom 73 % were IgG-positive and four (2.6%) B19 positive in BM (Heegaard et al. 2002). These figures are coherent with our results. One question is evident, though: What is the sensitivity of the PCR method? In Heegaard`s study, the sensitivity was stated as 200-2000 viral copies /ml sample volume, and in our laboratory, it has later specified as 1000 copies/ ml. Consequently, one can not exclude that the true general prevalence of B19 DNA in BM is actually higher than in those studies and that only high viral loads have been detected so far. Disregarding these aspects, our main conclusions from this study were that B19 DNA is not a general finding in seropositive individuals and that persistence of B19 DNA in BM is not a common course after primary infection.

4.3 PAPER III: HIGH FREQUENCY OF PARVOVIRUS B19 DNA IN BONE MARROW SAMPLES FROM RHEUMATIC PATIENTS

We previously presented a case report, Paper I, describing patients with various chronic symptoms and with persistent findings of B19 DNA in BM. Paper II included 100 patients with haematological disorders, and from the results we concluded that viral persistence is not a general finding in seropositive individuals. Next, because arthropathy is a well-established clinical symptom of B19 infection, especially in adults (Torok 1992), and numerous reports have associated this virus with rheumatic manifestations (Moore et al. 1999; Nikkari et al. 1994; Nigro et al. 1994; Pugliese et al. 2006; Ferri et al. 2005), we were inspired to investigate the frequency of B19 in patients with rheumatic diseases. PCR analysis of BM samples was used, since we know that erythropoetic cells are the predominant targets for B19 infection. Of patients who visited the Department of Rheumatology at Södra Älvsborg Hospital, Borås, Sweden, during the years 1997 to 1999, 130 were randomly chosen and invited to participate in this study. The various diagnoses of the 50 patients who accepted are summarized in Table 1.

B19 IgG serology was positive in 41 of those 50 patients (86 %), but all were B19 IgM negative. By PCR, 13 of the 50 patients (26%) were B19 DNA-positive in BM, and the frequency was higher, i.e., seven of 22 (32%), in the subset of patients with RA. When compared to our previous results in the study of haematological patients (4% positive), the difference was significant (Figure 6).
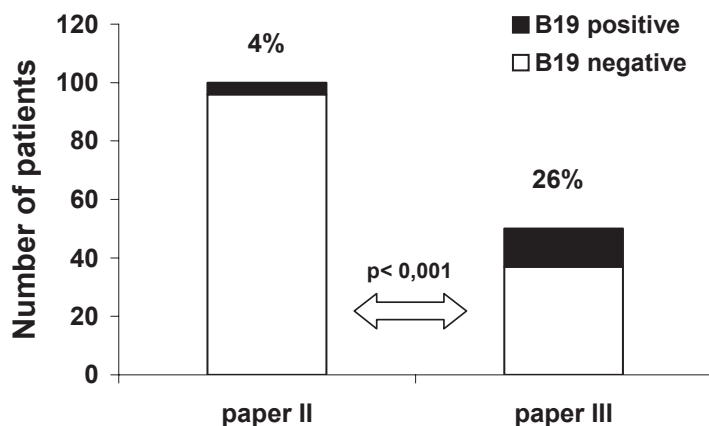

Figure 6.
The figure illustrates the difference between the results in Paper II including patients with haematological diseases in which four of 100 patients (4%) were B19 DNA positive and in Paper III in which B19 DNA was positive in 13 of 50 patients (26%) with rheumatological diseases. All results refer to B19 DNA in bone marrow analysed by PCR.

Regretfully, we retrospectively are aware that a contemporary study comparing the two study groups had been more powerful when interpreting results. However, the BM samples were collected under similar conditions, and the PCR technique used was mainly identical. In the haematology study, though, two PCR assays were used to detect DNA representing structural (VP) as well as non-structural proteins (NS1), and in the present study just the latter one was applied that might have underestimated the figures. Furthermore, the parameters of gender and age differed, with female dominance and younger patients evaluated in the rheumatology study. In our attempt to look for B19 DNA in patients with various diagnoses, broad inclusion criteria were used within the rheumatology spectrum, and the heterogeneous population was a disadvantage, making statistical analysis doubtful. However, of 22 patients with RA enrolled in the study, 19 were rheumatoid factor-positive, and 20 had erosive arthritis in X-ray. B19 DNA in BM was positive in seven of 22 RA patients and interestingly, in spite of the small population, the frequency of rheumatoid factor differed significantly between B19-positive and -negative patients, as shown in Table 3. A difference was also noted in that erosive arthritis was less common in the subset of RA patients with B19 DNA in BM. The difference was not significant, but of interest, since it is known that patients with established B19 arthropathy sometimes fulfil the criteria for RA but do not develop joint destruction. Neither were other differences significant but cannot be excluded since the number of patients was small. A larger study investigating B19 DNA in BM relative to erosive arthritis and rheumatoid factor in RA patients would be worthwhile.

Clearly, laboratory tests that detect B19 DNA are insufficient to prove viral etiology in diseases of different kind, since B19 DNA is also found in healthy controls. In general, the principle difference in diagnosing infections is dividing microbes into those that colonize or persist in healthy individuals (E. coli, Candida, herpesvirus) and those that are more obligatorily associated with disease (Legionella, Pneumocystis jiroveci, HIV). In the latter group, detection of the microbe is usually enough for diagnosis, but in the former, one must implement indirect methods like IgM serology, pathological preparations and, most important, clinical picture. Previously, B19 was classified as an abortive virus that causes an acute infection and is subsequently eliminated from the body by the immune defence. Accordingly, detection of the virus would be diagnostic. However, several studies have now demonstrated that B19 can persist irrespective of clinical symptoms (Söderlund et al. 1997). Consequently, other diagnostic tools are required for establishing a viral etiology such as immuno-histochemistry and quantitative PCR. The origin of the sample, in which virus is detected, is probably of importance when assessing B19 DNA presence and the relevance has to be established organ by organ. With the methodology available, we concluded that in a large proportion of rheumatic patients BM samples were B19 DNA-positive yet that these B19-positive RA-patients tested positive for RA-factor less often than B19-negative individuals. Only with larger studies will such results yield a statistically valid interpretation.

Table 3.
Characteristics of a subgroup of patients presented in Paper III, the 22 RA patients, and related to the presence of B19 DNA in bone marrow.

Parameters in 22 RA patients	B19 DNA pos n=7		B19 DNA neg n=15		p-value
	n	%	n	%	
Erosive arthritis in X-ray	5	71	15	100	ns (0,09)
Rheumatoid factor	4	57	15	100	0,023
Anti-nuclear antibodies	2	29	2	13	ns
Steroids	5	71	8	53	ns
Cytostatics	3	43	9	60	ns

Fisher's exact test was used and demonstrated that presence of rheumatoid factor was more common in RA patients without B19 DNA in BM than in patients with positive B19 DNA samples. The differences in other parameters were not significant.

RA   = rheumatoid arthritis
ns   = not significant

4.4 PAPER IV: CYTOKINE RESPONSES IN ACUTE AND PERSISTENT HUMAN PARVOVIRUS B19 INFECTION

This paper is divided into two parts. The first records the cytokine response after primary B19 infection in eight acutely infected individuals over a period of 2.5 years. The second describes 22 individuals with signs of persistent B19 infection studied for immunological parameters. This latter part is particularly important in the context of the present thesis, which questions whether persistent B19 infection can be referred to as a general immune deficiency or a selective impairment in immunological competence to eliminate B19. We also included 18 healthy seropositive individuals as controls for comparison of cytokine responses.

Dominance of a Th1 cytokine response in acute B19 infection

The cytokine profile associated with acute B19 infection was studied in eight female patients, followed for about 20-130 weeks. Diagnosis was determined by typical clinical symptoms associated with positive B19 IgM serology and detection of B19 DNA in serum. In only two of the eight patients, pro-inflammatory cytokines (IL1ß, GM-CSF, IL6, TNFα, and chemokine IL8 were elevated within the first weeks of acute infection. Thereafter, the concentrations decreased to low or undetectable levels. With respect to immunologic Th1 and Th2 responses, all eight patients had elevated levels of one or more Th1-related cytokines (IL2, IL12 and IL15) but no rise of IFNγ (except in one patient at weeks 1-3). This is surprising and notable, since IFNγ is usually associated with the Th1 response. The possibility that a technical artefact caused our results was considered but is not probable, since we demonstrated elevated levels of IFNγ in patients with persistent infection. The Th2 response, defined as detection of the cytokines IL4, IL5 and IL10, was low during the entire study period. Th2 cytokines are regarded as important in the immune system for development of immunoglobulin production; however, the levels of B19 IgG were normal. Evidently, then, the production of IgG antibodies may be induced by other mechanisms not studied here. Results from previous studies of the cytokine profile in acute B19 infection contrast with ours. Kerr investigated 84 patients with acute B19 infection and found a mixed Th1/Th2 response with elevated levels of IFNγ, TNFα and IL4 (Kerr et al. 2004). In that study, however, sampling was done only once and at different time points after infection than we used, perhaps accounting for the discrepancy. Production of IFNγ is crucial for the cellular immune response, and we previously noted that B19 antigen evoked IFNγ responses in CD8 cells of five acutely B19 infected patients (Norbeck et al. 2005). The present study shows elevated levels of IL12 and IL15, both known to be important in cellular immunity. IL12 stimulates IFNγ production by CD8 cells and Th1 cells and IL15 differentiation of CD8 cells into mature effector and memory cells (Kidd 2003; Yajima et al. 2006).

Since we had demonstrated persistent infection of parvovirus B19 DNA in numerous patients on more than one occasion during a period of several years, we were interested in investigating different immunological parameters for general aberrations that might be responsible. Initially 25 persistently infected individuals were included in the study. They were deemed chronically infected with parvovirus B19 on the basis of B19 DNA detected in BM and with long-lasting symptoms such as chronic fatigue, arthralgia and myalgia. Some were recruited after being diagnosed in routine clinical practice at the Clinic of Infectious Diseases, Södra Älvsborg Hopital, Borås, Sweden (n=16), and others from the study presented in Paper III (n=9). These were included consecutively, regardless of diagnosis or other parameters until the number reached 25. Later, we altered the criteria to be more restrictive, and two B19 positive BM samples taken at least six months apart were required for our own definition of persistent infection. In this way three patients were excluded and 22 remained. No general criteria exist for a precise definition of chronic B19 infection, and the natural course of B19 presence in BM following acute infection is unknown. However, B19 DNA in BM is not a general in seropositive individuals according to other studies (Paper II; Heegaard et al. 2002). By revising our criteria to demand two positive samples taken at an interval of more than six months, the presence and persistence of B19 virus were confirmed, and the risk of accidental occurrence could be minimised. Characteristics of included patients are briefly shown in Table 4.

Recent infection might account for viral presence in BM, and as much as 25-68% of B19 infections are reported to be asymptomatic (Heegaard et al. 2002; Noyola et al. 2004). In the present study six patients were IgM-positive indicating recent infection. In four of them the probable point of disease onset could be approximated and associated with symptoms consistent with B19 infection. The other two patients had no recent indicators as to the start of disease; furthermore, they were IgM-positive more than once in samples taken at intervals of several years. Usually B19 IgM serology is negative within a couple of months after primary infection (Anderson et al. 1985). Persistent IgM may illustrate chronic infection but sometimes represents a false positive reaction, which is fairly common in IgM assays in general, and particularly in patients with rheumatologic diseases (Johnson et al. 2004). One of our patients who was positive for IgM in 1999 as well as in 2003 suffered from SLE; still, she was B19 DNA-positive in BM on both occasions and viral relevance must be considered. In several reports, SLE has been associated with B19 infection (Trapani et al. 1999; Hsu et al. 2001), but contradictory results have also been published (Bengtsson et al. 2000). Nevertheless, B19 infection and SLE share clinical features, and in individual cases, the connection may be real. This patient with B19 infection and SLE was recruited from the study investigating B19 DNA in BM from rheumatology patients (Paper III) and illustrates a weakness of the study presented in Paper IV. The population of the study was heterogeneous, and the patients enrolled had different origins, clinical practise and a clinical study, respectively. A larger study with homogenous groups of patients positive for B19 DNA in BM compared with matched patients with negative B19 DNA would have been desirable but was at this time not possible to perform.

Table 4.
Clinical and laboratory findings in 22 subjects with persistent B19 infection presented in paper IV.

Study subject	Sex	Age	From R[a]	Diagnosis[b]	Fatigue[c]	CFS[d]	Arthralgia	Myalgia	IgM	PCR[e]	PCR time[f]	IgG-PCR time[g]
1	F	61	Y	RA	N	N	Y	N	N	2	12	12
2	F	45	N		N	N	Y	Y	N	5	47	48
3	F	43	N	Sjögren	Y	Y	Y	Y	N	6	67	77
4	F	26	N		Y	Y	Y	Y	N	4	24	25
5	F	59	N		Y	Y	Y	Y	Y	2	21	46
6	F	36	N		Y	Y	Y	Y	Y	7	110	115
7	M	46	N		Y	Y	N	Y	Y	3	53	56
8	M	45	N		Y	Y	Y	Y	N	3	21	27
9	F	40	N		Y	Y	N	N	N	2	18	20
10	F	40	N		Y	Y	Y	Y	N	2	6	21
11	F	38	N		Y	Y	Y	Y	N	2	36	37
12	F	41	N		Y	Y	Y	Y	N	2	60	121
13	F	62	N		Y	N	Y	Y	N	2	23	23
14	F	31	N		Y	Y	Y	Y	N	2	22	46
15	M	42	N		Y	Y	Y	Y	N	2	12	20
16	M	57	N		Y	Y	Y	Y	N	2	27	29
17	F	39	Y		Y	Y	Y	Y	N	2	41	41
18	F	37	Y	RA	N	N	Y	Y	N	2	16	16
19	F	60	Y	RA	Y	N	Y	Y	N	2	44	44
20	F	60	Y	RA	N	Y	Y	Y	N	2	17	17
21	F	43	Y		Y	Y	Y	Y	N	2	14	41
22	F	39	Y	SLE	Y	N	Y	Y	Y	2	42	42

a) Rheumatology patient. B19 positive in bone marrow in a previously performed study (paper III).
b) Subjectively experienced unnatural fatigue.
c) Definite rheumatic diagnosis.
d) Fulfilling criteria for CFS (Chronic Fatigue Syndrome) (Fukuda, Straus et al. 1994).
e) Number of B19 positive bone marrow samples.
f) Number of months from first to latest B19 PCR positive bone marrow sample.
g) Number of months from first positive B19 IgG in serum to latest positive B19 PCR in BM.

MHC class I molecules (HLA A, B and C) are presented on the surface of nearly all cells in the human body, whereas MHC class II (HLA DP, DQ, DR) are seen on lymphocytes and dendritic cells, the latter presenting antigens to lymphocytes following phagocytosis. Briefly, MHC class II, associated with antigens, incite immunological activity in lymphocytes whereas MHC class I, likewise associated with antigens, presented on infected cells designate them as targets for components of the activated immune system. Viral antigens are presented on the surface of cells they infect by binding to a groove in the MHC molecule, and the binding strength may vary for different HLA type. Furthermore, some antigens are HLA-restricted and presented exclusively in individuals with specific HLA types (Tolfvenstam et al. 2001). Consequently individuals who are homozygous in HLA type may provoke a more narrow antiviral immune reaction than seen in those who are HLA heterozygotic, since the infected cells in the latter group may present more antigen epitopes.. In conclusion, the MHC system is of great importance in immune defence, and the HLA type might be related to our questions concerning B19 persistence in a subset of individuals. Previously, Kerr described a relationship between symptomatic B19 infection and HLA DR4 (Kerr et al. 2002), which is of interest since that allele is associated with RA.

Lacking access to matched controls, we compared the distribution of HLA class I antigens in our patients to that in previously unpublished data from the Tobias registry, which contained information for 40928 Swedish BM donors. The great number of possible results in each variable makes a large control group of great value but BM donors represent a selective part of the general population. Even so, HLA A9 was shown to be more common in the 22 individuals, persistently infected with B19, than in the 40928 controls (36% and 18% respectively, p=0.047, Fisher's exact test), an association that not has been described before. No differences were shown for HLA B. Results concerning HLA DR did not show any significant differences when compared with a previous study including a healthy Swedish control group (Berlin et al. 1997).

Immune phenotyping of lymphocytes revealed deviating results according to reference values in some patients, but no uniform aberration in lymphocyte count was found to explain viral persistence (Table 5). Six patients had subnormal levels of B-cells (CD19) but their levels of immunoglobulin were normal. The finding of subnormal lymphocyte levels in some patients was not obviously related to viral persistence, considering inhomogeneous results in different patients and that most patients did not have any aberration. Alternatively, the pathogenetic pathway of persistence may differ among individuals. However, our more functional assay, measuring the production of IFNγ after antigenic stimulation by concanavalin A, failed to demonstrate any functional deficiency in T-cell response in these 22 persistently B19 infected individuals.

An explanation for viral persistence could be an imbalance of Th1/Th2 cytokines as proposed by Spanakis concerning hepatitis C (Spanakis et al. 2002). Our measurements of cytokine levels, though, did not reveal any definitive difference when samples from the B19-infected patients were compared to those from healthy seropositive individuals. The exception was IFNγ-level, which was higher in B19 infected subjects than in the controls, but this outcome could be an expression of disease rather than B19 infection *per se*.

Although we have not demonstrated any uniform deficiency in immunological function in patients with persistent B19 DNA in BM, on the other hand, our results have not excluded that possibility. Our methods to measure and describe the complicated system of defence against infectious diseases are limited, and sometimes we are studying the map rather than reality. Nevertheless, one of the major issues of my thesis is the question whether viral persistence is related to B19-specific immunodeficiency or to a general immunodeficiency of the host, and the former option is still without counter-evidence. Further support for this former theory is also described in Paper V.

Table 5.
Absolute size of lymphocyte subpopulations (10^9/l) in the 22 patients presented in Paper IV.

CD	Lymphocyte	Mean	Median	Reference
CD3	T	1,215	1,350	0,7-2,1
CD3 and CD4	Th	0,807	0,820	0,3-1,4
CD3 and CD8	Tc	0,443	0,435	0,2-0,9
CD16 and CD56	NK	0,215	0,205	0,09-0,6
CD19	B	0,168	0,135	0,1-0,5

CD   = Cluster of differention
NK   = Natural killer cells
Th   = T helper cells
Tc   = T cytotoxic cells
B   = B cells

Table 6.
ELISpot results, IFNγ production in SFC per million PBMC with and without concanavalin A stimulation in patients presented in paper IV.

	Study patients n=22		Controls n=19		Significance
	Mean	Median	Mean	Median	
Baseline	5,6	0,5	1,4	0	ns
Con A	4082	2150	4917	1500	ns

Con A   = concanavalin A
ns   = not significant according to Mann-Whitney

4.5 PAPER V: ABERRANT CELLULAR IMMUNE RESPONSES IN HUMANS INFECTED PERSISTENTLY WITH PARVOVIRUS B19

In our previous studies, the persistence of B19 DNA in BM was demonstrated in patients with chronic symptoms. We also concluded that B19 DNA in BM is not a general finding in seropositive individuals. Additionally, patients with viral persistence tested immunologically have not had uniform general deficiencies in immune function. Yet, irrespective of its relevance for clinical manifestations, the immunological background of persistence is of interest. Broader implications are also possible; for example, after hepatitis B infection in human adults, about 5% develop a persistent disease, possibly related to low levels of cytotoxic T-lymphocytes directed to hepatitis B antigen, indicating a selective immune deficiency (Nayersina et al. 1993). A similar pathogenetic mechanism of B19 infection might be responsible for viral persistence and such a mechanism would be well worth investigating.

Therefore, the study presented in Paper V was initiated and included nine subjects persistently infected with B19. The criteria for persistent infection were defined as at least two B19 DNA-positive BM samples identified at least six months apart. No other selection criteria were used when choosing among subjects in the larger group of patients with B19 persistence described in Paper IV. Fourteen healthy individuals were also enrolled in the study as a control group. They were B19 IgG-positive but IgM- and PCR-negative in serum. Achieving BM samples from these individuals to analyse by PCR would have been of great importance but wasn't possible for ethical reasons. Additionally three individuals who were B19 IgG-seronegative were used as controls for assay specificity.

The ELISpot technique was used to analyze T-cell responses *ex vivo* to stimulation by several antigens of B19 virus. First, 210 peptides consisting of sequences with 15-20 amino acids were synthesized. The peptides overlapped by 10 amino acids and covered about 92% of the entire translated proteins of the B19 genome such as NS1, VP1 and VP2. Peptides were then mixed in diverse pools, each pool covering a sequence of about 60-65 amino acids. PBMC were separated from blood by Ficoll-Paque and assayed within 8 hours after venesection. A more extended delay would have the cells' functionality. Triplicates of 2×10^5 PBMCs from each individual were incubated with different pools of peptides, and T-cell responses were measured as IFNγ production expressed by the number of spot-forming cells (SFC) per million PBMC counted in a stereomicroscope. At the same time, SFC in wells with non-stimulated cells was counted, and the values subtracted from results of cells exposed to antigen to eliminate non-specific IFNγ production from the final results.

The baseline for a positive response was defined by values exceeding two standard deviations given by values of SFC in all wells of a 96-well plate (Millipore) in an experiment with samples from one patient. Negative controls were excluded as well as the three highest and three lowest to rule out outliers. The cut-off line for a positive response was defined by values exceeding baseline with 20 SFC. This value was chosen on the basis of previous results in healthy individuals and acutely infected

patients and was coherent to reproducible results in new experiments and when pools of peptides were spliced in separate wells for each peptide. The choice of a model with an accurately defined cut-off level was crucial in this study. An alternative choice would have been responses in negative controls, but after careful evaluation of relevant previous studies the present model was chosen.

The results revealed T-cell responses in 24 of 196 NS1 pools from healthy seropositive individuals (14 pools x 14 subjects = 196) representing 12 of 14 pools and 8 of 14 individuals who reacted to 1-4 pools each. Comparably, the same subjects were positive in 5 of 126 VP pools (9 pools x 14 subjects = 126) representing responses to 4 of 9 pools in three individuals reacting to 1-2 pools each. When the relative numbers of pools with positive responses were compared, a difference was registered, showing a more common reaction to NS peptides than to VP peptides in healthy seropositive individuals. The opposite was found when T-cell responses were analysed in persistently infected individuals. Positive results were obtained in 9 of 126 pools (14 pools x 9 subjects = 126) with NS peptides and in 14 of 81 pools with VP peptides (9 pools x 9 subjects = 81). Consequently, the T-cell response was relatively more common against VP peptides than NS peptides in these individuals with persistent B19 infection. When comparing healthy seropositive individuals with persistently infected patients, a significantly higher relative number of T-cell responses against VP peptides, but not against NS1 peptides, were shown in the persistently infected individuals. However, the mean level of specific IFNγ responses to NS1 peptides, but not to VP peptides, was significantly higher in healthy seropositive individuals.

Therefore, the T-cell response in persistently B19 infected individuals seems to be skewed towards VP antigens compared to that in seropositive healthy individuals. When mean levels of B19 IgG were studied, a significantly higher value (97 IU/ ml, $p = 0.011$) was achieved in the nine B19 infected individuals than in the 14 healthy seropositive controls (61 IU/ ml). One interpretation of these results could be that an NS1 response is crucial for viral clearance, and when it fails, the virus continuously stimulates and enhances the humoral VP response. In individuals whose HLA types differ from one another, different antigens may be exposed and yield variable immune responses, but no such variation between the groups was noted that would indicate HLA related non-responsiveness as an explanation for the results. Furthermore, all study subjects responded to challenge with the control substance, phytohemagglutinin, excluding general intrinsic IFNγ deficiency. An alternative explanation to the T-cell responses obtained could be that some unknown mechanism of persistence causes secondary exhaustion of the T-cell response to NS1 protein. That interpretation is contradictory to the point that responses to VP peptides remained unaffected. Obviously, the mechanisms of viral clearance or persistence are far more complicated than we have been able to investigate in this study. For example, six of 14 healthy seropositive individuals did not respond to NS peptides at all, and 11 of 14 did not react to any of the VP peptide pools. Explanations for this result might be deficient viability of the PBMC:s used here or another technical difficulty. Viral persistence in BM of the 14 healthy seropositive individuals could not be excluded, since samples from their BM were not available, but that explanation is less probable, since our previous studies have shown low frequencies of B19 DNA in BM from a of haematology patients (Paper 2) and healthy BM donors (Heegaard et al. 2002).

5 CONCLUSIONS AND FUTURE POSSIBILITIES

We have studied persistent infection of parvovirus B19 in human BM and tried to determine (1) if it is related to the normal course of B19 infection, (2) if it is of clinical significance and (3) if there is immunological explanations for persistence. Since arthralgia and arthritis are well-established manifestations of B19 infection, our noteworthy discovery is the significantly higher frequency of B19 DNA in patients with rheumatologic diseases than among patients with haematological diseases. Additionally, for several years, we have followed patients with persistent B19 infection in BM and described their symptomatology. In some of them, according to clinical parameters, B19 infection has been obvious; in others, the simple coexistence of this infection and chronic disease has challenged us to confirm or reject the hypothesis of an etiological connection between them.

We also have tried to explain why virus persists in some individuals and not in others, which could be of interest regardless of clinical consequences. We have not been able to refute our hypothesis that chronic B19 infection may be related to a specific immunologic reaction related to B19 rather than general immune deficiency. The variability in clinical manifestations of parvovirus B19 infection is well established and, probably, the individual pattern of immune reaction is the cause of diversity in presentation of related diseases and disposition of chronic infection. With these ideas in mind and with the knowledge of the high seroprevalence of B19 virus in the population, undoubtedly small proportions of chronic infection and aberrant clinical presentation may lead to large total numbers of affected individuals. We don't know if findings of B19 DNA in BM are seldom, sometimes or always related to clinical symptoms. To advance in these efforts, we need better clinical methods and further studies. Initially, we have used a qualitative PCR technique, but the development of quantitative methods will make it possible to investigate if clinical significance is related to viral load. Ongoing studies are also needed to assess the proportion of persistent B19 infection in larger samples from patients with various diseases. Traditionally, the presence of virus has been investigated to diagnose the cause of dysfunction in affected organs. However, if the immune reaction to an infection underlies the pathogenesis of such diseases, the causative virus may be situated elsewhere, e.g., in the BM or lymphatic system, and from that location stimulate autoimmune manifestations. Perhaps, therefore, it might be more relevant to detect B19 DNA in BM of arthritic patients than to analyze their synovial fluid.

Since B19 also may persist in healthy individuals we need indirect methods other than DNA-detection to prove a relationship between virus and disease. Classically, IgM serology has been used to diagnose disease, but in our patients, B19 specific IgM usually was negative. Immunohistochemistry may be used to demonstrate B19 affected cells in specific organs and, hopefully, this method can be further developed. Ultimately, the immune system is the never-ending story that is decisive for our understanding of infectious diseases. Added to its importance in defence against and modulation of clinical manifestations, expanded knowledge of immune reactions in infectious diseases, including B19, may help us to get closer to accurate diagnosis and treatment as well as to establish if a positive PCR is truly relevant.

6 PARVOVIRUS B19 – INTE BARA FEMTE SJUKAN

Parvovirus B19 (B19) är ett av våra vanligare virus och mer än 80 % av befolkningen smittas någon gång i livet, vanligen i skolåldern. Den typiska symtombilden är barnsjukdomen "femte sjukan" som kännetecknas av hudutslag i ansikte och efterhand på armar och ben. Hudutslagen föregås ofta av lättare feber och initiala luftvägssymtom även om många som smittas förblir symtomfria. I vuxen ålder kan symtomen te sig annorlunda och framförallt hos kvinnor är ledbesvär vanligt. Ledbesvären kan ibland bli långvariga och ge misstanke om reumatisk sjukdom varför det kan vara värdefullt att ställa diagnos. Misstanke om reumatism är ju oroande för patienten men vid B19-infektion är ledbesvären oftast övergående. Hos individer med nedsatt immunförsvar, t ex HIV-patienter eller transplanterade kan infektionen bli mera allvarlig och orsaka exempelvis svår blodbrist eller hjärtmuskelinflammation. Om man smittas för första gången när man är gravid finns en ökad risk för allvarlig påverkan på fostret och missfall, även sent i graviditeten.

I början av nittiotalet hade jag en patient som utreddes pga flera månaders besvär av värk, trötthet och feber. Blodproverna visade på en störd funktion i benmärgen med lågt antal vita blodkroppar och blodbrist. I samband med benmärgsprovtagning skickades ett prov för DNA-analys gällande B19 och provet visade sig vara positivt. Vi ställdes inför frågan om fyndet var uttryck för sjukdom eller enbart förekomst av virus-DNA utan praktisk betydelse. Vi vet sedan tidigare att t ex herpesvirus av olika slag härbärgeras livslångt i kroppen efter smitta och att de ibland ger förnyad infektion på olika sätt. Exempelvis kan man få bältros när man blir äldre av det vattenkoppsvirus man smittades av som barn. Andra virus t ex influensa ger en akut infektion men viruset elimineras sedan från kroppen av immunförsvaret. När det gäller B19 har vi tidigare haft bristfällig kännedom om virusets naturliga förlopp hos människa och fyndet av B19 hos denna första patient blev början till min forskning.
Initialt började vi använda DNA-diagnostik av benmärg som en del i utredningen av patienter med kroniska symtom med oklar men misstänkt infektiös orsak. Symtomen innefattade ofta långvarig trötthet, oklar feber och värk. I avhandlingens första arbete beskrivs symtom och laboratoriefynd hos fem patienter som hade DNA från B19 i benmärgsprov.

För att värdera hur vanligt det var med förekomst av B19 i benmärg i allmänhet undersökte vi benmärgsprover från 100 patienter på en avdelning för blodsjukdomar. Eftersom benmärgsprovtagning kan vara smärtsamt passade vi på att ta prov för analys på patienter som ändå skulle genomgå denna undersökning av medicinska skäl. Av 100 undersöka patienter hade fyra av dem DNA från B19 i benmärgen som tecken på kvarvarande infektion medan 58 av 98 undersökta (59%) hade antikroppar som visade att de någon gång smittats av viruset. Vi kunde då dra slutsatsen att kvarvarande DNA från viruset inte är allmänt förekommande hos patienter som en gång i livet har smittats och att det ter sig som att en relativt begränsad andel av befolkningen blir kroniskt infekterad.

Som nämnts är ledbesvär ett vanligt fenomen om man smittas i vuxen ålder och i enstaka fall kan sjukdomsbilden likna ledgångsreumatism. När det gäller reumatologiska sjukdomar i allmänhet har det funnits teorier om att olika infektioner skulle kunna vara utlösande faktor för den överaktivitet i immunförsvaret som ledsjukdomar kan vara uttryck för. Det var därför av intresse att undersöka förekomsten av B19 hos denna patientgrupp. Vi analyserade benmärgsprover från 50 patienter med olika reumatiska sjukdomar och kunde konstatera att förekomsten av DNA från B19 var betydligt större än hos patienterna med blodsjukdomar. Av 50 patienter hade 13 B19-DNA i benmärgen (26 %), att jämföra med 4% hos patienterna med blodsjukdom. Skillnaden är av intresse antyder ett samband mellan B19 och reumatologisk sjukdom. Orsakssamband är däremot svårt att fastställa men resultaten motiverar fortsatta studier.

Eftersom alla som smittas uppenbarligen inte blir kroniska bärare av viruset har vi också intresserat oss för det immunologiska reaktionsmönstret hos patienter med kronisk virusförekomst. Vi har kunnat konstatera att patienter med kroniskt bärarskap av B19 vanligen inte har någon allmän nedsättning av immunförsvarets funktion vilket diskuteras i arbete IV. Vår hypotes är i stället att de generellt sett har ett normalt immunförsvar som dock har en liten lucka när det gäller att försvara sig mot B19. Den kroniska överlevnaden av viruset kan sedan leda till en kronisk stimulering av immunförsvaret vilket skulle kunna vara orsaken till långvariga symtom. En viktig faktor som påverkar symtomen vid alla infektioner är immunförsvarets egen aktivitet. Vi har också kunnat se att immunförsvarets specifika reaktion mot B19 skiljer sig något från den hos friska vilket stöder vår hypotes. Bakgrunden till den individuella variationen i reaktionsmönstret mot B19 skulle kunna ligga på ett genetiskt plan och vara relaterat till ärftlig benägenhet vilket återstår att undersöka.

Fortsättningsvis hoppas vi att tydligare kunna värdera om förekomst av virus har betydelse för olika sjukdomar eller inte och i så fall undersöka nyttan av behandling. Sedan tidigare vet vi att patienter med nedsatt immunförsvar och svår infektion med B19 kan behandlas med gammaglobulin och i framtiden kommer sannolikt också ett vaccin bli tillgängligt.

Sammanfattningsvis är B19 ett av våra vanligaste virus som också uppvisar en stor variation i hur det yttrar sig hos olika personer. Att en del individer tycks vara benägna till kronisk infektion är av intresse eftersom kronisk virusförekomst skulle kunna vara orsaken till kronisk immunstimulering och därmed också kroniska symtom.

7 ACKNOWLEDGEMENTS

This chapter cannot be large enough. A lot of persons have contributed to the production of the present thesis, and I gratefully want to thank all of you.

First, Kristina Broliden, my supervisor, has to be praised. Her positive and supportive attitude has been the difference between giving up and keeping on. Initially there was no intention from my point of view to graduate with a PhD, but Kristina was convinced and suddenly I found myself on the train and couldn't stop it. She has all through the journey been very encouraging and never been negative even if there have been reasons. The time schedule has not been followed especially tightly, but Kristina has respected my lack of time. However, her talent to put pressure in a positive, effective and gentle way is impressive. I am proud to be a part of her group.

Thank you to my other supervisors, Thomas Tolfvenstam and Adiba Isa, for being helpful and interested in explaining laboratory mysteries for my clinical brain. Also, as well as my other collaborators in the group, Oscar Norbeck, and Taha Hirbod, they should be admired for your willingness to take the very early morning plane to Borås, eager to collect samples for fast transport to the laboratory in Stockholm. I know that some of these days ended up in the laboratory early the next morning. A lot of other skillful scientists in Sweden and abroad have been included in our work and I want to give my thanks to Jennifer Bostic, Maria Söderlund, Mia Brytting, Klaus Hedman, Anna Lindblom, Vicky Kasprowicz, Paul Bowness, Paul Klenerman, Ulla Eriksson, Jan Konar, Bengt Andersson, and associated laboratory personnel.

I also want to express my gratitude to the staff at Södra Älvsborg Hospital in Borås for a well-balanced mix of support and stimulating skepticism.

- Present and former colleagues at Clinic of Infectious Diseases. Thank you for taking care of the patients during my scientific holidays.
- Nurses and other personnel in the same clinic, always helpful and positive when I have needed help and assistance in collecting samples from blood or bone marrow.
- Carl Magnus Stolt for teaching me how to puncture crista iliaca, diagnosing B19 infection in a pancytopenic patient 1991 and for cooperation in the study described in Paper II.
- Göran Kvist for cooperation in the study described in Paper III.
- Anna Lindqvist at Photo Media Department, for excellent help with graphic design and figures.

The financial support has of course been necessary and several organizations have been very generous; Fokus Borås Foundation, Alice Swenson Foundation for Clinical Research, Research and Development Council in Södra Älvsborg, Research and Development at Södra Älvsborg Hospital, the Tobias Foundation, the Swedish Children's Cancer Foundation, the Swedish Cancer Foundation, the Swedish Research Council, the Wellcome Trust, the Medical Research Council, UK, and the Commission of the European Communities.

Finally, I want to express my deepest gratitude to the basic support club of my life:

- My parents, Bengt and Barbro. You have always been behind all my decisions in life. I know my roots and I'm proud of them.
- My sisters Ingela, Eva and Sara with families for always being there. Thank you Eva and Lasse for your hospitality during my stays in Stockholm.
- My children Malin, Petter, Karin and Elin for making me happy.
- Inger, I love You.

8 REFERENCES

Abkowitz, J. L., K. E. Brown, R. W. Wood, N. L. Kovach, S. W. Green and N. S. Young (1997). "Clinical relevance of parvovirus B19 as a cause of anemia in patients with human immunodeficiency virus infection." J Infect Dis **176**(1): 269-73.

Ager, E. A., T. D. Chin and J. D. Poland (1966). "Epidemic erythema infectiosum." N Engl J Med **275**(24): 1326-31.

Aguilar-Bernier, M., J. Bassas-Vila, J. I. Torne-Gutierrez, G. Martinez-Garcia, H. Aragoneses-Fraile and A. Miranda-Romero (2006). "Presence of perineuritis in a case of papular purpuric gloves and socks syndrome associated with mononeuritis multiplex attributable to B19 parvovirus." J Am Acad Dermatol **54**(5): 896-9.

Anderson, M. J., P. G. Higgins, L. R. Davis, J. S. Willman, S. E. Jones, I. M. Kidd, J. R. Pattison and D. A. Tyrrell (1985). "Experimental parvoviral infection in humans." J Infect Dis **152**(2): 257-65.

Anderson, M. J., E. Lewis, I. M. Kidd, S. M. Hall and B. J. Cohen (1984). "An outbreak of erythema infectiosum associated with human parvovirus infection." J Hyg (Lond) **93**(1): 85-93.

Astell, C. R., L. Weixing, J. Brunstein and A. J. St (1997). Human Parvovirus B19. Basel, Karger.

Ballou, W. R., J. L. Reed, W. Noble, N. S. Young and S. Koenig (2003). "Safety and immunogenicity of a recombinant parvovirus B19 vaccine formulated with MF59C.1." J Infect Dis **187**(4): 675-8.

Barah, F., P. J. Vallely, G. M. Cleator and J. R. Kerr (2003). "Neurological manifestations of human parvovirus B19 infection." Rev Med Virol **13**(3): 185-99.

Barker, E., S. F. Fujimura, M. B. Fadem, A. L. Landay and J. A. Levy (1994). "Immunologic abnormalities associated with chronic fatigue syndrome." Clin Infect Dis **18 Suppl 1**: S136-41.

Bengtsson, A., A. Widell, S. Elmstahl and G. Sturfelt (2000). "No serological indications that systemic lupus erythematosus is linked with exposure to human parvovirus B19." Ann Rheum Dis **59**(1): 64-6.

Berg, A. M., S. J. Naides and R. W. Simms (1993). "Established fibromyalgia syndrome and parvovirus B19 infection." J Rheumatol **20**(11): 1941-3.

Berlin, M., A. Fogdell-Hahn, O. Olerup, A. Eklund and J. Grunewald (1997). "HLA-DR predicts the prognosis in Scandinavian patients with pulmonary sarcoidosis." Am J Respir Crit Care Med **156**(5): 1601-5.

Bluth, M. H., K. B. Norowitz, S. Chice, V. N. Shah, M. Nowakowski, A. S. Josephson, H. G. Durkin and T. A. Smith-Norowitz (2003). "Detection of IgE anti-parvovirus B19 and increased CD23+ B cells in parvovirus B19 infection: relation to Th2 cytokines." Clin Immunol **108**(2): 152-8.

Broliden, K. (2001). "Parvovirus B19 infection in pediatric solid-organ and bone marrow transplantation." Pediatr Transplant **5**(5): 320-30.

Broliden, K., T. Tolfvenstam, S. Ohlsson and J. I. Henter (1998). "Persistent B19 parvovirus infection in pediatric malignancies." Med Pediatr Oncol **31**(2): 66-72.

Brown and Young (1997). "The Simian Parvoviruses." Rev Med Virol **7**(4): 211-218.

Brown, K. E., S. M. Anderson and N. S. Young (1993). "Erythrocyte P antigen: cellular receptor for B19 parvovirus." Science **262**(5130): 114-7.

Brown, K. E. and N. S. Young (1996). "Parvoviruses and bone marrow failure." Stem Cells **14**(2): 151-63.

Brown, K. E., N. S. Young and J. M. Liu (1994). "Molecular, cellular and clinical aspects of parvovirus B19 infection." Crit Rev Oncol Hematol **16**(1): 1-31.

Cassinotti, P., G. Burtonboy, M. Fopp and G. Siegl (1997). "Evidence for persistence of human parvovirus B19 DNA in bone marrow." J Med Virol **53**(3): 229-32.

Cassinotti, P., G. Siegl, B. A. Michel and P. Bruhlmann (1998). "Presence and significance of human parvovirus B19 DNA in synovial membranes and bone marrow from patients with arthritis of unknown origin." J Med Virol **56**(3): 199-204.

Chia, J. K. (2005). "The role of enterovirus in chronic fatigue syndrome." J Clin Pathol **58**(11): 1126-32.

Chisari, F. V. and C. Ferrari (1995). "Hepatitis B virus immunopathology." Springer Semin Immunopathol **17**(2-3): 261-81.

Chorba, T., P. Coccia, R. C. Holman, P. Tattersall, L. J. Anderson, J. Sudman, N. S. Young, E. Kurczynski, U. M. Saarinen, R. Moir and et al. (1986). "The role of parvovirus B19 in aplastic crisis and erythema infectiosum (fifth disease)." J Infect Dis **154**(3): 383-93.

Cohen, B. J., S. Beard, W. A. Knowles, J. S. Ellis, D. Joske, J. M. Goldman, P. Hewitt and K. N. Ward (1997). "Chronic anemia due to parvovirus B19 infection in a bone marrow transplant patient after platelet transfusion." Transfusion **37**(9): 947-52.

Cohen, B. J., M. M. Buckley, J. P. Clewley, V. E. Jones, A. H. Puttick and R. K. Jacoby (1986). "Human parvovirus infection in early rheumatoid and inflammatory arthritis." Ann Rheum Dis **45**(102): 832-8.

Corcoran, A. and S. Doyle (2004). "Advances in the biology, diagnosis and host-pathogen interactions of parvovirus B19." J Med Microbiol **53**(Pt 6): 459-75.

Cossart, Y. E., A. M. Field, B. Cant and D. Widdows (1975). "Parvovirus-like particles in human sera." Lancet **1**(78982): 72-3.

De Re, V., S. De Vita, V. Battistella, A. Marzotto, M. Libra, G. Ferraccioli and M. Boiocchi (2002). "Absence of human parvovirus B19 DNA in myoepithelial sialadenitis of primary Sjogren's syndrome." Ann Rheum Dis **61**(9): 855-6.

De Renzo, A., A. Azzi, K. Zakrzewska, L. Cicoira, R. Notaro and B. Rotoli (1994). "Cytopenia caused by parvovirus in an adult ALL patient." Haematologica **79**(3): 259-61.

De Stefano, R., S. Manganelli, E. Frati, E. Selvi, A. Azzi, K. Zakrzewska and R. Marcolongo (2003). "No association between human parvovirus B19 infection and Sjogren's syndrome." Ann Rheum Dis **62**(1): 86-7.

Deiss, V., J. D. Tratschin, M. Weitz and G. Siegl (1990). "Cloning of the human parvovirus B19 genome and structural analysis of its palindromic termini." Virology **175**(1): 247-54.

Dobec, M., A. Juchler, A. Flaviano and F. Kaeppeli (2006). "Prolonged Parvovirus B19 Viremia in Spite of Neutralizing Antibodies after Erythema Infectiosum in Pregnancy." Gynecol Obstet Invest **63**(1): 53-54.

Eichhorn, R. F., E. J. Buurke, P. Blok, M. J. Berends and C. L. Jansen (1999). "Sickle cell-like crisis and bone marrow necrosis associated with parvovirus B19 infection and heterozygosity for haemoglobins S and E." J Intern Med **245**(1): 103-6.

Eis-Hubinger, A. M., U. Reber, T. Abdul-Nour, U. Glatzel, H. Lauschke and U. Putz (2001). "Evidence for persistence of parvovirus B19 DNA in livers of adults." J Med Virol **65**(2): 395-401.

Enders, M., A. Weidner, I. Zoellner, K. Searle and G. Enders (2004). "Fetal morbidity and mortality after acute human parvovirus B19 infection in pregnancy: prospective evaluation of 1018 cases." Prenat Diagn **24**(7): 513-8.

Erdman, D. D., B. C. Anderson, T. J. Torok, T. H. Finkel and L. J. Anderson (1997). "Possible transmission of parvovirus B19 from intravenous immune globulin." J Med Virol **53**(3): 233-6.

Erdman, D. D., E. L. Durigon, Q. Y. Wang and L. J. Anderson (1996). "Genetic diversity of human parvovirus B19: sequence analysis of the VP1/VP2 gene from multiple isolates." J Gen Virol **77 (Pt 11)**: 2767-74.

Erdman, D. D., M. J. Usher, C. Tsou, E. O. Caul, G. W. Gary, S. Kajigaya, N. S. Young and L. J. Anderson (1991). "Human parvovirus B19 specific IgG, IgA, and IgM antibodies and DNA in serum specimens from persons with erythema infectiosum." J Med Virol **35**(2): 110-5.

48

Eriksson, B. M., A. Stromberg and A. Kreuger (1988). "Human parvovirus B19 infection with severe anemia affecting mother and son." Scand J Infect Dis **20**(3): 335-7.

Ferri, C., A. Azzi and C. M. Magro (2005). "Parvovirus B19 and systemic sclerosis." Br J Dermatol **152**(4): 819-20.

Foto, F., K. G. Saag, L. L. Scharosch, E. J. Howard and S. J. Naides (1993). "Parvovirus B19-specific DNA in bone marrow from B19 arthropathy patients: evidence for B19 virus persistence." J Infect Dis **167**(3): 744-8.

Fukuda, K., S. E. Straus, I. Hickie, M. C. Sharpe, J. G. Dobbins and A. Komaroff (1994). "The chronic fatigue syndrome: a comprehensive approach to its definition and study. International Chronic Fatigue Syndrome Study Group." Ann Intern Med **121**(12): 953-9.

Gendi, N. S., K. Gibson and B. P. Wordsworth (1996). "Effect of HLA type and hypocomplementaemia on the expression of parvovirus arthritis: one year follow up of an outbreak." Annals of the Rheumatic Diseases **55**(1): 63-5.

Gillespie, S. M., M. L. Cartter, S. Asch, J. B. Rokos, G. W. Gary, C. J. Tsou, D. B. Hall, L. J. Anderson and E. S. Hurwitz (1990). "Occupational risk of human parvovirus B19 infection for school and day-care personnel during an outbreak of erythema infectiosum." Jama **263**(15): 2061-5.

Golstein, M. A., P. Semaille and S. Steinfeld (1996). "Rheumatic manifestations due to human parvovirus B19. A report of four cases." Rev Rhum Engl Ed **63**(11): 854-8.

Hayakawa, F., K. Imada, M. Towatari and H. Saito (2002). "Life-threatening human parvovirus B19 infection transmitted by intravenous immune globulin." Br J Haematol **118**(4): 1187-9.

Heegaard, E. D. and K. E. Brown (2002). "Human parvovirus B19." Clin Microbiol Rev **15**(3): 485-505.

Heegaard, E. D., B. L. Petersen, C. J. Heilmann and A. Hornsleth (2002). "Prevalence of parvovirus B19 and parvovirus V9 DNA and antibodies in paired bone marrow and serum samples from healthy individuals." J Clin Microbiol **40**(3): 933-6.

Heegaard, E. D., S. Rosthoj, B. L. Petersen, S. Nielsen, F. Karup Pedersen and A. Hornsleth (1999). "Role of parvovirus B19 infection in childhood idiopathic thrombocytopenic purpura." Acta Paediatr **88**(6): 614-7.

Hokynar, K., J. Brunstein, M. Soderlund-Venermo, O. Kiviluoto, E. K. Partio, Y. Konttinen and K. Hedman (2000). "Integrity and full coding sequence of B19 virus DNA persisting in human synovial tissue." J Gen Virol **81**(Pt 4): 1017-25.

Holtzman, M. J., E. Y. Kim, M. S. Lo, J. W. Tyner, L. P. Shornick, K. C. Sumino and Y. Zhang (2005). "Defining and adjusting divergent host responses to viral infection." Immunol Res **32**(1-3): 123-41.

Horton, H., N. Russell, E. Moore, I. Frank, R. Baydo, C. Havenar-Daughton, D. Lee, M. Deers, M. Hudgens, K. Weinhold and M. J. McElrath (2004). "Correlation between interferon- gamma secretion and cytotoxicity, in virus-specific memory T cells." J Infect Dis **190**(9): 1692-6.

Hsu, T. C. and G. J. Tsay (2001). "Human parvovirus B19 infection in patients with systemic lupus erythematosus." Rheumatology (Oxford) **40**(2): 152-7.

Hsu, T. C., W. J. Wu, M. C. Chen and G. J. Tsay (2004). "Human parvovirus B19 non-structural protein (NS1) induces apoptosis through mitochondria cell death pathway in COS-7 cells." Scand J Infect Dis **36**(8): 570-7.

Ilaria, R. L., Jr., A. L. Komaroff, L. R. Fagioli, W. C. Moloney, C. A. True and S. J. Naides (1995). "Absence of parvovirus B19 infection in chronic fatigue syndrome." Arthritis Rheum **38**(5): 638-41.

Isa, A., V. Kasprowicz, O. Norbeck, A. Loughry, K. Jeffery, K. Broliden, P. Klenerman, T. Tolfvenstam and P. Bowness (2005). "Prolonged activation of virus-specific CD8+T cells after acute B19 infection." PLoS Med **2**(12): e343.

Jacobson, S. K., J. S. Daly, G. M. Thorne and K. McIntosh (1997). "Chronic parvovirus B19 infection resulting in chronic fatigue syndrome: case history and review." Clin Infect Dis **24**(6): 1048-51.

Jensen, I. P., P. Thorsen, B. Jeune, B. R. Moller and B. F. Vestergaard (2000). "An epidemic of parvovirus B19 in a population of 3,596 pregnant women: a study of sociodemographic and medical risk factors." Bjog **107**(5): 637-43.

Johnson, A. H. and A. Gough (2004). "False positive parvovirus serology." J Rheumatol **31**(3): 625-6.

Jones, L. P., D. D. Erdman and L. J. Anderson (1999). "Prevalence of antibodies to human parvovirus B19 nonstructural protein in persons with various clinical outcomes following B19 infection." J Infect Dis **180**(2): 500-4.

Kawai, K. and A. Kawai (1992). "Studies on the relationship between chronic fatigue syndrome and Epstein-Barr virus in Japan." Intern Med **31**(3): 313-8.

Kerr, J. R., F. Barah, M. L. Chiswick, G. V. McDonnell, J. Smith, M. D. Chapman, J. B. Bingham, P. Kelleher and M. N. Sheppard (2002). "Evidence for the role of demyelination, HLA-DR alleles, and cytokines in the pathogenesis of parvovirus B19 meningoencephalitis and its sequelae." J Neurol Neurosurg Psychiatry **73**(6): 739-46.

Kerr, J. R., F. Barah, D. L. Mattey, I. Laing, S. J. Hopkins, I. V. Hutchinson and D. A. Tyrrell (2001). "Circulating tumour necrosis factor-alpha and interferon-gamma are detectable during acute and convalescent parvovirus B19 infection and are associated with prolonged and chronic fatigue." J Gen Virol **82**(Pt 12): 3011-9.

Kerr, J. R., J. Bracewell, I. Laing, D. L. Mattey, R. M. Bernstein, I. N. Bruce and D. A. Tyrrell (2002). "Chronic fatigue syndrome and arthralgia following parvovirus B19 infection." J Rheumatol **29**(3): 595-602.

Kerr, J. R., J. P. Cartron, M. D. Curran, J. E. Moore, J. R. Elliott and R. A. Mollan (1995). "A study of the role of parvovirus B19 in rheumatoid arthritis." Br J Rheumatol **34**(92): 809-13.

Kerr, J. R., P. V. Coyle, R. J. DeLeys and C. C. Patterson (1996). "Follow-up study of clinical and immunological findings in patients presenting with acute parvovirus B19 infection." J Med Virol **48**(1): 68-75.

Kerr, J. R. and V. S. Cunniffe (2000). "Antibodies to parvovirus B19 non-structural protein are associated with chronic but not acute arthritis following B19 infection." Rheumatology (Oxford) **39**(8): 903-8.

Kerr, J. R., V. S. Cunniffe, P. Kelleher, R. M. Bernstein and I. N. Bruce (2003). "Successful intravenous immunoglobulin therapy in 3 cases of parvovirus B19-associated chronic fatigue syndrome." Clin Infect Dis **36**(9): e100-6.

Kerr, J. R., V. S. Cunniffe, P. Kelleher, A. J. Coats and D. L. Mattey (2004). "Circulating cytokines and chemokines in acute symptomatic parvovirus B19 infection: negative association between levels of pro-inflammatory cytokines and development of B19-associated arthritis." J Med Virol **74**(1): 147-55.

Kerr, J. R., D. L. Mattey, W. Thomson, K. V. Poulton and W. E. Ollier (2002). "Association of symptomatic acute human parvovirus B19 infection with human leukocyte antigen class I and II alleles." J Infect Dis **186**(4): 447-52.

Kerr, J. R. and D. A. Tyrrell (2003). "Cytokines in parvovirus B19 infection as an aid to understanding chronic fatigue syndrome." Curr Pain Headache Rep **7**(5): 333-41.

Kerr, S., G. O'Keeffe, C. Kilty and S. Doyle (1999). "Undenatured parvovirus B19 antigens are essential for the accurate detection of parvovirus B19 IgG." J Med Virol **57**(2): 179-85.

Kidd, P. (2003). "Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease." Altern Med Rev **8**(3): 223-46.

Koch, W. C. and S. P. Adler (1989). "Human parvovirus B19 infections in women of childbearing age and within families." Pediatr Infect Dis J **8**(2): 83-7.

Kurtzman, G., N. Frickhofen, J. Kimball, D. W. Jenkins, A. W. Nienhuis and N. S. Young (1989). "Pure red-cell aplasia of 10 years' duration due to persistent parvovirus B19 infection and its cure with immunoglobulin therapy." N Engl J Med **321**(8): 519-23.

Kurtzman, G. J., B. Cohen, P. Meyers, A. Amunullah and N. S. Young (1988). "Persistent B19 parvovirus infection as a cause of severe chronic anaemia in children with acute lymphocytic leukaemia." Lancet **2**(8621): 1159-62.

Lachmann, P. J. and A. Davies (1997). "Complement and immunity to viruses." Immunol Rev **159**: 69-77.

Lalvani, A., R. Brookes, S. Hambleton, W. J. Britton, A. V. Hill and A. J. McMichael (1997). "Rapid effector function in CD8+ memory T cells." J Exp Med **186**(6): 859-65.

Lamparter, S., M. Schoppet, S. Pankuweit and B. Maisch (2003). "Acute parvovirus B19 infection associated with myocarditis in an immunocompetent adult." Hum Pathol **34**(7): 725-8.

Larsson, M., X. Jin, B. Ramratnam, G. S. Ogg, J. Engelmayer, M. A. Demoitie, A. J. McMichael, W. I. Cox, R. M. Steinman, D. Nixon and N. Bhardwaj (1999). "A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1-positive individuals." Aids **13**(7): 767-77.

Lehmann, H. W., A. Plentz, P. Von Landenberg, E. Muller-Godeffroy and S. Modrow (2004). "Intravenous immunoglobulin treatment of four patients with juvenile polyarticular arthritis associated with persistent parvovirus B19 infection and antiphospholipid antibodies." Arthritis Res Ther **6**(1): R1-R6.

Leventhal, L. J., S. J. Naides and B. Freundlich (1991). "Fibromyalgia and parvovirus infection." Arthritis Rheum **34**(10): 1319-24.

Lim, L. C. (2005). "Acquired red cell aplasia in association with the use of recombinant erythropoietin in chronic renal failure." Hematology **10**(3): 255-9.

Lindblom, A., A. Isa, O. Norbeck, S. Wolf, B. Johansson, K. Broliden and T. Tolfvenstam (2005). "Slow clearance of human parvovirus B19 viremia following acute infection." Clin Infect Dis **41**(8): 1201-3.

Liu, W., M. Ittmann, J. Liu, R. Schoentag, P. Tierno, M. A. Greco, G. Sidhu, M. Nierodzik and R. Wieczorek (1997). "Human parvovirus B19 in bone marrows from adults with acquired immunodeficiency syndrome: a comparative study using in situ hybridization and immunohistochemistry." Hum Pathol **28**(7): 760-6.

Lowenthal, E. A., A. Wells, P. D. Emanuel, R. Player and J. T. Prchal (1996). "Sickle cell acute chest syndrome associated with parvovirus B19 infection: case series and review." Am J Hematol **51**(3): 207-13.

Lundqvist, A., T. Tolfvenstam, M. Brytting, C. M. Stolt, K. Hedman and K. Broliden (1999). "Prevalence of parvovirus B19 DNA in bone marrow of patients with haematological disorders." Scand J Infect Dis **31**(2): 119-22.

Maquet, D., C. Demoulin and J. M. Crielaard (2006). "Chronic fatigue syndrome: a systematic review." Ann Readapt Med Phys **49**(6): 337-47, 418-27.

Modrow, S. and S. Dorsch (2002). "Antibody responses in parvovirus B19 infected patients." Pathol Biol (Paris) **50**(5): 326-31.

Moffatt, S., N. Yaegashi, K. Tada, N. Tanaka and K. Sugamura (1998). "Human parvovirus B19 nonstructural (NS1) protein induces apoptosis in erythroid lineage cells." J Virol **72**(4): 3018-28.

Moore, T. L., R. Bandlamudi, S. M. Alam and G. Nesher (1999). "Parvovirus infection mimicking systemic lupus erythematosus in a pediatric population." Semin Arthritis Rheum **28**(5): 314-8.

Mori, Y., H. Yamashita, Y. Umeda, Y. Uchiyama-Tanaka, A. Nose, N. Kishimoto, Y. Kijima, T. Nagata, M. Mori, H. Matsubara, H. Yoshida and T. Iwasaka (2002). "Association of parvovirus B19 infection with acute glomerulonephritis in healthy adults: case report and review of the literature." Clin Nephrol **57**(1): 69-73.

Mosley, J. W., D. M. Reisler, D. Brachott, D. Roth and J. Weiser (1968). "Comparison of two lots of immune serum globulin for prophylaxis of infectious hepatitis." Am J Epidemiol **87**(3): 539-50.

Mouthon, L., L. Guillevin and Z. Tellier (2005). "Intravenous immunoglobulins in autoimmune- or parvovirus B19-mediated pure red-cell aplasia." Autoimmun Rev **4**(5): 264-9.

Munakata, Y., T. Saito-Ito, K. Kumura-Ishii, J. Huang, T. Kodera, T. Ishii, Y. Hirabayashi, Y. Koyanagi and T. Sasaki (2005). "Ku80 autoantigen as a cellular coreceptor for human parvovirus B19 infection." Blood **106**(10): 3449-56.

Murray, J. C., P. K. Kelley, W. R. Hogrefe and K. L. McClain (1994). "Childhood idiopathic thrombocytopenic purpura: association with human parvovirus B19 infection." Am J Pediatr Hematol Oncol **16**(4): 314-9.

Musiani, M., M. Zerbini, G. Gentilomi, M. Plazzi, G. Gallinella and S. Venturoli (1995). "Parvovirus B19 clearance from peripheral blood after acute infection." J Infect Dis **172**(5): 1360-3.

Mylonakis, E., B. P. Dickinson, M. D. Mileno, T. Flanigan, F. J. Schiffman, A. Mega and J. D. Rich (1999). "Persistent parvovirus B19 related anemia of seven years' duration in an HIV-infected patient: complete remission associated with highly active antiretroviral therapy." Am J Hematol **60**(2): 164-6.

Naides, S. J. and E. H. Field (1988). "Transient rheumatoid factor positivity in acute human parvovirus B19 infection." Arch Intern Med **148**(12): 2587-9.

Naides, S. J., L. L. Scharosch, F. Foto and E. J. Howard (1990). "Rheumatologic manifestations of human parvovirus B19 infection in adults. Initial two-year clinical experience." Arthritis Rheum **33**(92): 1297-309.

Nakamura, H., K. Eguchi, T. Nakamura, A. Mizokami, S. Shirabe, A. Kawakami, N. Matsuoka, K. Migita, Y. Kawabe and S. Nagataki (1997). "High prevalence of Sjogren's syndrome in patients with HTLV-I associated myelopathy." Ann Rheum Dis **56**(3): 167-72.

Nayersina, R., P. Fowler, S. Guilhot, G. Missale, A. Cerny, H. J. Schlicht, A. Vitiello, R. Chesnut, J. L. Person, A. G. Redeker and et al. (1993). "HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection." J Immunol **150**(10): 4659-71.

Newkirk, M. M., K. N. Watanabe Duffy, J. Leclerc, N. Lambert and J. B. Shiroky (1994). "Detection of cytomegalovirus, Epstein-Barr virus and herpes virus-6 in patients with rheumatoid arthritis with or without Sjogren's syndrome." Br J Rheumatol **33**(4): 317-22.

Nguyen, Q. T., C. Sifer, V. Schneider, X. Allaume, A. Servant, F. Bernaudin, V. Auguste and A. Garbarg-Chenon (1999). "Novel human erythrovirus associated with transient aplastic anemia." J Clin Microbiol **37**(8): 2483-7.

Nguyen, Q. T., S. Wong, E. D. Heegaard and K. E. Brown (2002). "Identification and characterization of a second novel human erythrovirus variant, A6." Virology **301**(2): 374-80.

Nigro, G., P. D'Eufemia, M. Zerbini, A. Krzysztofiak, R. Finocchiaro and O. Giardini (1994). "Parvovirus B19 infection in a hypogammaglobulinemic infant with neurologic disorders and anemia: successful immunoglobulin therapy." Pediatr Infect Dis J **13**(11): 1019-21.

Nigro, G., M. Zerbini, A. Krzysztofiak, G. Gentilomi, M. A. Porcaro, T. Mango and M. Musiani (1994). "Active or recent parvovirus B19 infection in children with Kawasaki disease." Lancet **343**(8908): 1260-1.

Nikkari, S., R. Luukkainen, T. Mottonen, O. Meurman, P. Hannonen, M. Skurnik and P. Toivanen (1994). "Does parvovirus B19 have a role in rheumatoid arthritis?" Ann Rheum Dis **53**(2): 106-11.

Nikkari, S., J. Mertsola, H. Korvenranta, R. Vainionpaa and P. Toivanen (1994). "Wegener's granulomatosis and parvovirus B19 infection." Arthritis Rheum **37**(11): 1707-8.

Nikkari, S., A. Roivainen, P. Hannonen, T. Mottonen, R. Luukkainen, T. Yli-Jama and P. Toivanen (1995). "Persistence of parvovirus B19 in synovial fluid and bone marrow." Ann Rheum Dis **54**(7): 597-600.

Norbeck, O., A. Isa, C. Pohlmann, K. Broliden, V. Kasprowicz, P. Bowness, P. Klenerman and T. Tolfvenstam (2005). "Sustained CD8+ T-cell responses induced after acute parvovirus B19 infection in humans." J Virol **79**(18): 12117-21.

Norbeck, O., N. Papadogiannakis, K. Petersson, T. Hirbod, K. Broliden and T. Tolfvenstam (2002). "Revised clinical presentation of parvovirus B19-associated intrauterine fetal death." Clin Infect Dis **35**(9): 1032-8.

Noyola, D. E., M. L. Padilla-Ruiz, M. G. Obregon-Ramos, P. Zayas and B. Perez-Romano (2004). "Parvovirus B19 infection in medical students during a hospital outbreak." J Med Microbiol **53**(Pt 2): 141-6.

Olerup, O. and H. Zetterquist (1992). "HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation." Tissue Antigens **39**(5): 225-35.

Pattison, J. R., S. E. Jones, J. Hodgson, L. R. Davis, J. M. White, C. E. Stroud and L. Murtaza (1981). "Parvovirus infections and hypoplastic crisis in sickle-cell anaemia." Lancet **1**(82212): 664-5.

Penna, A., F. V. Chisari, A. Bertoletti, G. Missale, P. Fowler, T. Giuberti, F. Fiaccadori and C. Ferrari (1991). "Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen." J Exp Med **174**(6): 1565-70.

Pugliese, A., T. Beltramo, D. Torre and D. Roccatello (2006). "Parvovirus B19 and immune disorders." Cell Biochem Funct.

Rouse, B. T. and S. Deshpande (2002). "Viruses and autoimmunity: an affair but not a marriage contract." Rev Med Virol **12**(2): 107-13.

Rugolotto, S., E. M. Padovani, A. Sanna, G. P. Chiaffoni, P. L. Marradi and C. Borgna-Pignatti (1999). "Intrauterine anemia due to parvovirus B19: successful treatment with intravenous immunoglobulins." Haematologica **84**(7): 668-9.

Selbing, A., A. Josefsson, L. O. Dahle and R. Lindgren (1995). "Parvovirus B19 infection during pregnancy treated with high-dose intravenous gammaglobulin." Lancet **345**(8950): 660-1.

Serjeant, B. E., I. R. Hambleton, S. Kerr, C. G. Kilty and G. R. Serjeant (2001). "Haematological response to parvovirus B19 infection in homozygous sickle-cell disease." Lancet **358**(9295): 1779-80.

Servant, A., S. Laperche, F. Lallemand, V. Marinho, G. De Saint Maur, J. F. Meritet and A. Garbarg-Chenon (2002). "Genetic diversity within human erythroviruses: identification of three genotypes." J Virol **76**(18): 9124-34.

Shade, R. O., M. C. Blundell, S. F. Cotmore, P. Tattersall and C. R. Astell (1986). "Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis." J Virol **58**(3): 921-36.

Skjoldebrand-Sparre, L., T. Tolfvenstam, N. Papadogiannakis, B. Wahren, K. Broliden and M. Nyman (2000). "Parvovirus B19 infection: association with third-trimester intrauterine fetal death." Bjog **107**(4): 476-80.

Smith-Whitley, K., H. Zhao, R. L. Hodinka, J. Kwiatkowski, R. Cecil, T. Cecil, A. Cnaan and K. Ohene-Frempong (2004). "Epidemiology of human parvovirus B19 in children with sickle cell disease." Blood **103**(2): 422-7.

Smith, T. J., J. W. Cullen, S. D. Peck, K. E. Brown and N. S. Young (1995). "Pancytopenia secondary to parvovirus B19 infection in a child with acute lymphoblastic leukemia, mimicking leukemic relapse." Am J Hematol **50**(3): 226-7.

Soderlund, M., C. S. Brown, B. J. Cohen and K. Hedman (1995). "Accurate serodiagnosis of B19 parvovirus infections by measurement of IgG avidity." J Infect Dis **171**(3): 710-3.

Soderlund, M., R. von Essen, J. Haapasaari, U. Kiistala, O. Kiviluoto and K. Hedman (1997). "Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy." Lancet **349**(9058): 1063-5.

Sokal, E. M., M. Melchior, C. Cornu, A. T. Vandenbroucke, J. P. Buts, B. J. Cohen and G. Burtonboy (1998). "Acute parvovirus B19 infection associated with fulminant hepatitis of favourable prognosis in young children." Lancet **352**(9142): 1739-41.

Spanakis, N. E., G. A. Garinis, E. C. Alexopoulos, G. P. Patrinos, P. G. Menounos, A. Sklavounou, E. N. Manolis, V. G. Gorgoulis and D. Valis (2002). "Cytokine serum levels in patients with chronic HCV infection." J Clin Lab Anal **16**(1): 40-6.

Takahashi, Y., C. Murai, S. Shibata, Y. Munakata, T. Ishii, K. Ishii, T. Saitoh, T. Sawai, K. Sugamura and T. Sasaki (1998). "Human parvovirus B19 as a causative agent for rheumatoid arthritis." Proc Natl Acad Sci U S A **95**(14): 8227-32.

Tolfvenstam, T., A. Oxenius, D. A. Price, B. L. Shacklett, H. M. Spiegel, K. Hedman, O. Norbeck, M. Levi, K. Olsen, M. Kantzanou, D. F. Nixon, K. Broliden and P. Klenerman (2001). "Direct ex vivo measurement of CD8(+) T-lymphocyte responses to human parvovirus B19." J Virol **75**(1): 540-3.

Tolfvenstam, T., N. Papadogiannakis, O. Norbeck, K. Petersson and K. Broliden (2001). "Frequency of human parvovirus B19 infection in intrauterine fetal death." Lancet **357**(9267): 1494-7.

Torok, T. J. (1992). "Parvovirus B19 and human disease." Adv Intern Med **37**: 431-55.

Trapani, S., M. Ermini and F. Falcini (1999). "Human parvovirus B19 infection: its relationship with systemic lupus erythematosus." Semin Arthritis Rheum **28**(5): 319-25.

Trepo, C. and L. Guillevin (2001). "Polyarteritis nodosa and extrahepatic manifestations of HBV infection: the case against autoimmune intervention in pathogenesis." J Autoimmun **16**(3): 269-74.

Tsujimura, M., K. Matsushita, H. Shiraki, H. Sato, K. Okochi and Y. Maeda (1995). "Human parvovirus B19 infection in blood donors." Vox Sang **69**(3): 206-12.

Tyndall, A., W. Jelk and H. H. Hirsch (1994). "Parvovirus B19 and erosive polyarthritis." Lancet **343**(8895): 480-1.

Valeur-Jensen, A. K., C. B. Pedersen, T. Westergaard, I. P. Jensen, M. Lebech, P. K. Andersen, P. Aaby, B. N. Pedersen and M. Melbye (1999). "Risk factors for parvovirus B19 infection in pregnancy." Jama **281**(12): 1099-105.

van Elsacker-Niele, A. M., H. T. Weiland, A. C. Kroes and M. C. Kappers-Klunne (1996). "Parvovirus B19 infection and idiopathic thrombocytopenic purpura." Ann Hematol **72**(3): 141-4.

Vartdal, F., G. Gaudernack, S. Funderud, A. Bratlie, T. Lea, J. Ugelstad and E. Thorsby (1986). "HLA class I and II typing using cells positively selected from blood by immunomagnetic isolation--a fast and reliable technique." Tissue Antigens **28**(5): 301-12.

Weigel-Kelley, K. A., M. C. Yoder and A. Srivastava (2003). "Alpha5beta1 integrin as a cellular coreceptor for human parvovirus B19: requirement of functional activation of beta1 integrin for viral entry." Blood **102**(12): 3927-33.

Wen, S., N. Shimizu, H. Yoshiyama, Y. Mizugaki, F. Shinozaki and K. Takada (1996). "Association of Epstein-Barr virus (EBV) with Sjogren's syndrome: differential EBV expression between epithelial cells and lymphocytes in salivary glands." Am J Pathol **149**(5): 1511-7.

White, D. G., P. P. Mortimer, D. R. Blake, A. D. Woolf, B. J. Cohen and P. A. Bacon (1985). "Human Parvovirus Arhtropathy." Lancet: 419-421.

Wolfe, F., H. A. Smythe, M. B. Yunus, R. M. Bennett, C. Bombardier, D. L. Goldenberg, P. Tugwell, S. M. Campbell, M. Abeles, P. Clark and et al. (1990). "The American College of Rheumatology 1990 Criteria for the Classification of Fibromyalgia. Report of the Multicenter Criteria Committee." Arthritis Rheum **33**(2): 160-72.

von Poblotzki, A., A. Hemauer, A. Gigler, E. Puchhammer-Stockl, F. X. Heinz, J. Pont, K. Laczika, H. Wolf and S. Modrow (1995). "Antibodies to the nonstructural protein of parvovirus B19 in persistently infected patients: implications for pathogenesis." J Infect Dis **172**(5): 1356-9.

Wong, T. Y., P. K. Chan, C. B. Leung, C. C. Szeto, J. S. Tam and P. K. Li (1999). "Parvovirus B19 infection causing red cell aplasia in renal transplantation on tacrolimus." Am J Kidney Dis **34**(6): 1132-6.

Woolf, A. D., G. V. Campion, A. Chishick, S. Wise, B. J. Cohen, P. T. Klouda, O. Caul and P. A. Dieppe (1989). "Clinical manifestations of human parvovirus B19 in adults." Arch Intern Med **149**(52): 1153-6.

Wu, C. G., B. Mason, J. Jong, D. Erdman, L. McKernan, M. Oakley, M. Soucie, B. Evatt and M. Y. Yu (2005). "Parvovirus B19 transmission by a high-purity factor VIII concentrate." Transfusion **45**(6): 1003-10.

Vuorinen, T., K. Lammintausta, P. Kotilainen and S. Nikkari (2002). "Presence of parvovirus B19 DNA in chronic urticaric and healthy human skin." J Clin Virol **25**(2): 217-21.

Yaegashi, N. (2000). "Pathogenesis of nonimmune hydrops fetalis caused by intrauterine B19 infection." Tohoku J Exp Med **190**(2): 65-82.

Yaegashi, N., T. Niinuma, H. Chisaka, T. Watanabe, S. Uehara, K. Okamura, S. Moffatt, K. Sugamura and A. Yajima (1998). "The incidence of, and factors leading to, parvovirus B19-related hydrops fetalis following maternal infection; report of 10 cases and meta-analysis." J Infect **37**(1): 28-35.

Yajima, T., K. Yoshihara, K. Nakazato, S. Kumabe, S. Koyasu, S. Sad, H. Shen, H. Kuwano and Y. Yoshikai (2006). "IL-15 regulates CD8+ T cell contraction during primary infection." J Immunol **176**(1): 507-15.

Yeh, S. P., C. F. Chiu, C. C. Lee, C. T. Peng, C. Y. Kuan and K. C. Chow (2004). "Evidence of parvovirus B19 infection in patients of pre-eclampsia and eclampsia with dyserythropoietic anaemia." Br J Haematol **126**(3): 428-33.

Yoto, Y., T. Kudoh, K. Haseyama, N. Suzuki, T. Oda, T. Katoh, T. Takahashi, S. Sekiguchi and S. Chiba (1995). "Incidence of human parvovirus B19 DNA detection in blood donors." Br J Haematol **91**(4): 1017-8.

Zinkernagel, R. M. (1996). "Immunology taught by viruses." Science **271**(5246): 173-8.

I

ORIGINAL ARTICLE

Clinical and Laboratory Findings in Immunocompetent Patients with Persistent Parvovirus B19 DNA in Bone Marrow

ANDERS LUNDQVIST[1,2], THOMAS TOLFVENSTAM[2], JENNIFER BOSTIC[3], MARIA SÖDERLUND[4] and KRISTINA BROLIDEN[2]

From the [1]Department of Infectious Diseases, Borås Hospital, Borås, [2]Division of Clinical Virology, Huddinge University Hospital, Karolinska Institute, Huddinge, Stockholm, Sweden, [3]MedImmune Inc., Gaithersburg, MD, USA, and the [4]Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland

The clinical relevance of parvovirus B19 DNA persistence in bone marrow was examined in 10 immunocompetent individuals undergoing examinations for unexplained fever, arthralgia or chronic leukopenia. Common causes of these symptoms had been ruled out and bone marrow aspiration was indicated at this stage of investigation. In addition to morphological analysis of the bone marrow, a test for B19 DNA was performed with 2 nested PCRs. Five of these 10 selected patients had detectable B19 DNA in their bone marrow, whereas no viraemia was observed. Additional bone marrow samples were collected at least 6 months after the first sample from the B19 DNA-positive patients, of whom 3 were found to be still positive. Indeed, 2 of the patients have been positive for more than 5 y of follow-up. Sera from all patients with persistent B19 DNA in bone marrow could neutralize the virus. One patient responded to treatment with immunoglobulin but later relapsed. No other cause of the symptoms was found, despite extensive investigations, and at least some of the prolonged disease manifestations may be due to parvovirus B19.

K. Broliden, MD, PhD, Division of Clinical Virology, F68, Karolinska Institute, Huddinge University Hospital, SE-141 86 Huddinge, Sweden

INTRODUCTION

Human parvovirus B19 can cause a common childhood disease with exanthema and fever, known as erythema infectiosum (fifth disease); polyarthritis or arthralgia, which in rare cases can persist for years; and transient aplastic crises in patients with underlying haemolytic disorders; while vertical transmission can cause hydrops foetalis and foetal death (1).

The primary site of B19 replication is erythroid precursor cells, and B19 DNA can be found in the bone marrow with or without viraemia. The virus is usually cleared from the bone marrow within a few months after primary infection. Signs of bone marrow failure in connection with persistent B19 infection have been reported in various groups of immunocompromised patients, usually leading to severe chronic anaemia (2–6). Cases of immunocompetent patients with chronic B19 infection and bone marrow dysfunction have also been reported, including single cases of recurrent, granulocytic aplasia (7), pure red cell anaemia (8), and a picture mimicking the myelodysplastic syndrome (9). However, persistent B19 infections without anaemia have also been seen in a few immunocompetent individuals (7, 10). The prevalence of chronic, subclinical infections is not known. In the present study we retrospectively summarized the clinical and laboratory findings in patients with persistent B19 DNA in bone marrow.

MATERIAL AND METHODS

Study groups

This retrospective study summarizes 10 patients who had been tested for the presence of parvovirus B19 DNA in bone marrow during the years 1993–98. Follow-up samples were also available from some of the B19 DNA positive patients. All patients had been referred to the Department of Infectious Diseases at Borås Hospital, Borås, Sweden, on account of unclear symptoms with suspected infectious etiology. The median age of the patients, all women, was 43 y (range 25–75 y).

Immunoglobulin preparations

Three patients with persistence of B19 were given immunoglobulins in order to clear the infection. Gammonativ (Pharmacia & Upjohn, Stockholm, Sweden) was used for intravenous administration, Gammanorm (Pharmacia & Upjohn) for subcutaneous infusion (11).

PCR

Serum and bone-marrow samples were tested in 2 different nested PCRs, representing the structural (VP) and the non-structural (NS) proteins of the B19 virus, as described previously (12). Briefly, in each PCR, the result was considered positive if at least 2 out of 3 separate amplifications gave identical results and all controls were correctly positive or negative. At least 1 of the 2 PCRs (VP/NS) was required for positivity of the patient. Both serum and bone-marrow samples were analysed after heat treatment at 94°C for 10 min, followed by centrifugation at 12,000 rpm for 5 min. The following primers were used:

VP-PCR. First amplification: nucleotide 2955–2974, 5′-GGA CTG TAG CAG ATG AAG AG-3′ and reverse primer 3364–3349, 5′-TAT GGG ACT GAT GGT G-3′. The nested round:

nucleotide 3002–3020, 5′-GGG TTT CAA GCA CAA GTA G-3′ and reverse primer 3291–3272, 5′-CCT TAT AAT GGT GCT CTG GG-3′.

NS-PCR. First amplification: nucleotide 1355–1374; 5′-GGC AGC ATG TGT TAA GTG G-3′ and reverse primer 1723–1703, 5′-CAG TTG TTG TAG TGT TCC C-3′. The nested round: nucleotide 1399–1422; 5′-AAT ACA CTG TGG TTT TAT GGG CCG-3′ and reverse primer 1682–1659, 5′-CCA TTG CTG GTT ATA ACC ACA GGT-3′.

The PCRs specific for CMV, EBV and HHV-6 DNA have been described elsewhere (13).

Serological assays

Specific B19 IgG in serum was detected using an EIA (DAKO, Glostrup, Denmark), and IgG titration was performed using another EIA (Eurodiagnostica, Malmö, Sweden). In our hands in the latter EIA, 5% of normal individuals ($n = 120$, ages 10–86 y) have a titer higher than 1,000. B19 IgM in serum was analysed in an indirect immunofluorescence assay (IFA) (Biotrin, Dublin, Ireland) and in an EIA (DAKO, Glostrup, Denmark).

The EIA methods for IgG avidity and epitope type specificity (ETS) have been described elsewhere (14, 15). ETS ratio > 5 and avidity score > 25, respectively, indicates past infection (> 6 months ago).

Neutralizing antibodies were analysed in serum by a recently described method (16). Briefly, a megakaryocytic cell line (UT-7) was grown in 2 units/ml Epo (erythropoetin), which renders the cells mildly permissive for B19 infection. Dilutions of serum samples (1:200, 1:500, 1:1000) were added to 3×10^6 UT-7/Epo cells in a 1 ml culture volume. Viremic serum (Campbell A stock) was then added at a 1:25.000 final volume. The cells were harvested after 36 h of culture in 37°C. Total RNA was collected using a commercially available kit (Promega). RT-PCR was then performed to detect spliced viral transcript. A beta-actin transcript was used as a control. Serum with previously defined B19 neutralizing titers was included in each test; random donors will usually neutralize B19 at a dilution of 1:200 to 1:1000.

RESULTS

Parvovirus B19 DNA in patients with fever, arthralgia or leukopenia

A total of 10 patients were tested for B19 DNA in serum and bone marrow for diagnostic purposes. They suffered from various symptoms, including prolonged fever, arthralgia and/or leukopenia. Routine clinical and laboratory examinations, including B19 serology, had failed to explain the etiology of their symptoms and therefore bone marrow examinations were indicated. In addition to morphological studies of the bone marrow (which failed to give a diagnosis), B19 DNA was analysed by PCR; 5 of the 10 patients were positive. B19 DNA was also analysed in corresponding serum samples but no viraemia could be demonstrated in any of the 10 patients. No other cause of the symptoms was found. In follow-up studies, after at least 6 months, bone marrow samples from 3 of these 5 patients were still B19 DNA positive, whereas 2 were B19 DNA negative. Indeed, B19 DNA could still be detected more than 5 y after onset of symptoms in cases 1 and 2 (Tables I and II, respectively). In view of the persistent symptoms and B19 DNA as the only finding during the investigation, they were treated with immunoglobulin preparations. Their laboratory findings and treatment schedules are given in Tables I–III, respectively.

The patients with persistent B19 DNA findings were PCR negative for EBV, CMV and HHV-6 DNA and their serological responses to these viruses were unexceptional (data not shown). All of them were HIV-antibody negative. None of them had a history of repeated severe bacterial or viral infections.

Table I. *Treatment schedule and laboratory parameters for case 1*

Treatment	Months after onset of symptoms						
	6	14	19	30	40	52	63
Immunoglobulin, i.v. (month)			[a](27)	[a](32)	[a](36)		
Immunoglobulin, s.c. (month)						[a](43–50)	
B19-specific parameters							
DNA by PCR in bone marrow			pos	pos	pos	pos	pos
DNA by PCR in serum	neg	neg		neg	neg	neg	
S-IgM	pos	neg		neg	neg	neg	neg
S-IgG (titre)	>1000	>1000		>1000	>1000	>1000	>1000
S-ETS	4	4		9	13		
S-Avidity	28	24		45	53		
S-Neutralization (titre)				>1000		500	

[a] Immunoglobulin, i.v. (400 mg/kg) administered for 1, 3 and 5 d, respectively; immunoglobulin s.c. (85 mg/kg) administered twice a month during the indicated time. pos: positive; neg: negative; S: serum

Table II. *Treatment schedule and laboratory parameters for case 2*

Treatment	Months after onset of symptoms							
	8	19	23	35	45	48	57	62
Immunoglobulin, i.v. (month)			[a](21)	[a](26)				
Immunoglobulin, s.c. (month)					[a](37–44)			
B-19 specific parameters								
DNA by PCR in bone marrow		pos	pos	pos	neg	neg		pos
DNA by PCR in serum	neg	neg	neg	neg	neg		neg	
S-IgM	pos/neg	neg	neg	neg	neg	neg	neg	neg
S-IgG (titre)	450	600	500	700	650	500	500	650
S-ETS	21	18	6	23				
S-Avidity	nd	57	56	52				
S-Neutralization (titre)	200	>1000						

[a] Immunoglobulin, i.v. (400 mg/kg) administered for 1 and 3 d respectively; immunoglobulin s.c. (100 mg/kg) administered twice a month during the indicated time. pos: positive; neg: negative; pos/neg: positive in an EIA, negative in an IFA; S: serum; nd: not done

Case 1. A 36-y-old female working at a day-care centre was previously healthy, apart from periods of irritable colon. She was admitted to our clinic after 6 months of recurrent, long-lasting periods of fever, fatigue, myalgia and symmetric arthralgia located mainly in the wrists. An outbreak of erythema infectiosum at her day-care centre was noted the same year as the onset of clinical symptoms. Physical examination and blood chemistry at presentation were normal, except for a leukopenia $(1.9 \times 10^9/l)$, with a normal differential count, and a mild anaemia (haemoglobin 112 g/l). Bone marrow examination revealed megaloblastoid erythropoesis. B19 IgM was only present in the first serum sample, whereas B19 IgG was constantly high in that sample and during follow-up (Table I). Increasing B19 avidity and ETS ratio indicated that primary infection had taken place at least some months before the study started. Sera from months 30 and 52 were able to neutralize B19 virus. However, the possibility that the rises in ETS and avidity, as well as the presence of neutralizing activity, were due to the immunoglobulin given (as described below) cannot be ruled out. The presence of B19 DNA was demonstrated by PCR in all bone marrow samples, but never in the serum samples.

The symptoms and the leukopenia of the patient persisted and immunoglobulin treatment was initiated at month 27 (Table I). At the end of the study the symptoms, except the fever, and the leukopenia persisted.

Case 2. A 43-y-old female working at a day-care centre fell ill with upper respiratory-tract symptoms, cough and high fever (month 0). She was treated with phenoximethyl-penicillin and the airway symptoms disappeared, whereas the fever (about 38°C) and fatigue persisted. She had had symptoms of irritable colon for several years but otherwise had been healthy. Repeated analyses of blood chemistry during the study period were normal. An extensive investigation was performed without reaching a diagnosis. A serum sample 8 months after onset of symptoms was B19

PCR negative, whereas B19 IgM was positive in 1 test (EIA) and negative in another (IFA) (Table II). The B19 IgG titre and ETS were high, indicating past immunity. Sera from month 8 and 19 were able to neutralize the B19 virus (prior to immunoglobulin treatment). One year later, the bone marrow was found B19 DNA positive.

Treatment was initiated in an attempt to clear the infection at month 21 (Table II). The patient temporarily experienced an effect of treatment, with months of normal temperature and asymptomatic periods. Interestingly, after the last therapy session the bone marrow samples were B19 DNA negative (at months 45 and 48). However, at month 62 the bone marrow was again B19 DNA positive and the fever and chronic fatigue had reappeared.

Case 3. A 44-y-old female working at a day-care center had been suffering from unspecified motor and sensory neuropathy since childhood when she was admitted to the clinic (month 0). She also had, for 20 y, had chronic arthralgia in a number of joints, facial erythema and recurrent erythema nodosum. At the beginning of the study, high titres of rheumatoid factor and antinuclear antibodies were found, as well as circulating immune complexes. The erythrocyte-sedimentation rate was between 20 and 40 during the study period. She was eventually diagnosed with Sjögren's syndrome at the end of our study.

Serum samples were available from months 0, 9, 16 and 18, showing high B19 IgG titres, negative B19 IgM and negative B19 DNA by PCR (Table III). B19 DNA was detected in all of the 4 consecutive bone-marrow samples. Morphological bone-marrow examinations showed normal results, except for signs of iron deficiency, which was judged to be menstrually related. The patient was treated with immunoglobulin, starting at month 2, in an attempt to clear the virus. No alteration in her clinical status has, however, been noted.

Cases 4–10. Cases 4–10 represented women aged 25–75 y (median 35 y). They were all B19 IgG positive, B19 IgM

negative and B19 DNA negative in serum. Case 4 had fever and arthralgia for 2 months and was B19 DNA positive in bone marrow. A follow-up sample 7 months later was B19 DNA negative. Case 5 had fever and fatigue for 10 months when B19 DNA in bone marrow was positive. A follow-up sample 11 months later was negative and she was asymptomatic at that time. Cases 6–10 were all B19 DNA negative in bone marrow and suffered from fatigue, leukopenia, fever, exanthema (case 6), chronic leukopenia for many years (case 7), unexplained fever and anemia (case 8), prolonged arthralgia (case 9), and recurrent fever and myalgia for 3 y (case 10), respectively.

DISCUSSION

An acute B19 infection is characterized by B19 viraemia and B19 IgM followed by B19 IgG seroconversion. The viraemia and IgM response are usually cleared within a few weeks or months (17). In a few cases, B19 DNA can persist in bone marrow cells and synovial membranes even without viraemia (18, 19). In 1 study, the prevalence of B19 PCR positivity in bone marrow of healthy individuals was found to be 9% (20). Comparisons are complicated by the different sensitivities of PCR assays, but in our hands about 10% of children receiving chemotherapy for various malignancies (12) and 4% of unselected adult patients investigated at a haematology clinic have been found to be B19-PCR positive in bone marrow (21). The rate of infection in otherwise healthy adults is probably lower than in children on account of immunocompetence and a higher degree of immunity. These figures are of interest in relation to the present study group, which was highly selected. All patients included in this retrospective study were women who were investigated for an infectious aetiology of their varying symptoms. B19 DNA was found by PCR in bone marrow from 5 of the 10 patients. Three of these patients had a persistent infection and their first and last B19 isolates have recently been sequenced (unpublished results). Interestingly, the persis-

tently infected patients all worked in day-care centres, where B19 infections are known to be common. Frequent exposure to the virus might lead to a higher risk of chronic infection. The shedding of right titres of virus in small children with primary B19 infection together with close physical contact could result in exposure to high viral doses for people working in day-care centres, and thereby possibly further increase the risk of chronic infection. Chronically infected patients have been noted to produce IgG antibodies without the ability to neutralize the virus (22). However, our chronically B19-infected patients had B19-specific neutralising antibodies and were apparently immunocompetent.

B19 DNA, demonstrable by PCR in bone marrow and/or serum, can be found in both the presence and the absence of B19 IgG and B19 IgM (7, 10, 23, 24). All of the B19 DNA positive patients in the present study already had B19 IgG antibodies in normal or high titres at presentation. Cases 1 and 2 also had B19 IgM antibodies in the first of several, consecutive, serum samples. The sera were tested for epitope type-specific antibodies and avidity (14, 15) and were found to be equivocal for case 1 and to exclude acute or recent (within 2–3 months) infection for case 2. The source and date of primary infection for case 3 are not known and ETS and avidity results indicated past infection (> 6 months ago).

A wide variety of clinical symptoms have been associated with B19 infections, including neurological (25–27) and rheumatological manifestations (28). Our patients with persistence of B19 DNA in bone marrow suffered from various symptoms, not classical for parvovirus B19, even if fever, fatigue, arthralgia, leukopenia and anaemia as seen in case 1 are well-known manifestations of B19 infection. Case 2 presented with upper respiratory-tract symptoms and fever and has during the study fulfilled the criteria for the chronic fatigue syndrome. Chronic fatigue has been described in association with persistent B19 infections in 2 patients (29). Clinically, the defined syndrome resembles an

Table III. *Treatment schedule and laboratory parameters for case 3*

Treatment	Month[a]					
	0	2	4	9	16	18
Immunoglobulin, i.v. (month)		[b](2) [b](3–9)				
B19-specific parameters						
DNA by PCR in bone marrow		pos	pos	pos		pos
DNA by PCr in serum	neg			neg	neg	neg
S-IgM	neg			neg	neg	neg
S-IgG (titre)	>1000			>1000	>1000	>1000
S-ETS	7			7		
S-Avidity	42			59		
S-Neutralization (titre)	500					>1000

[a] Month 0: admitted to our clinic, symptomatic since many years
[b] Immunoglobulin, i.v. (400 mg/kg) administered for 5 days at month 2 and once per month during months 3–9, respectively. pos: positive; neg: negative; S: serum

infection and a wide range of possible etiological agents, including viral, have been considered. The role of the B19 virus in chronic fatigue syndrome needs further investigation. Case 3 was eventually diagnosed with Sjögren's syndrome. This syndrome is an autoimmune disease of possible viral aetiology (30). Whether the finding of B19 DNA in her bone marrow was merely coincidental, or the infection worsened her disease, is unclear. Treatment with immunoglobulin often cures B19 infection in both immunosuppressed and immunocompetent patients (31). However, the effects of immunoglobulin treatment in our chronically infected patients were only transient.

It cannot be proven that B19 is the cause of the symptoms in our cases, but the findings merit attention. Future case reports and epidemiological studies in frequently exposed populations may clarify the role of persistent B19 infections in immunocompetent individuals.

ACKNOWLEDGEMENTS

REFERENCES

1. Anderson LJ. Human Parvoviruses. J Infect Dis 1990; 161: 603–8.
2. Kurtzman GJ, Ozawa K, Cohen B, Hanson G, Oseas R, Young NS. Chronic bone marrow failure due to persistent B19 parvovirus infection. N Engl J Med 1987; 317: 287–94.
3. Kurtzman GJ, Cohen B, Meyers P, Amunullah A, Young NS. Persistent B19 parvovirus infection as a cause of severe chronic anemia in children with acute lymphocytic leukemia. Lancet 1988; 2: 1159–62.
4. Graeve JLA, de Alarcon PA, Naides SJ. Parvovirus B19 infection in patients receiving cancer chemotherapy: the expanding spectrum of disease. Am J Pediatr Hematol Oncol 1989; 11: 441–4.
5. Weiland HT, Salimans MM, Fibbe WE, Kluin PM, Cohen BJ. Prolonged parvovirus B19 infection with severe anaemia in a bone marrow transplant patient. Br J Haematol 1989; 71: 300.
6. Frickhofen N, Abkowitz JL, Safford M, Berry JM, Antunez-deMayolo J, Astrow A, et al. Persistent B19 parvovirus infection in patients infected with human immunodeficiency virus type 1 (HIV-1): a treatable cause of anemia in AIDS. Ann Intern Med 1990; 113: 926–33.
7. Pont J, Puchhammer-Stöckl E, Chott A, Popow-Kraupp T, Kienzer H, Postner G, et al. Recurrent granulocytic aplasia as clinical presentation of a persistent parvovirus B19 infection. Br J Hematol 1992; 80: 160–5.
8. Kurtzman G, Frickhofen N, Kimball J, Jenkins DW, Nienhuis AW, Young NS. Pure red-cell aplasia of 10 years' duration due to persistent parvovirus B19 infection and its cure with immunoglobulin. N Engl J Med 1989; 321: 519–23.
9. Baurmann H, Schwarz TF, Oertel J, Serke S, Roggendorf M, Huhn D. Acute parvovirus B19 infection mimicking

10. Faden H, Gary GW, Anderson LJ. Chronic parvovirus infection in a presumably immunologically healthy woman. Clin Inf Dis 1992; 15: 595–7.
11. Hammarström L, Samuelsson J, Grimfors G. Subcutaneous gammaglobulin for patients with secondary hypogammaglobulinemia. Lancet 1995; 345: 382–3.
12. Broliden K, Tolfvenstam T, Ohlsson S, Henter. JI. Persistent B19 Parvovirus infection in pediatric malignancies. Med Ped Oncol 1998; 31: 66–72.
13. Wang FZ, Dahl H, Linde A, Brytting M, Ehrnst A, Ljungman P. Lymphotropic herpesviruses in allogeneic bone marrow transplantation. Blood 1996; 88: 3615–20.
14. Söderlund M, Brown CS, Spaan WJM, Hedman L, Hedman K. Epitope-type specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995; 172: 1431–6.
15. Söderlund M, Brown CS, Cohen BJ, Hedman K. Accurate serodiagnosis of B19 parvovirus infections by measurements of IgG avidity. J Infect Dis 1995; 171: 710–3.
16. Bostic J, Koenig S. Quantitative analysis of parvovirus B19 neutralizing responses in immune sera by an RT-PCR based assay. J Infect Dis 1999, In press.
17. Anderson MJ, Higgins PG, Davis LR, Willman JS, Jones SE, Kidd IM, et al. Experimental parvoviral infection in humans. J Infect Dis 1985; 152: 257–65.
18. Liu W, Ittmann M, Liu J, et al. Human parvovirus B19 in bone marrows from adults with aquired immunodeficiency syndrome: a comparative study using in situ hybridization and immunohistochemistry. Hum Pathol. 1997; 28: 760–6.
19. Söderlund M, von Essen R, Haapasaari J, Kiistala U, Kiviluoto O, Hedman K. Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. Lancet 1997; 349: 1063–5.
20. Cassinotti P, Burtonboy G, Fopp M, Siegl G. Evidence for persistence of human parvovirus B19 DNA in bone marrow. J Med Virol 1997; 53: 229–32.
21. Lundqvist A, Tolfvenstam T, Brytting M, Stolt CM, Hedman K, Broliden K. Prevalence of parvovirus B19 DNA in bone marrow of patients with hematologic disorders. Scand J Infect Dis 1999, In press.
22. Kurtzman GJ, Cohen BJ, Field AM, Oseas R, Blaese RM, Young NS. Immune response to B19 parvovirus and an antibody defect in persistent viral infection. J Clin Invest 1989; 84: 1114–23.
23. Cassinotti P, Weitz M, Siegl G. Human parvovirus B19 infections: routine diagnosis by a new nested polymerase chain reaction assay. J Med Virol 1993; 40: 228–34.
24. Foto F, Saag KG, Scharosch LL, Howard EJ, Naides SJ. Parvovirus B19-specific DNA in bone marrow from B19 arthropathy patients: evidence for B19 virus persistence. J Infect Dis 1993; 167: 744–8.
25. Walsh KJ, Armstrong RD, Turner AM. Brachial plexus neuropathy associated with human parvovirus infection. Br Med J 1988; 296: 896.
26. Faden H, Gary GW, Korman M. Numbness and tingling of fingers associated with parvovirus B19 infection. J Infect Dis 1990; 161: 354–5.
27. Denning DW, Amos A, Rudge P, Cohen BJ. Neuralgic amyotrophy due to parvovirus infection. J Neurol Neurosurg Psychiatr 1987; 50: 641–2.
28. Finkel T, Török T, Ferguson P, Durigon EL, Zaki SR, Leung DYM, et al. Chronic parvovirus B19 infection and systemic necrotising vasculitis: opportunistic infection or etiological agent? Lancet 1994; 343: 1255–8.

myelodysplastic syndrome of the bone marrow. Ann Hematol 1992; 64: 43.

29. Kerr JR, Curran MD, Moore JE, Coyle PV, Ferguson WP. Persistent parvovirus B19 infection. Lancet 1995; 345: 1118.
30. Moutsopoulos H, Papadopoulos G, Youinou P. Gougerot-Sjo-gren syndrome: the viruses in the box of the accused. Ann Med Interne 1994; 145: 25–31.
31. Young NS. Parvovirus infection and its treatment. Clin Exp Immunol 1996; 104 Suppl 1: 26–30.

II

ORIGINAL ARTICLE

Prevalence of Parvovirus B19 DNA in Bone Marrow of Patients with Haematological Disorders

ANDERS LUNDQVIST[1,2], THOMAS TOLFVENSTAM[2], MARIA BRYTTING[3], CARL MAGNUS STOLT[1], KLAUS HEDMAN[4] and KRISTINA BROLIDEN[2]

From the [1]Department of Infectious Diseases, Borås Hospital, Borås, Sweden, [2]Department of Virology, Haartman Institute, University of Helsinki, Helsinki, Finland, [3]Division of Clinical Virology, Huddinge University Hospital, Karolinska Institute, Stockholm, and the [4]Department of Clinical Virology, Swedish Institute for Infectious Disease Control, Stockholm, Sweden

Patients with haematological disorders ($n = 100$) were examined for prevalence of parvovirus B19 DNA in the bone marrow and serum, irrespective of B19-related symptoms. B19 DNA was studied using 2 nested PCRs and the serum samples were further analysed with B19-specific IgG, IgM and avidity as well as seroreactivity against linear and conformational epitopes of the B19 VP2 antigen. The latter assays specify whether the IgG antibody response represents acute or past B19 infection. B19 DNA was detected in 4 of the 100 bone marrow samples, whereas all the serum samples were B19 DNA negative. None of the 4 B19 DNA positive patients had symptoms typical of B19 infection and serology showed past infection. Furthermore, 2 were still B19 DNA positive in bone marrow more than 1 y after the first sample indicating virus persistence. The seroprevalence for B19 IgG was 59% and 2 patients were B19 IgM positive. Thus, presence of B19 DNA in bone marrow from patients with haematological disorders is not a general finding in seropositive patients. B19 DNA can persist in bone marrow, but in our material this finding showed no clear correlation with symptomatic B19 infection.

K. Broliden, MD, Division of Clinical Virology, F68, Huddinge University Hospital, Karolinska Institute, SE-141 86 Huddinge, Sweden

INTRODUCTION

Parvovirus B19 was first associated with human disease in 1981, when it was connected with aplastic crisis in children with haemolytic anaemia; later it was described to be the aetiology of the common childhood disease erythema infectiosum. Parvovirus B19 may also cause hydrops foetalis and foetal death when non-immune pregnant women are infected. The virus has been associated with several other haematological disorders, e.g. chronic anaemia in congenital or acquired immunodeficiencies, as well as with arthropathy of varying duration. The seroprevalence is usually stated to be 60–80% in adults (1).

B19 infections in haematologically healthy persons may lead to transient anaemia and reticulocytopenia, as well as to varying degrees of neutropenia (2, 3) and thrombocytopenia (4, 5). In experimental infection of normal human volunteers, bone marrow samples have revealed an almost total loss of erythroid precursors at all stages of development with the appearance of giant pronormoblasts (6). B19 can infect human tissue and cell types expressing its cellular receptor, the P-antigen (7). However, only erythroid progenitor cells have been shown to be permissive for B19 replication. The tropism of the virus for these cells explains why B19 DNA is readily detected in bone marrow samples.

Acute and persistent B19 infections have been diagnosed by using B19 DNA-specific PCR in bone marrow and/or serum samples. PCR is a useful complement to B19 serology, especially in immunodeficient patients who might have difficulties in mounting an adequate immune response to the infection. However, the relevance of a B19 DNA finding in bone marrow in the absence of B19 DNA in serum and/or B19 specific serology is not clear (8–10). This question is especially relevant in patients with haematological disorders, where B19 infection is an important differential diagnosis. In this study we therefore evaluated the prevalence and clinical significance of B19 DNA in this patient category.

MATERIALS AND METHODS

Study group

A total of 100 patients were enrolled in this prospective study during the period October 1994 to April 1996 at the Hematology Clinic, Department of Internal Medicine, Borås Hospital, Borås, Sweden. The patients were diagnosed as follows (number of patients): acute lymphatic leukaemia (1); acute myeloic leukaemia (17); chronic lymphoproliferative disease (23); chronic myeloproliferative disease (15); plasma cell disease (13); anaemia (11); other cytopenias (11); adenocarcinoma (3); other (6). Follow-up samples were collected from some of the patients after this study period. Bone marrow examinations were performed as a part of the clinical investigation, either for diagnostic purposes or as a follow-up after chemotherapy. These unselected samples were collected irrespective of parvovirus-related symptoms. No parvovirus B19 epidemics had been noted by the virology laboratories in Sweden during the study period (data not shown). The mean age in the study group was 63 y (range 20–88 y); 54 men and 46 women were included.

Serology

B19 IgG in serum was detected using an EIA (Eurodiagnostica, Malmö, Sweden) and B19 IgM using an indirect immunofluorescence test (IFA) (Biotrin International Ltd, Dublin, Ireland). B19

IgM positive samples were also tested in an EIA (DAKO, Glostrup, Denmark) with comparable results.

For improved timing of B19 infection, IgG reactivities with conformational and linear VP2 epitopes (epitope-type specificity; ETS) were measured, using native and denatured VP2 capsid antigens (DAKO, Glostrup, Denmark) as described (11).

Measurement of IgG avidity further improves timing of recent infections (12) and our method using the B19 VP1 protein as antigen has been described previously (13).

PCR

Serum and bone-marrow samples were tested in 2 different nested PCRs, representing the structural (VP) and the non-structural (NS) proteins of the B19 virus, as described previously (14). Briefly, in each PCR, the result was considered positive if at least 2 out of 3 separate amplifications gave identical results and all controls were correctly positive or negative. At least 1 of the 2 PCRs (VP/NS) was required for positivity of the patient. Both serum and bone-marrow samples were analysed after heat treatment at 94°C for 10 min, followed by centrifugation at 12,000 rpm for 5 min. The following primers were used.

VP-PCR. First amplification: nucleotides 2955–2974, 5′-GGA CTG TAG CAG ATG AAG AG-3′ and reverse primer 3364 to 3349, 5′-TAT GGG ACT GAT GGT G-3′. The nested round: nucleotides 3002–3020, 5′-GGG TTT CAA GCA CAA GTA G-3′ and reverse primer 3291–3272, 5′-CCT TAT AAT GGT GCT-CTG GG-3′.

NS-PCR. First amplification: nucleotides 1355–1374; 5′-GGC AGC ATG TGT TAA GTG G-3′ and reverse primer 1723–1703, 5′-CAG TTG TTG TAG TGT TCC C-3′. The nested round: nucleotides 1399–1422; 5′-AAT ACA CTG TGG TTT TAT GGG-CCG-3′ and reverse primer 1682–1659, 5′-CCA TTG CTG GTT-ATA ACC ACA GGT-3′.

RESULTS

B19 DNA was detected by PCR in 4 out of 100 bone marrow samples. Of these 3 were positive in both the VP- and NS-PCR and 1 was positive only in the NS-PCR. The 4 corresponding serum samples were all B19 IgG positive, but B19 DNA and IgM negative. B19 IgG ETS and avidity scores indicated past infection (>6 months previously) (Table I).

The B19 IgG seroprevalence was 58 out of 98 (59%) (2 serum samples were not available). Interestingly, only 1 of 9 patients with myeloma had B19 IgG whereas presence of B19 IgG was distributed more equally in the other patient categories.

B19 IgM was detected in 2 samples without B19 DNA in either serum or bone marrow (Table I). These 2 patients had polyclonal IgM elevation due to their haematological disease. The samples were B19 IgM positive in both EIA and IF assays. Case 1 was still B19 IgM positive in a serum sample collected 19 months after the first serum sample. The ETS and avidity results were borderline in case 1 and pointed to acute (<6 months earlier) B19 infection in case 2.

Cases 1 and 2 were patients with B19 DNA negative bone marrow and B19 IgM positive serum. Case 1 had chronic lymphatic leukaemia and a history of long-lasting periodic anaemia with reduced erythropoiesis not obviously explained by the leukaemia. He also had periods of arthralgia. Case 2 had myelodysplastic syndrome but no clinical signs compatible with B19 infection.

Table I. *Characterization of patients with positive B19 IgM or detectable B19 DNA by PCR*

Case	Sex	Age (y)	Diagnosis	Bone marrow B19 DNA	Serum B19 DNA	B19-specific serology			
						B19 IgG	B19 IgM	ETS ratio	Avidity (%)
1	M	58	Chronic lymphatic leukemia	Neg	Neg	Pos	Pos	4	22
			Follow-up sample 19 months later	nd	Neg	Pos	Pos	6	30
2	M	82	Myelodysplastic syndrome	Neg	Neg	Pos	Pos	4	1
3	M	48	Myeloma	Pos	Neg	Pos	Neg	8	27
			Follow-up sample 18 months later:	Pos	Neg	Pos	Neg	nd	nd
4	M	47	Polycythemia	Pos	Neg	Pos	Neg	10	64
5	M	68	Unexplained anemia and elevated sedimentation rate	Pos	Neg	Pos	Neg	14	43
			Follow-up sample 12 months later	Pos	Neg	Pos	Neg	nd	nd
6	M	51	Acute lymphatic leukemia	Pos	Neg	Pos	Neg	10	23
			Follow-up sample 30 months later	Neg	Neg	Pos	Neg	nd	nd

ETS >5 and avidity $>25\%$ indicate past infection (>6 months earlier); nd: not done.

Cases 3–6 were patients with B19 DNA positive bone marrow and B19 IgM negative serum.

Case 3 had had myeloma for many years and exanthema and facial rash 1 y before inclusion. His bone marrow was suppressed, but this could be explained by the myeloma and the resultant chemotherapy. B19 DNA was detected also in a bone-marrow sample 18 months later. He had been treated for 1 y with high doses of intravenous immunoglobulin because of his myeloma. Case 4 had polycythemia of unspecified type with unknown aetiology. He had no skin or joint symptoms. Case 5 was examined because of anaemia (haemoglobin count 103 g/l) and elevated sedimentation rate (ESR 110). No aetiology was found in spite of an extensive investigation. His laboratory parameters normalised spontaneously in 3 months. Follow-up PCR of B19 DNA in bone marrow after 12 months was positive. Case 6 had acute lymphatic leukaemia and had suffered for many years from morning stiffness in multiple joints but had no rheumatological diagnosis. Rheumatoid factor and antinuclear antibodies were negative and sedimentation rate was normal. Follow-up analysis of B19 DNA in bone marrow was negative 30 months after inclusion in this study.

DISCUSSION

The prevalence of parvovirus B19 DNA was studied by PCR in bone marrow and serum samples from patients attending a haematological clinic. A total of 100 consecutive patients were included, irrespective of parvovirus-related symptoms. B19 DNA was detected in bone marrow in 4 of the 100 patients, whereas all corresponding serum samples were B19 DNA negative. Two patients were B19 IgM positive without detectable B19 DNA in either bone marrow or serum. Both patients had an increased level of polyclonal IgM, and thus a false positive IgM reactivity cannot be excluded. Although a previous symptomatic B19 infection was possible in 3 of the 4 B19 DNA positive cases, the persistence of B19 DNA was not associated with any B19-related clinical signs or symptoms at the time of bone marrow sampling, except for prolonged arthralgia in 1 case. Follow-up bone marrow samples were available from 3 patients and 2 of those were still B19 DNA positive 12 and 18 months later, respectively.

B19 virus has been implicated as a possible aetiological agent for many haematological disorders (15–22). B19 DNA is normally cleared from serum shortly after acute infection and the IgM response disappears as a rule within a few months (23). Persistence of B19 DNA has been described primarily in immunodeficient patients and can in some cases be associated with a prolonged, symptomatic B19 infection with or without presence of B19 IgG antibodies (8, 15, 24). B19 DNA can also persist in presumably healthy immunocompetent individuals in bone marrow (9) or serum (25–27). Persistence of B19 DNA in bone marrow

in the absence of viraemia, as seen in our patients, has previously been associated with complicated B19 infection in other patient categories, such as chronic arthropathy in adults or paediatric malignancies (10, 14). In a group of patients with leukopenia, prolonged fever, fatigue or arthropathy, we recently found B19 DNA persistence in bone marrow, without viraemia, in 3 out of 10 cases. No other cause of the symptoms was found in spite of extensive investigation (28). However, in the present study group, representing unselected adults with diverse haematological disorders, we can not clearly associate persistent B19 DNA findings in bone marrow with ongoing B19-associated disease. B19 DNA has been detected in the synovial membranes of patients both with and without chronic arthropathy (29) and in skin biopsies (30). B19 DNA may also persist in macrophages and monocytes for a long time after acute infection. It must, however, be pointed out that PCR makes it possible to detect B19 genomes irrespective of virus viability and also degraded DNA. Culture systems for documentation of B19 viability are not readily available.

The seroprevalence in the study group, 59%, corresponds with the expected figure in an adult population (1). However, the frequency may be falsely low because of low levels of total IgG in a subset of the study group. This is illustrated by the fact that only 1 of 9 patients with myeloma was B19 IgG positive. Also other patients in our study group may have difficulties in mounting a relevant antibody response due to their immunosuppressive chemotherapy or haematological disorder. These examples illustrate the diagnostic difficulties with B19 serology in patients with underlying disorders affecting the immune system. DNA detection by PCR in serum and bone marrow may thus be a valuable complement. If a B19 infection can be diagnosed correctly in cases of, for example, unclear anaemia in a patient with leukaemia, unnecessary cessation of chemotherapy and expensive investigations can be avoided. Furthermore, B19 infection can be treated in some cases with immunoglobulin preparations. Immunoglobulin is the only known treatment of chronic B19 infection but has also been described as a possible mode for transmission of the B19 virus (31). Many patients with haematological disorders are also immunosuppressed and might be more prone to develop a persistent B19 infection than are immunocompetent individuals, who usually clear their infection within a few weeks (23).

The present study thus showed that neither B19 DNA positivity in bone marrow nor viraemia is a general finding in this patient category. The 4 patients with B19 DNA in bone marrow had no obvious B19-associated symptoms at the time of bone marrow puncture. However, bone marrow suppression caused by their haematological disorder might be worsened by persistent B19 infection. Further studies are needed to elucidate the mechanism of viral persistence in patients with and without haematological disorders.

REFERENCES

1. Anderson LJ, Young NS, editors. Monographs in virology: human parvovirus B19. Vol. 20, 1997, Karger, Basel.
2. Saunders PW, Ried MM, Cohen BJ. Human parvovirus induced cytopenias: a report of five cases. Br J Hematol 1986; 63: 407–10.
3. Doran HM, Teall AJ. Neutropenia accompanying erythroid aplasia in human parvovirus infection. Br J Hematol 1988; 69: 287–8.
4. Nagai K, Morohoshi T, Kudoh T, Yoto Y, Suzuki N, Matsunaga Y. Transient erythroblastopenia of childhood with megakaryocytopenia associated with human parvovirus B19 infection. Br J Hematol 1992; 80: 131–2.
5. Inoue S, Kinra NK, Mukkamala SR, Gordon R. Parvovirus B-19 infection: aplastic crisis, erythema infectiosum and idiopathic thrombocytopenic purpura. Pediatr Infect Dis J 1991; 10: 251–3.
6. Potter CG, Potter AC, Hatton CS, Chapel HM, Anderson MJ, Pattson JR, et al. Variation of erythroid and myeloid precursors in the marrow and peripheral blood of volunteer subjects infected with human parvovirus (B19). J Clin Invest 1987; 79: 1486–92.
7. Brown KE, Anderson SM, Young NS. Erythrocyte P antigen: cellular receptor for B19 Parvovirus. Science 1993; 262: 114–7.
8. Liu W, Ittmann M, Liu J, et al. Human parvovirus B19 in bone marrows from adults with acquired immunodeficiency syndrome: a comparative study using in situ hybridization and immunohistochemistry. Hum Pathol. 1997; 28: 760–6.
9. Cassinotti P, Burtonboy G, Fopp M, Siegl G. Evidence for persistence of human parvovirus B19 DNA in bone marrow. J Med Virol. 1997; 53: 229–32.
10. Foto F, Saag KG, Scharosch LL, Howard EJ, Naides SJ. Parvovirus B19-specific DNA in bone marrow from B19 arthropathy patients: evidence for B19 virus persistence. J Infect Dis 1993; 167: 744–8.
11. Söderlund M, Brown CS, Spaan WJM, Hedman L, Hedman K. Epitope-type specific IgG responses to capsid proteins VP1 and VP2 of human parvovirus B19. J Infect Dis 1995; 172: 1431–6.
12. Hedman K, Lappalainen M, Söderlund M, Hedman L. Avidity of IgG in serodiagnosis of infectious diseases. Rev Med Microbiol. 1993; 4: 123–9.
13. Söderlund M, Brown CS, Cohen BJ, Hedman K. Accurate serodiagnosis of B19 parvovirus infections by measurements of IgG avidity. J Infect Dis 1995; 171: 710–3.
14. Broliden K, Tolfvenstam T, Ohlsson S, Henter. JI. Persistent B19 Parvovirus infection in pediatric malignancies. Med Ped Oncol 1998; 31: 66–72.
15. Kurtzman GJ, Ozawa K, Cohen B, Hanson G, Oseas R, Young NS. Chronic bone marrow failure due to persistent B19 parvovirus infection. N Engl J Med 1987; 317: 287–94.
16. Kurtzman GJ, Cohen B, Meyers P, Amunullah A, Young NS.

17. Graeve JLA, de Alarcon PA, Naides SJ. Parvovirus B19 infection in patients receiving cancer chemotherapy: the expanding spectrum of disease. Am J Pediatr Hematol Oncol 1989; 11: 441–4.
18. Weiland HT, Salimans MM, Fibbe WE, Kluin PM, Cohen BJ. Prolonged parvovirus B19 infection with severe anaemia in a bone marrow transplant patient. Br J Haematol 1989; 71: 300.
19. Frickhofen N, Abkowitz JL, Safford M, Berry JM, AntunezdeMayolo J, Astrow A, et al. Persistent B19 parvovirus infection in patients infected with human immunodeficiency virus type 1 (HIV-1): a treatable cause of anemia in AIDS. Ann Intern Med 1990; 113: 926–33.
20. Pont J, Puchhammer-Stöckl E, Chott A, Popow-Kraupp T, Kienzer H, Postner G, et al. Recurrent granulocytic aplasia as clinical presentation of a persistent B19 parvovirus infection. Br J Haematol 1992; 80: 160–5.
21. Kurtzman G, Frickhofen N, Kimball J, Jenkins DW, Nienhuis AW, Young NS. Pure red-cell aplasia of 10 years' duration due to persistent parvovirus B19 infection and its cure with immunoglobulin. N Engl J Med 1989; 321: 519–23.
22. Baurmann H, Schwarz TF, Oertel J, Serke S, Roggendorf M, Huhn D. Acute parvovirus B19 infection mimicking myelodysplastic syndrome of the bone marrow. Ann Hematol 1992; 64: 43.
23. Anderson MJ, Higgins PG, Davis LR, Willman JS, Jones SE, Kidd IM, Pattison JR, Tyrrell DA. Experimental parvoviral infection in humans. J Infect Dis 1985; 152: 257–65.
24. Gahr M, Pekrun A, Eiffert H. Persistence of parvovirus B19-DNA in blood of a child with severe combined immunodeficiency associated with chronic pure red cell aplasia. Eur J Ped 1991; 150: 470–2.
25. Faden H, Gary GW, Anderson LJ. Chronic parvovirus infection in a presumably immunologically healthy woman. Clin Inf Dis 1992; 15: 595–7.
26. Söderlund M, Ruutu P, Ruutu T, Asikainen K, Franssila R, Hedman K. Primary and secondary infections by human parvovirus B19 following bone marrow transplantation: characterization by PCR and B-cell molecular immunology. Scand J Infect Dis 1997; 29: 129–35.
27. Sasaki T, Murai C, Muryoi T, Takahashi Y, Munakata Y, Sugamura K, Abe K. Persistent infection of human parvovirus B19 in a normal subject. Lancet 1995; 346: 851.
28. Lundqvist A, Tolfvenstam T, Söderlund M, Broliden K. Clinical and laboratory findings in patients with persistent Parvovirus B19 DNA in bone marrow. Scand J Inf Dis 1999; 31: 11–6.
29. Söderlund M, von Essen R, Haapasaari J, Kiistala U, Kiviluoto O, Hedman K. Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. Lancet 1997; 349: 1063–5.
30. Nikkari S, Lappalainen H, Saario R, Lammintausta K, Kotilainen P. Detection of Parvovirus B19 in skin biopsy, serum, and bone marrow of a patient with fever, rash, and polyarthritis followed by pneumonia, pericardial effusion, and hepatitis. Eur J Clin Microbiol Infect Dis 1996; 15: 954–7.
31. Erdman DD, Anderson BC, Török TJ, Finkel TH, Anderson LJ. Possible transmission of Parvovirus B19 from intravenous immune globulin. J Med Virol 1997; 53: 233–6.

III

High frequency of parvovirus B19 DNA in bone marrow samples from rheumatic patients

Anders Lundqvist[a], Adiba Isa[b], Thomas Tolfvenstam[b], Göran Kvist[c], Kristina Broliden[b],*

[a] *Clinic of Infectious Diseases, Södra Älvsborg Hospital, S-50182 Borås, Sweden*
[b] *Division of Clinical Virology, F68 Karolinska Institutet, Karolinska University Hospital, Huddinge, S-14186 Stockholm, Sweden*
[c] *Department of Rheumatology, Clinic of Internal Medicine, Södra Älvsborg Hospital, S-50182 Borås, Sweden*

Abstract

Background: Human parvovirus B19 (B19) polymerase chain reaction (PCR) is now a routine analysis and serves as a diagnostic marker as well as a complement or alternative to B19 serology. The clinical significance of a positive B19 DNA finding is however dependent on the type of tissue or body fluid analysed and of the immune status of the patient.
Objectives: To analyse the clinical significance of B19 DNA positivity in bone marrow samples from rheumatic patients.
Study design: Parvovirus B19 DNA was analysed in paired bone marrow and serum samples by nested PCR technique. Serum was also analysed for B19-specific IgG and IgM antibodies and the results were compared with clinical and epidemiological data.
Results and conclusions: B19 IgG was found in 41 of 50 patients (82%) whereas none was B19 IgM positive. The serologic evaluation showed that none of the patients had acute B19 infection. However, B19 DNA was detected by PCR in 13 of 50 (26%) bone marrow samples from these patients indicating a high frequency of persistent infection compared with previous reports of patient groups and healthy controls. In the study, 22 patients had rheumatoid arthritis (RA) and 7 of these RA patients were B19 DNA positive in bone marrow. Rheumatoid factor was positive in 4 of the 7 B19 DNA positive RA patients as compared with Rheumatoid factor positivity in all of the 15 B19 DNA negative RA patients. Erosive arthritis in X-ray was less common in the B19 DNA positive group than in the B19 DNA negative group. A high frequency of parvovirus B19 DNA was thus detected in bone marrow samples in rheumatic patients. The clinical data does not support a direct association between B19 PCR positivity and rheumatic disease manifestation. Therefore, the clinical significance of B19 DNA positivity in bone marrow samples from rheumatic patients must be interpreted with caution.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Parvovirus B19; Persistence; Bone marrow; Arthritis; Diagnosis; PCR

1. Introduction

Detection of human parvovirus B19 (B19) DNA as a diagnostic tool in various tissues has been evaluated in many different clinical settings. These include analysis of B19 DNA by polymerase chain reaction (PCR) in placental and fetal tissues in intrauterine fetal deaths and in amniotic fluid or cord blood in hydrops fetalis (Tolfvenstam et al., 2001; Skjoldebrand-Sparre et al., 2000), in serum from bone marrow transplant recipients (Soderlund et al., 1997a), in heart tissue samples from multiorgan donors to assess transplant related complications (Mantke et al., 2004), and in myocardial tissue in fatal myocarditis (Papadogiannakis et al., 2002). While analysis of B19 DNA by PCR is a useful routine diagnostic tool in certain clinical settings and specific tissue samples, it is also essential to evaluate in which cases the finding of B19 DNA is not linked to disease. To learn more about the relevance of B19 DNA findings, the present study evaluates the frequency of B19 DNA positivity in bone marrow in patients attending a rheumatology clinic.

2. Materials and methods

2.1. Study group

Among all patients in the population ($n = 996$) who had visited the Department of Rheumatology at Södra Älvsborg Hospital, Borås, Sweden, between 1997 and 1999, 130 were selected (randomly selected from a list of all patients sorted by age) and were offered to participate in the study. A total of 50 of these accepted and were included and sampled between October 1997 and December 2001. The reason for not wanting to participate was usually because of the invasivity of bone marrow sampling. The diagnosis of patients in the study group ($n = 50$) and population ($n = 996$) respectively, are presented in Table 1. In the study group, 45 of 50 (90%) patients were women compared with 798 of 996 (80%) in the population. The mean age was 54 (median 55, range 33–79) and 57 (median 52, range 17–89) in the study group and population, respectively. Diagnostic criteria for diagnosis when applicable were in accordance with American College of Rheumatology (ACR).

2.2. Laboratory monitoring

Bone marrow puncture was performed in local anaesthesia in the posterior superior iliac spine and at the same occasion venous blood was drawn for routine analysis.

B19 viral DNA in serum and bone marrow samples was detected using a nested PCR described previously (Broliden et al., 1998), with the exception of the use of a modified outer forward primer (GGC AGC ATG TGT TAA AGT GG). The assay amplified a 284 bp fragment in the gene encoding the B19 NS-1 protein. Both serum and bone marrow samples were analysed after heat treatment at 94 °C for 10 min, followed by centrifugation at 12,000 rpm for 10 min.

B19 IgG and IgM were analysed using an enzyme immunosorbent assay (Biotrin International Ltd., Dublin, Ireland).

2.3. Ethics

Informed consent was obtained from the study participants. Ethical permission for the study was obtained from the Ethical Committees at the University of Gothenburg and Karolinska Institutet, respectively.

2.4. Statistical analysis

The chi-square test was used for statistical analysis.

3. Results and discussion

B19 IgG serology was positive in 41 of 50 (82%) randomly selected patients from a Rheumatology Unit, whereas B19 IgM was negative in all samples. B19 DNA was positive in 13 of 50 (26%) bone marrow samples from the same patients whereas all serum samples were B19 DNA negative. In the subset of rheumatoid arthritis (RA) patients, B19 DNA was positive in 7 of 22 patients (32%). Furthermore, B19 DNA positivity was scored as follows for the different diagnosis: 1 of 8 (systemic lupus erythematosus), 1 of 4 (Sjögren's disease), 0 of 1 (Wegener's granulomatosis, giant cell arthritis, mixed connective tissue disease and myalgia, respectively), 2 of 7 (arthralgia), and 2 of 6 (undefined connective tissue disease).

In the study group no significant difference was demonstrated between B19 DNA positive and B19 DNA negative patients regarding hematology variables, autoantibodies, X-ray findings and treatments (data not shown). Rheumatoid factor was significantly less common in B19 DNA positive RA patients than in B19 DNA negative RA patients: four of seven B19 DNA positive RA patients were Rheumatoid factor positive compared with 15 B19 DNA negative RA patients who were Rheumatoid factor positive ($p = 0.023$). The frequency of erosive arthritis as defined by X-ray was also less common in B19 DNA positive RA patients but the difference was not significant ($p = 0.09$) (Table 2). Radiological findings with rheumatoid joint destruction were seen in 5 of 7 B19 DNA positive RA patients and in 15 of 15 B19 DNA

Table 1

Distribution of diagnoses in the study group as compared with all patients attending the clinic during the study period

Diagnosis	Study group		Population	
	n	%	n	%
Patients with a defined systemic/rheumatic disease				
RA	22	44	592	59
SLE	8	16	43	4.3
Sjögren's disease	3	6	47	4.7
Wegener's granulomatosis	1	2	4	0.4
Giant cell arthritis	1	2	15	1.4
MCTD	1	2	9	0.9
Patients with isolated arthralgias, myalgias and UCTD				
Arthralgia	7	14	102	10.2
Myalgia	1	2	49	4.9
UCTD	6	12	25	2.5
Total number	50	100	996	100

RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; UCTD, undefined connective tissue disease.

Table 2

Characteristics of the 22 RA patients related to the presence of B19 DNA in bone marrow (ns: not significant)

Parameters in 22 RA patients	B19 DNA		p-Value
	Positive	Negative	
Erosive arthritis in X-ray	5/7	15/15	0.09, ns
Rheumatoid factor	4/7	15/15	0.023
Anti-nuclear antibodies	2/5	2/12	ns
Steroids	5/7	8/15	ns
Cytostatics	3/7	9/15	ns

negative RA patients. Steroid treatment was ongoing in 13 patients and cytostatics in 12 patients but no difference in B19 DNA frequency was demonstrated in various treatment groups or to treatment as such.

Infection of immunocompetent subjects with B19 was previously assumed to take a self-limited course followed by clearance of the virus from the host, except in a few cases where the virus persists for a longer time. In immunosuppressed individuals, B19 persistence is however a common outcome following acute infection and B19 DNA can be found in bone marrow samples for many years (reviewed in Heegaard and Brown, 2002a) with our without symptomatic anemia (LaMonte et al., 2004). B19 DNA has by now been found in other locations, such as synovial tissue both in B19-related arthritic patients and in healthy seropositive controls in rather high frequencies (Soderlund et al., 1997b). B19 DNA in synovial tissue is thus not diagnostic for B19 infection in patients with rheumatic disorders. We therefore wanted to evaluate the significance of B19 DNA in bone marrow samples in rheumatic patients since the bone marrow harbor the predominant target cells for the virus, namely the erythropoietic precursor cells. Bone marrow is therefore of interest for studying viral persistence. In our present group of 50 rheumatic subjects, none was B19 DNA positive in serum. As a reference value, the frequency of B19 viremia in voluntary blood donors has been estimated to range between 1/260 and 1/40000 (McOmish et al., 1993; Cohen et al., 1990; Yoto et al., 1995). In contrast to the B19 DNA negative serum samples, the detection of B19 DNA in bone marrow samples from our patients was high (26%). This can be compared with studies including healthy seropositive individuals (2%) (Heegaard et al., 2002b) and patients attending a hematology clinic (4%) (Lundqvist et al., 1999). The latter study was performed by us using the same sampling technique under comparable conditions as the present but is, however, a historical control. The average age of patients was 63 in the haematology study and 54 in the present group. The female/male quotient was 46/54 in the first study and 45/5 in the present. The PCR methods used were mainly identical but in the hematology study (showing 4% B19 DNA positivity) we used two PCR assays, which may underestimate the prevalence in the present rheumatology group (showing 26% B19 DNA positivity).

It is evident that laboratory tests detecting B19 DNA is not enough to prove the viral etiology in different diseases since viral DNA is also found in healthy seropositive controls. However, a correlation between B19 DNA in bone marrow and rheumatological disease cannot be excluded. The possibility of immune suppression by steroids or chemotherapy resulting in deficient clearance of virus and making the patients susceptible to infection was considered as an explanation to the B19 DNA persistence. However, no difference in treatment regimens was seen between B19 DNA positive and negative groups, respectively. There was no difference in other clinical parameters and B19 prevalence in different groups of diagnosis although the number of study subjects

did not allow statistical evaluation for all parameters. In the subgroup of RA patients, however, presence of B19 DNA in bone marrow was reversely correlated with presence of rheumatoid factor. B19 infection shares with many other viral infections an ability to induce the production of autoantibodies with and without organ specificity. This production is usually short-lived and is not associated with chronic symptoms. For example, autoantibodies such as rheumatoid factor may be positive after acute B19 infection and can misleadingly suggest early RA. We also found that erosive arthritis in X-ray was more common in B19 DNA negative RA patients than in positive. The difference, however, was not significant but might support previous reports describing definite B19 arthropathy as not erosive and perhaps a relation between B19 and RA with a less severe prognosis (Moore, 2000).

Attribution of a case of a specific rheumatic disorder to parvovirus B19 infection is difficult in the absence of serological confirmation of acute infection at onset. This is becoming even more apparent with the rapid development of new molecular technologies leading to the discovery of new viruses or previously defined viruses in new locations of the human body. For each of these new discoveries, relevance to disease and pathogenicity, persistence and the availability of diagnostic tests needs to be assessed. Long-term persistence of B19 DNA in locations such as peripheral blood, bone marrow and synovial tissue has been found in both RA and non-arthritic B19 seropositive individuals. Thus, the clinical implications of B19 DNA-positivity for each sample type (i.e. synovia, bone marrow, serum) must be settled.

Acknowledgements

References

Broliden K, Tolfvenstam T, Ohlsson S, Henter JI. Persistent B19 parvovirus infection in pediatric malignancies. Med Pediatr Oncol 1998;31:66–72.

Cohen BJ, Field AM, Gudnadottir S, Beard S, Barbara JA. Blood donor screening for parvovirus B19. J Virol Methods 1990;30:233–8.

Heegaard ED, Brown KE. Human parvovirus B19. Clin Microbiol Rev 2002a;15:485–505.

Heegaard ED, Petersen BL, Heilmann CJ, Hornsleth A. Prevalence of parvovirus B19 and parvovirus V9 DNA and antibodies in paired bone marrow and serum samples from healthy individuals. J Clin Microbiol 2002b;40:933–6.

LaMonte AC, Paul ME, Read JS, Frederick MM, Erdman DD, Han LL, Anderson LJ, Women and Infants Transmission Study. Persistent parvovirus B19 infection without the development of chronic anemia in HIV infected and uninfected children: the Women and Infants Transmission Study. J Infect Dis 2004;189:847–51.

Lundqvist A, Tolfvenstam T, Brytting M, Stolt CM, Hedman K, Broliden K. Prevalence of parvovirus B19 DNA in bone marrow of patients with haematological disorders. Scand J Infect Dis 1999;31:119–22.

Mantke OD, Nitsche A, Meyer R, Klingel K, Niedrig M. Analysing myocardial tissue from explanted hearts of heart transplant recipients and multi-organ donors for the presence of parvovirus B19 DNA. J Clin Virol 2004;31:32–9.

McOmish F, Yap PL, Jordan A, Hart H, Cohen BJ, Simmonds P. Detection of parvovirus B19 in donated blood: a model system for screening by polymerase chain reaction. J Clin Microbiol 1993;31: 323–8.

Moore TL. Parvovirus-associated arthritis. Curr Opin Rheumatol 2000;12:289–94.

Papadogiannakis N, Tolfvenstam T, Fischler B, Norbeck O, Broliden K. Active, fulminant, lethal myocarditis associated with parvovirus B19 infection in an infant. Clin Infect Dis 2002;35:1027–31.

Skjoldebrand-Sparre L, Tolfvenstam T, Papadogiannakis N, Wahren, Broliden K, Nyman M. Parvovirus B19 infection: association with third-trimester intrauterine fetal death. BJOG 2000;107:476–80.

Soderlund M, Ruutu P, Asikainen K, Franssila R, Hedman K. Primary and secondary infections by human parvovirus B19 following bone marrow transplantation: characterisation by PCR and B-cell molecular immunology. Scand J Infect Dis 1997a;29:129–35.

Soderlund M, von Essen R, Haapasaari J, Kiistala U, Kiviluoto O, Hedman K. Persistence of parvovirus B19 DNA in synovial membranes of young patients with and without chronic arthropathy. Lancet 1997b;349:1063–5.

Tolfvenstam T, Papadogiannakis N, Norbeck O, Petersson K, Broliden K. Frequency of human parvovirus B19 infection in intrauterine fetal death. Lancet 2001;357:1494–7.

Yoto Y, Kudoh T, Haseyama K, et al. Incidence of human parvovirus B19 DNA detection in blood donors. Br J Haematol 1995;91:1017–8.

IV

Cytokine responses in acute and persistent human parvovirus B19 infection

Adiba Isa, [1*] Anders Lundqvist, [1,2] Anna Lindblom,[1] Thomas Tolfvenstam,[1] and Kristina Broliden[1]

[1] Department of Medicine, Infectious Disease Unit, Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, Solna, Sweden

[2] Clinic of Infectious Diseases, Södra Älvsborg Hospital, Borås, Sweden

Running title: Cytokine profile in Parvovirus B19 infection

Keyword: human parvovirus B19, acute infection, persistent infection, cytokine

*Corresponding author

Adiba Isa, Department of Medicine, Solna, Infectious Disease Unit, Center for Molecular Medicine, CMM L8:03, Karolinska Institutet, Karolinska University Hospital, Solna,

SE-171 76 Stockholm, Sweden. Phone: +46-8-51 77 67 43, Fax: +46-8-313147, adiba.isa@ki.se

Abstract

The aim of this study was to characterize the pro-inflammatory and Th1/Th2 cytokine responses during acute parvovirus B19 (B19) infection and determine whether an imbalance of the Th1/Th2 cytokine pattern is related to persistent B19 infection. Cytokines were quantified by multiplex beads immunoassay in serum from B19 infected patients and controls. The cytokine responses were correlated to B19 serology, quantitative B19 DNA levels and clinical symptoms. In addition to a pro-inflammatory response, elevated levels of the Th1 type of cytokines IL-2, IL-12 and IL-15 were evident at time of the initial peak of B19 viral load in a few patients during acute infection. This pattern was seen in the absence of an IFNγ response. During follow-up (20-130 weeks post acute infection) some of these patients had a sustained Th1 cytokine response. The Th1 cytokine response correlated with the previously identified sustained CD8$^+$ T cell response and viremia. A cross-sectional study on patients with persistent B19 infection showed no apparent imbalance of their cytokine pattern except for an elevated level of IFNγ response. No general immunodeficiency was diagnosed as an explanation for the viral persistence in this later group. Neither the acutely infected nor the persistently infected patients demonstrated a Th2 cytokine response. In conclusion, the acutely infected patients demonstrated a sustained Th1 cytokine response whereas the persistently infected patients did not exhibit an apparent imbalance of their cytokine pattern except for an elevated IFNγ response.

Introduction

Parvovirus B19 (B19), a non-enveloped, single stranded DNA virus, is one of the smallest viruses known to infect mammalian cells. The viral genome (5kbp) encodes for three major proteins, the non-structural protein 1 (NS1) and the viral capsid proteins (VP1 and VP2). B19 is associated with a variety of clinical manifestations. The infection can be asymptomatic, or give a mild febrile illness (erythema infectiosum), associated with transient anaemia, exanthema and arthralgia. The clinical manifestations can also be more severe like cytopenia in immunocompromised individuals, and hydrops fetalis or intrauterine fetal death in pregnant women. B19 is classified as a lytic non-persistent virus that is normally transmitted through the respiratory route [1].

Until recently, it has thus been believed that B19 was cleared from the blood circulation shortly after production of neutralising antibodies, a few weeks to months post acute infection. However, by introducing a highly sensitive B19 DNA PCR quantitative assay, we have previously shown that viral clearance is much slower than earlier believed and viral DNA can be detected in peripheral blood of infected individuals more than two years post acute infection [2]. Interestingly, a sustained, activated and mature antigen-specific CD8^{+}T cell response accompanies this viremia and is also present in the circulation more than two years post primary infection [3, 4]. Thus, the classification of B19 as a rapidly clearing lytic infection in immunocompetent subjects should be re-considered. Consequently, since infected individuals eventually clear the infection in blood but still maintain a low CD8^{+}T cell response [2, 4], the virus may persist in other compartments and only replicate intermittently or at a low level in remotely infected individuals. A B19-specific T helper cell mediated response has also been detected in remotely infected individuals [5, 6]. It has been shown that B19 commonly persists in immunosuppressed individuals and in a few cases of immunocompetent individuals [1].

Cytokines mediate a number of important immunoregulatory functions and play a critical role in the modulation of the immune system. An aberrant pro-inflammatory cytokine profile or a later shift in the balance of the Th1/Th2 cytokine response may play a role in the control of the viral infection and lead to viral persistence [7]. Primary B19 infection has previously been associated with a mixed Th1/Th2 profile, with elevated levels of IFNγ, tumour-necrosis factor α (TNFα) and IL-6. An association between the symptoms related to B19 infection and elevation of several inflammatory cytokines was also shown [8, 9]. The objective of the present study was to characterize the kinetics of the pro-inflammatory and Th1/Th2 cytokine pattern in consecutively followed acutely infected patients and to determine whether an imbalance of this response predominates in individuals with persistent B19 infection. A better understanding of cytokine responses in relation to viral load, cellular and humoral immune responses as well as severity of infection in the individual patient may allow new therapeutic strategies for severe B19 infection.

3

Material and Methods

Study subjects

Group I, 'Acutely B19 infected patients'

Eight previously healthy adults (8 females, mean age 43 yrs, range 38-54 yrs) with B19 infection presenting with at least three out of the four symptoms fever, arthralgia, fatigue and rash, were included. They had early resolution of symptoms, except for two patients with mild transient arthralgia lasting for several months. All patients were prospectively identified after their serum samples had been referred to the Clinical Virology Laboratory at the Karolinska University Hospital, and were followed for 20-130 weeks (median 80 weeks). All patients had detectable B19 DNA in serum by PCR and anti-B19 IgM and IgG in serum at the first time point. Five of these patients have been presented in previous studies [2-4].

Group II, 'Persistently B19 infected patients':

Twenty-two (4 males, 18 females, mean age 45 yrs, range 31-62 yrs) individuals were included, based on persistent B19 DNA in bone marrow (BM). This was defined as at least two repeatedly B19 DNA positive BM samples collected with at least 6 month's interval. In total, 2-6 samples were collected per patient (mean time of follow-up were 33 months, range 6-110 months). In the patients followed for only a few months, a recent asymptomatic infection could not be excluded since the virus may not yet have been cleared after primary infection. Clinical data on the persistently infected individuals were as follow; fatigue 19/22; chronic fatigue syndrome 14/22; arthralgia 20/22; myalgia 18/22 and paresthesia 11/22. None of them had a history of repeated severe bacterial or other viral infections as a sign of a general immunodeficiency. All patients were B19 IgG positive and two were also B19 IgM positive for almost two years. Sixteen (seven and nine) of these patients have been presented previously, separately in two different study [10, 11].

Group III, 'Healthy control subjects'

Eighteen healthy B19 seropositive laboratory workers (12 male, 6 female, mean age 43 yrs, range 31-61 yrs) without any history of recent apparent symptoms were included in the study as controls. All were B19 IgG seropositive, B19 IgM seronegative and had no detectable B19 DNA in serum.

Ethical approval for the study was obtained from the local ethical Committees at the University of Gothenburg and Karolinska Institutet, Karolinska University Hospital, Sweden.

Parvovirus B19 Serology and PCR

B19 IgG and IgM were detected using Enzyme Immune Assay, EIA (Biotrin International Ltd, Dublin, Ireland) according to the manufacturer's instructions. Presence of B19 DNA in serum and BM was confirmed using a nested PCR with a sensitivity of 10^3 geq/ml [10] and a quantitative PCR with a sensitivity of 10^2 geq/ml [2].

Analysis of the general immune status of the persistently infected individuals

To determine whether the persistently infected individuals (group II) had an underlying immunodeficiency, an extended evaluation was performed according to routines at the Clinical Immunology Laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden.

HLA-typing: The HLA antigen expression was analysed by the Tissue Typing Laboratory (Sahlgrenska University Hospital, Gothenburg, Sweden). HLA class I antigen expression was determined by serology. Lymphocytes in peripheral blood were isolated and determinations of HLA antigens were done using a complement-dependent cytotoxicity technique (CDC). HLA-DR antigen genotyping was performed using a PCR-SSP technique [12].

Immunophenotype of peripheral blood mononuclear cells: FACS analysis was performed using PBMC stained with directly flurochrome-conjugated anti-human CD3, CD4, CD8, CD16, CD19, CD56 and HLA-DR (BD Biosciences, Mountain View, CA, USA). PBMC were incubated at 4° C for 15 minutes with antibodies. Cells were washed and later fixed with 1-2% formaldehyde. Cell acquisition was performed using FACS Calibur with CellQuest software (Becton Dickinson, Stockholm, Sweden). The absolute number of lymphocytes was determined using Sysmex K-4500 cell counter (TOA Medical Electronics Co, Japan). The results for each subpopulation were expressed as the percentage of lymphocytes and as the number of cells x 10^9/L.

Enzyme linked immunospost (ELISpot) assay: The ex vivo cellular immune responses were measured by IFNγ ELISpots. Briefly, 96-well nitrocellulose bottomed plates (Multiscreen-HA plates; Milipore, Mulsheim, France) were coated with100µl, 15 µg/ml anti-IFNγ monoclonal antibody overnight at 4^oC (Mabtech, Stockholm, Sweden). After washing 5 times, duplicates of different concentration of cells ($5x10^4$, $2x10^4$, 10^4 of cells)/ well were supplemented in 200µL medium. Con A was added in 25Mg/ml and the cells were incubated for 48h at 37° C with 5 % CO_2. 100µL of 1µg/ml biotin-conjugated anti-IFNγ (Mabtech, Stockholm, Sweden) was added and incubated at room temperature for 2h. 100µL streptavidin conjugated alkaline phosphatase (4µg/ml) was added per well. Plates were washed 5 times with 0.05M Tris buffer pH: 9.5 and the spots were developed with 100µL of BCIP-NBT (Life Technologies, Heidelberg). One spot was considered to represent an IFNγ secreting cells, and counted using a dissection microscope. The results were reported as mean spot-forming cells (SFC) per million PBMC.

Quantification of cytokines

Twelve different cytokines, IL-1beta, IL-2, -4, -5, -6, -8, -10, -12, -15, GM-CSF, INFγ and TNF-α, were quantified in serum from all individuals. A commercially available multiplex beads immunoassay, based on the Luminex platform (Biosource International, Inc, Camarillo, California, USA) was used according to the procedure of the manufacturer. All samples were run in duplicates. Briefly, beads with defined spectral property were conjugated to the analyte-specific capture antibodies. Beads, samples, standards and the controls were pipetted in a filter bottomed 96 well plate and incubated for 2h while shaking (550rpm). After three washes the biotinylated detector antibodies were added to the beads and incubated for one hour at the room temperature. Streptavidin conjugated to the R-Phycoerythrin (SA-PE) was added to the wells after several washings and incubated for 30 min. By monitoring the spectral properties of the beads and the amount of fluorescence associated with PE, the instrument measures the concentration of the analytes presented in the original specimens. The data (mean fluorescence intensity) was analyzed using a Luminex reader (Luminex, Austin, TX), and the mean concentration was calculated as pg/ml serum.

Statistical analysis

Statistical analysis was performed with Graph Pad Instat and Epi Info.

Results

Dominance of a Th1 cytokine response in acute B19 infection

Eight individuals (group I) were studied regarding B19 serology, B19 DNA and cytokine profile during and following acute B19 infection (20-130 weeks of follow-up). The first serum sample was collected within one to five weeks after onset of acute symptoms. B19 IgM was detected in all patients from study start and up to 17 weeks post primary infection, whereas B19 IgG was present in all serum samples throughout the study. B19 DNA was detected for the entire period for all but one patient who cleared the infection after 108 weeks.

The presence and concentration of cytokines were tested longitudinally in the serum samples representing all individuals. Several pro-inflammatory cytokines (IL-1ß, GM-CSF, IL-6, TNFα, and chemokine IL-8) were elevated about 2-65 fold within the first weeks of acute infection in two of eight individuals (Fig. 1). Thereafter the concentrations decreased to low or undetectable levels. The cytokine levels in the other six patients were <5 pg/ml during the entire follow-up, except for the chemokine IL-8 that rose transiently in three patients at one or two time points each without obvious relations to any other markers. All pro-inflammatory cytokines were very low in the healthy seropositive individuals, except for two subjects who showed high levels of IL-8 (data not shown).

The concentrations of the Th1 cytokines IL-2, IL-12 and IL-15 were elevated in the first weeks following infection in some of the patients (figure 2). These three Th1 cytokines peaked in patient, P2, in a sample collected at 3 weeks following onset of acute symptoms (Fig. 2a). IL-2 and IL-12 levels decreased at week 20, whereas IL-15 levels remained at about 600 pg/ml. Patient 3 was not sampled until 5 weeks post onset of symptoms but had higher levels of IL-2, IL-12 and IL-15 as compared with the sample collected at week 10 (Fig. 2b). Interestingly, patient, P1, suddenly peaked in viral load (from 10^4 to 1.5×10^5 DNA copies/ml) after about 1 year of stable titers. This coincided with a peak of IL-2, IL-12 and IL-15, followed by a loss of viremia some months later (Fig. 2c).

The Th1 cytokine responses in the other five patients were high and stable. IL-2, IL-12 and IL-15 were elevated in patients P4 and P7, IL-12 and IL-15 were elevated in patients, P6 and P8, and only IL-12 was elevated in patient, P5 (data not shown).

However, in contrast to the other Th1 cytokines, the levels of IFNγ (except P2 at weeks 1- 3) were not elevated. The Th2 cytokines IL-10, IL-4 and IL-5 were <5 pg/ml in all patients (except for a raised value of IL-10 in patient P4 at week 5) (data not shown).

Persistently B19 infected individuals do not have a general immunodeficiency

Extensive investigations were performed to evaluate the HLA pattern and immunological condition of the persistently B19 infected patients (group II). HLA class I and II typing, immunophenotyping of PBMC and also the capacity of producing IFNγ after Con A stimulation, were performed. The frequency of HLA distribution was compared with 40928 individuals in the Tobias Registry, the Swedish register of BM donors. No significant differences in frequencies of HLA I types were found with one exception. HLA A*9 was more common in persistently infected individuals (8 of 22, about 36%), compared to the Tobias registry (about 18%), a difference which was statistically significant (p value <0.047, Fisher's exact test). The frequency of HLA DR did not show any significant difference as compared to a study on healthy Swedish individuals (Fisher's exact test) [12]. The frequencies of lymphocytes and NK cells did not reveal any abnormalities as compared to a reference group [13]. Six study patients had subnormal levels of CD19$^+$B-cells ($< 0,1x10^9$) but gel electrophoresis did not reveal any deficiency in immunoglobulin distribution. The capacity of IFNγ production after Con A stimulation displayed no difference between persistent B19 infected individuals and controls (blood donors). All individuals mounted high IFNγ responses to ConA, more than 400 spots/ 10^6 PBMC.

These assays were performed at an accredited clinical immunology laboratory and the results were interpreted according to standard reference values. In summary, we could, not detect any general immunodeficiency, which could explain the viral persistence of B19 in this group.

Cytokine responses; some persistently B19 infected individuals have an elevated IFNγ response

All persistently B19 infected individuals (group II) had detectable B19 DNA in BM for at least 6-110 months as tested by nested PCR. B19 DNA in serum could only be detected in seven of the 22 individuals. Cytokine levels in serum showed significantly higher concentrations of IFNγ (p-value 0.004, t-test) as compared with healthy seropositive controls (group III) (Fig. 3). However, only 10 of 22 patients had levels above 100 pg/ml of IFNγ. The mean concentration of the other pro-inflammatory or Th1/Th2 associated cytokines did not differ significantly when comparing persistently infected individuals with healthy seropositive controls (data not shown).

Discussion

Acute B19 infection is associated with extensive replication of B19 virus in erythroid precursor cells in the bone marrow. We recently reported that this results in persistent viremia despite development of neutralizing antibodies and a B19-specific CD8^{+}T cell response [3]. The lack of viral resolution is contradictory and we have therefore dissected the fine-tuned cytokine immune response in consecutive samples from eight acutely infected patients and followed them for a minimum of 20 weeks. An initial peak of pro-inflammatory cytokines (IL-1b, TNFα, IL-6 and the chemokine IL-8) was found at onset of acute B19 infection in 2 of the 8 patients. This peak may have been missed in the other patients since the samples were collected within one to five weeks after onset of symptoms. However, an induction of the Th1 type of cytokines IL-2, IL-12 and IL-15 was seen already in the earliest available samples and this expression was sustained during the follow-up period in many patients. In contrast, cytokines associated with a Th2 type of immune response (IL-4, IL-5, IL-10) as well as an IFNγ responses remained low during the observation time. Despite the lack of these Th2 cytokines the patients developed normal B19-specific IgG levels. These antibodies may have been induced by a low-grade IL-6 response as well as other cytokines not studied here (i.e. TGF-ß).

With regard to the adaptive immune response, IL-12 was the first detectable Th1 type of cytokine followed by IL-2 and IL-15 elevations. The induction of these cytokines was linked in time to some of the patients who have previously been assessed for B19 specific CD8^{+}T cell responses which increased in magnitude, matured and remained activated over time [4]. Together with IL-7, IL-15 appear to be important in up-regulation of Bcl-2 in antigen-specific activated T cells and thus of critical importance for the survival of the effector CD8^{+} T cells during the contraction phase [14]. However, IL-15 does not seem to be mandatory for the expansion of the effector T-cell during a viral infection [15]. Together with the sustained IL-2 and IL-12 expression, the IL-15 expression presented here is thus in line with our previous results with the detection of an activated and mature B19-specific CD8^{+} effector T-cell (CD38^{+}CD57^{+}perforin^{+}) population, long after resolution of B19 related symptom, but during the time of a continuous low-level B19 viremia. This kind of vigorous cytokine and CD8^{+}T cell response has not previously been seen for lytic viruses in humans, but rather resembles antigen persistent virus infections such as EBV.

However, despite the presence of a Th1 cytokine response there was no detectable serum concentration of IFNγ. This was indeed a surprising finding since IL-12 and IL-2 production normally is linked to significant IFNγ synthesis. We also assessed samples from patients with chronic inflammatory conditions and found significantly increased IFNγ levels in these patients (data not shown). We therefore do not believe that lack of detectable IFNγ in our acutely infected patients were due to technical reasons. Our previous study showed that short time *in vitro* culture with B19-antigens induced a significant IFNγ production as measured by ELISpot during more than two years following acute B19 infection [3]. Together, these data indicate that the persistent B19 viremia in our acutely infected patients may be associated with an aberrant cytokine profile *in vivo* with a selective IFNγ deficiency despite the Th1 cytokine induction.

It has indeed been possible to link the cytokine responses to previous studies on the CD8^{+}T cell responses in detail in some of the patients. For example, one of the patients (P1) had a second viremic period (>1.5 log increase of viral load) about one year after the acute B19 infection. Interestingly, the peak of viremia coincided with a peak of the CD8^{+}T cell response [3] and the peak of IL-2, IL-12 and IL-15 levels. A few weeks before this peak, a loss of one of the three epitope-specific CD8^{+}T-cell responses were noted and the frequency of B19 specific CD8^{+}T cells were decreased to much lower levels around this time as defined by tetramer staining [4]. It is not possible to determine whether the increase of the viral load was due to a new infection or reactivation of the primary infection, the loss of one of three epitope specific CD8^{+}T cells or a result of a viral mutation to escape the immune response. Surprisingly, this patient was the only individual in this study that cleared the viremia during follow up (at 108 weeks), maybe as a result of the boosted immune response.

One previous study has correlated B19 infection with a mixed Th1/Th2 profile with elevated levels of IFNγ, TNFα and IL-6 at acute B19 infection [9]. Another study demonstrated expression of mRNA for IFNγ and IL-2 about two weeks post symptom presentation in a child with acute B19 infection [16]. These differences to our study could possibly be explained by the timing of the first sample in relation to onset of primary infection as well as to severity of acute infection and to the fact that we followed our patients with the consecutive samples for a long time period. Our patients were previously healthy and resolved their B19-associated clinical symptoms within two months time, thus representing the vast majority of acutely infected patients by not developing chronic fatigue or long-lasting severity arthralgia. The pathogenesis of B19 infection with transient symptoms but persistence of viremia for more then 2 years is unusual when compared to most systemic viral infections. In most settings, chronic viremia is associated with clinical symptoms or findings. For instance chronic Hepatitis B infection (deficiency in the cytotoxic T cell response) and chronic Hepatitis C infection (IFNα deficiency) results in liver damage, HIV-1 infection (lack of polyfunctional CD8^{+}T cells) in CD4^{+}T cell decline, EBV (lack of perforin and induction of viral IL-10) in lymphoproliferative conditions, and severe CMV (down-regulated MHC class I and cytotoxic T cells) in a broad spectrum of symptoms. In all these examples abnormal immune responses result in impaired T cell mediated cytotoxic response. All these viruses are associated with selective disturbances in their cytokine response pattern [7, 17-19]. Furthermore, complete induction of Th1 immune responses is normally associated with elimination of the pathogen via cytotoxic activity. Our finding of lack of IFNγ in vivo during a prolonged period from acute B19-infection is therefore surprising and abnormal in the context of other generalized viral infections.

We were not able to follow the primary B19 infected patients for more than 2 years and could thus not determine at which time point the Th1 cytokines, T cell responses and viremia decreased to the undetectable levels observed in seropositive healthy individuals [4]. Neither did we follow a large enough group of acutely infected patients to determine whether an imbalance of the cytokine pattern was correlated with a persistent status of the infection as seen in a few percent of infected patients [20, 21]. One may speculate that activated B19-specific CD8^{+}T cells do not migrate to the bone marrow that harbour the virus-infected cells or that the cells may not be fully functioning *in vivo*. Our and other studies [3, 5, 6] have demonstrated B19-specific cytotoxic and helper T cell activity after *in vitro* stimulation. In order to evaluate the relevance of these assays it is of vital interest to study these phenomena *in vivo* at the site of virus replication, in this case the bone marrow, since the concentration of cytokines at different sites my vary.

We next analysed the cytokine pattern in a group of persistently B19 infected individuals who remained B19 DNA positive in bone marrow samples for at least six months, several of them for years, following acute infection. Initially we performed an extensive immunological examination to determine whether these individuals suffered from a general immunodeficiency that could explain the B19 persistence. Some patients had subnormal levels of NK-cells and CD19^{+}B-cell in blood (as compared to established reference values) but without any immunoglobulin distribution deficiencies and the evaluation did not reveal any general underlying immunodeficiency disorders in the patients. However, HLA A9 was more common among persistently B19 infected patients than in controls. A relation between HLA-DR*4 and symptomatic acute B19 infection has been described previously. As seen for many other infections, individuals can be sensitive to microbial agents due to selective properties in their immunological repertoire. Our finding, overrepresentation of HLA A9 in persistent B19 infection has not been described before and is difficult to interpret. However, by extensive evaluations of genetic (unpublished data), humoral [22] and cellular [11] immune responses we have only found one type of defect that discriminates persistently infected individuals from healthy seropositive subjects: a shift in the *in vitro* T cell immune response from the B19 NS1 to the B19 viral capsid proteins [11]. In the present study we aimed to determine whether a difference in the cytokine response was further associated with the persistent status of the infection. An imbalance of Th1/Th2 cytokines has been suggested to be associated with the chronicity of hepatitis C virus (HCV) [7]. However, only higher levels of IFNγ as compared with acutely and remotely B19 infected patients were found in our persistently infected individuals. This probably indicated an unspecific response and cannot possibly explain the persistent status of the B19 infection or the aberrant T cell response as described above.

It seems clear that the previous designation of B19 as a lytic non-persistent virus needs modification since the infection neither leads to rapid viral clearance nor contraction of the initial T cell burst [23, 24]. The virus rather seems to have characteristics of both persistent and lytic non-persistent viruses by now also demonstrating that the Th1 cytokine levels were sustained at high levels. This further confirms our hypothesis of describing B19 infection as a new type of host-virus relationship not seen in any other "acute" human viral infection.

References

1. Young NS and Brown KE, *N Engl J Med* **350**, 586-97, 2004.
2. Lindblom A, Isa A, Norbeck O, Wolf S, Johansson B, Broliden K and Tolfvenstam T, *Clin Infect Dis* **41**, 1201-3, 2005.
3. Norbeck O, Isa A, Pohlmann C, Broliden K, Kasprowicz V, Bowness P, Klenerman P and Tolfvenstam T, *J Virol* **79**, 12117-21, 2005.
4. Isa A, Kasprowicz V, Norbeck O, Loughry A, Jeffery K, Broliden K, Klenerman P, Tolfvenstam T and Bowness P, *PLoS Med* **2**, e343, 2005.
5. Franssila R, Hokynar K and Hedman K, *J Infect Dis* **183**, 805-9, 2001.
6. Franssila R and Hedman K, *Vaccine* **22**, 3809-15, 2004.
7. Spanakis NE, Garinis GA, Alexopoulos EC, Patrinos GP, Menounos PG, Sklavounou A, Manolis EN, Gorgoulis VG and Valis D, *J Clin Lab Anal* **16**, 40-6, 2002.
8. Kerr JR, Cunniffe VS, Kelleher P, Coats AJ and Mattey DL, *J Med Virol* **74**, 147-55, 2004.
9. Kerr JR and Tyrrell DA, *Curr Pain Headache Rep* **7**, 333-41, 2003.
10. Lundqvist A, Isa A, Tolfvenstam T, Kvist G and Broliden K, *J Clin Virol* **33**, 71-4, 2005.
11. Isa A, Norbeck O, Hirbod T, Lundqvist A, Kasprowicz V, Bowness P, Klenerman P, Broliden K and Tolfvenstam T, *J Med Virol* **78**, 129-33, 2006.
12. Olerup O and Zetterquist H, *Tissue Antigens* **39**, 225-35, 1992.
13. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, Hooijkaas H and van Dongen JJ, *J Pediatr* **130**, 388-93, 1997.
14. Yajima T, Yoshihara K, Nakazato K, Kumabe S, Koyasu S, Sad S, Shen H, Kuwano H and Yoshikai Y, *J Immunol* **176**, 507-15, 2006.
15. Becker TC, Wherry EJ, Boone D, Murali-Krishna K, Antia R, Ma A and Ahmed R, *J Exp Med* **195**, 1541-8, 2002.
16. Bluth MH, Norowitz KB, Chice S, Shah VN, Nowakowski M, Josephson AS, Durkin HG and Smith-Norowitz TA, *Clin Immunol* **108**, 152-8, 2003.
17. De Rosa SC, Lu FX, Yu J, Perfetto SP, Falloon J, Moser S, Evans TG, Koup R, Miller CJ and Roederer M, *J Immunol* **173**, 5372-80, 2004.
18. Becker Y, *Virus Genes* **28**, 5-18, 2004.
19. Rico MA, Quiroga JA, Subira D, Castanon S, Esteban JM, Pardo M and Carreno V, *Hepatology* **33**, 295-300, 2001.
20. Heegaard ED, Petersen BL, Heilmann CJ and Hornsleth A, *J Clin Microbiol* **40**, 933-6, 2002.
21. Lundqvist A, Tolfvenstam T, Brytting M, Stolt CM, Hedman K and Broliden K, *Scand J Infect Dis* **31**, 119-22, 1999.
22. Lundqvist A, Tolfvenstam T, Bostic J, Soderlund M and Broliden K, *Scand J Infect Dis* **31**, 11-6, 1999.
23. Hou S, Hyland L, Ryan KW, Portner A and Doherty PC, *Nature* **369**, 652-4, 1994.
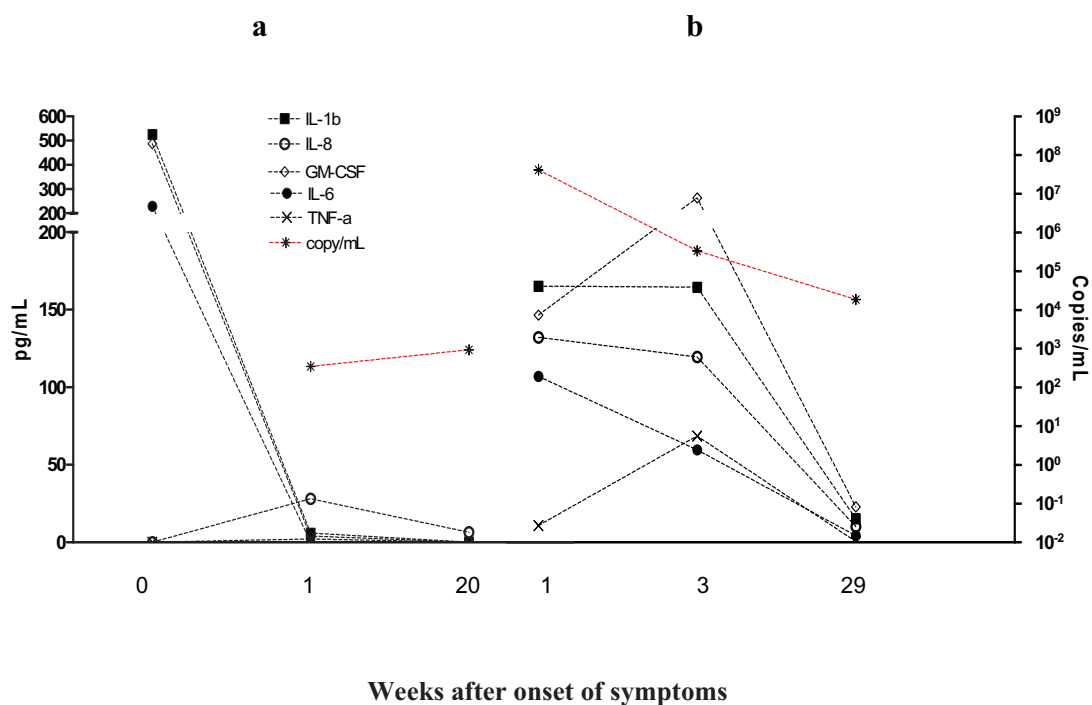24. Badovinac VP, Porter BB and Harty JT, *Nat Immunol* **3**, 619-26, 2002.

Figure 1. Pro-inflammatory cytokine levels in serum of acutely B19 infected individuals. Concentration of the pro-inflammatory cytokines TNF-α, IL-1ß, GM-CSF, IL-6, IFNγ and chemokine IL-8 as well as B19 viral load in serum is shown for two acutely B19 infected individuals, a) P7 and, b) P2. The time points designate number of weeks after onset of symptoms.
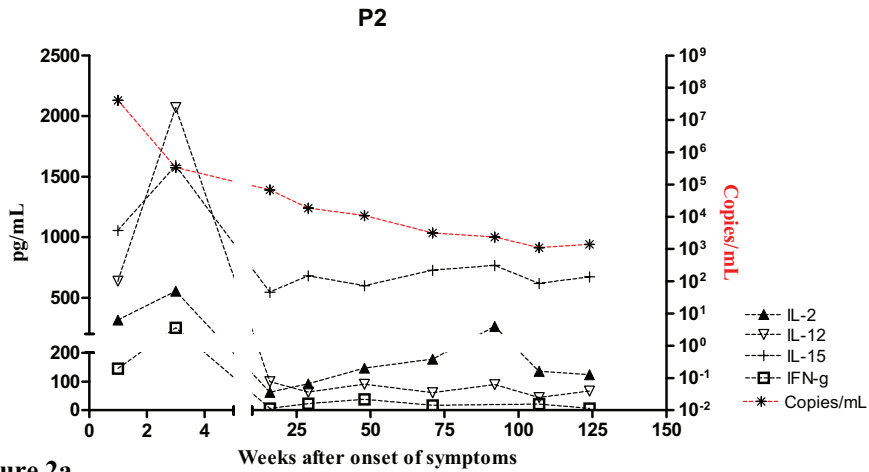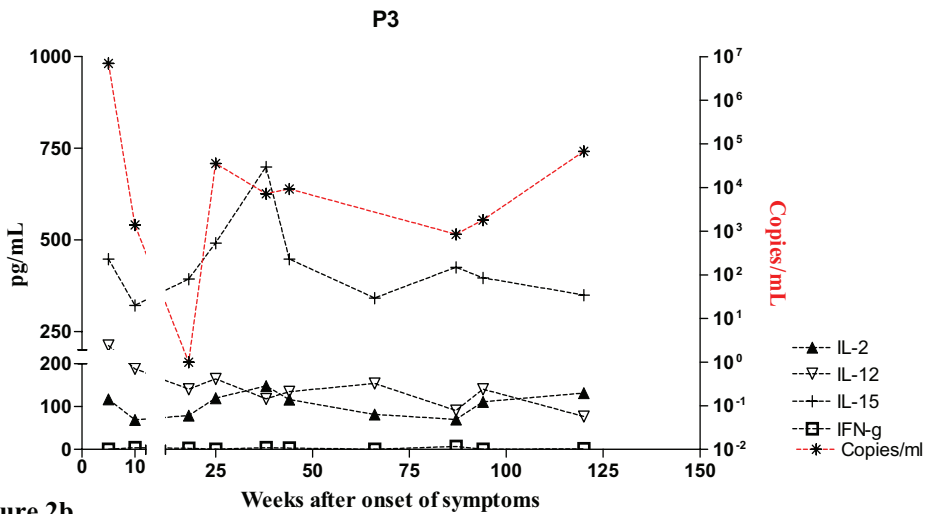
Figure 2a

Figure 2b

Figure 2. **Th1 cytokine levels in serum of acutely B19 infected individuals.**
Concentrations of the Th1cytokines IL-2, IL-12, IL-15 and IFNγ are shown for three
patients: a) P2 and b) P3 and c) P1. The B19 viral load in serum is shown as DNA
copies/ml. Mean levels of IL-2, IL-12, IL-15 and IFNγ for the healthy seropositive
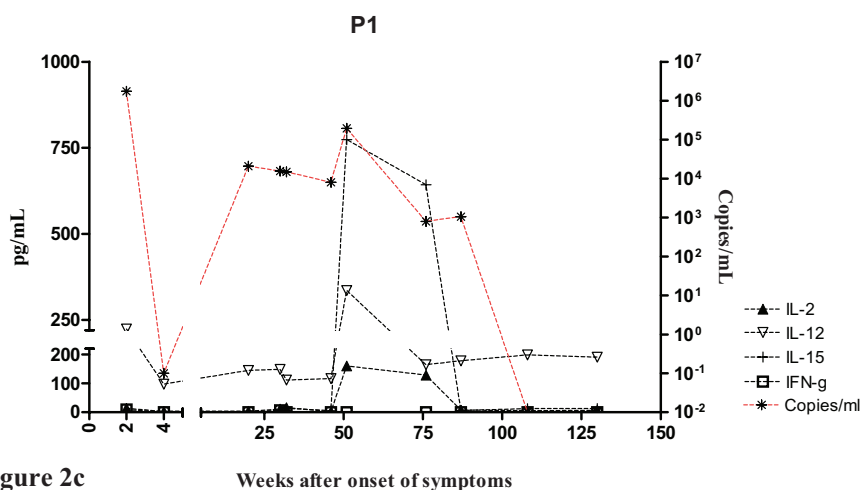controls were 106, 125, 505 and 5 pg/ml, respectively.

P1

Figure 2c

Weeks after onset of symptoms
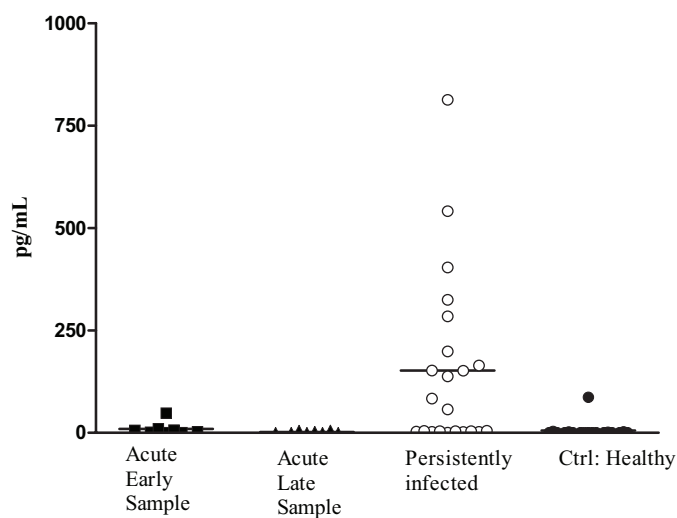
Legend: IL-2, IL-12, IL-15, IFN-g, Copies/ml

Figure 3. IFNγ levels of acutely and persistently B19 infected individuals.
The concentrations of IFNγ were compared between the different study groups.
"Acute": group I, acutely infected individuals (n=8). The "acute early sample" was
collected within one to five weeks of symptom appearance. The "acute late sample"
was collected after 20-130 weeks of follow up. "Persistently infected": group II,
persistently infected individuals (n=22). "Ctrl: Healthy": group III, healthy B19
seropositive individuals (n=18).

V

Aberrant Cellular Immune Responses in Humans Infected Persistently With Parvovirus B19

Adiba Isa,[1]* Oscar Norbeck,[1] Taha Hirbod,[1] Anders Lundqvist,[1,2] Victoria Kasprowicz,[3,4] Paul Bowness,[3] Paul Klenerman,[4] Kristina Broliden,[1] and Thomas Tolfvenstam[1,5]

[1]Infectious Disease Unit, Institution for Medicine, Center for Molecular Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden
[2]Department of Infectious Diseases, Borås Hospital, Borås, Sweden
[3]Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford University, Oxford, United Kingdom
[4]Peter Medawar Building for Pathogen Research, Nuffield Department of Medicine, Oxford University, Oxford, United Kingdom
[5]Division of Clinical Virology, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

A subset of parvovirus B19 (B19) infected patients retains the infection for years, as defined by detection of B19 DNA in bone marrow. Thus far, analysis of B19-specific humoral immune responses and viral genome variations has not revealed a mechanism for the absent viral clearance. In this study, ex-vivo cellular immune responses were assessed by enzyme linked immunospot assay mounted against the majority of the translated viral genome. Compared to seropositive healthy individuals, individuals with B19 persistence (2–8 years) showed larger number of responses to the structural proteins ($P = 0.0022$), whereas responses to the non-structural protein were of lower magnitude ($P = 0.012$). These observations provide the first findings of immunological discrepancies between individuals with B19 persistence and healthy individuals, findings that may reflect both failed immunity and antigenic exhaustion. *J. Med. Virol. 78:129–133, 2006.*
© 2005 Wiley-Liss, Inc.

KEY WORDS: erythrovirus; viral persistence; cellular immunity; T-lymphocyte responses

INTRODUCTION

Human parvovirus B19 (B19) is one of the smallest viruses known to infect mammalian cell and replicates primarily in erythrocyte precursors in bone marrow. Parvovirus B19 is a member of the *Parvoviridae* family and subfamily *Parvovirinae*. Since B19 replicates autonomously in erythroid progenitor cells it is classified in the third genus *Erythrovirus*. It is a single stranded DNA virus with icosahedral symmetry. The viral genome (5.5 kb) encodes of three main proteins,

the non-structural protein NS1, and the capsid proteins VP1 and VP2. The two-capsid proteins arise by alternative splicing with VP2 accounting for 96% of the viral capsid composition. B19 uses the erythrocyte P antigen receptor for infection of immature erythropoietic cells as well as an integrin co-receptor $\alpha5\beta1$. B19 is ubiquitous in society with a seroprevalence in the adult population of 70%–80%. The virus causes the self-limiting childhood disease erythema infectiosum with fever and rash. However, the infection can give severe complications in pregnant women causing fetal death or hydrops fetalis. It may also cause transient aplastic crisis in individuals with underlying hematological disorders and severe cytopenia in immunocompromised persons, reviewed by Heegaard and Brown [2002] and Weigel-Kelley et al. [2003]. Neutralizing antibodies are believed to mediate both clearance of the virus and long-term protective immunity after resolution of the infection. Both neutralizing long-term humoral and proliferative responses have been shown elicited by the virus structural proteins, in particularly the unique region of VP1 [Musiani et al., 2000; Franssila et al., 2001; Zuffi et al.,

2001]. Fewer studies have been carried out concerning the cytotoxic T-cell responses directed to the virus, but acutely infected patient exhibit strong responses targeting mainly the NS1 protein [Tolfvenstam et al., 2001; Klenerman et al., 2002; Norbeck et al., 2005].

A proportion of apparently immunocompetent persons retain B19 DNA in their bone marrow for years (hereafter referred to as "persistently infected") [Faden et al., 1992; Cassinotti et al., 1993; Sasaki et al., 1995; Lundqvist et al., 1999a]. This is reported rare in healthy B19 seropositive individuals, although the virus is probably less rapidly cleared in normal hosts than thought previously [Lundqvist et al., 1999b; Heegaard et al., 2002; Lindblom et al., 2005].

Assessment of viral and host factors in B19 persistently infected individuals have demonstrated B19 neutralizing antibodies with normal epitope distribution [Tolfvenstam et al., 2000] and a high viral sequence homology to that of B19 strains isolated from transient infections (unpublished data, GeneBank accession no: AY028223-57) [Servant et al., 2002]. The distribution of the cellular immune responses to B19 in persistent infected and healthy seropositive individuals was examined in an attempt to clarify the pathogenesis of the absent viral clearance.

MATERIALS AND METHODS

Study Subjects

The ex-vivo cellular immune responses in two different cohorts were investigated. Fourteen healthy seropositive individuals (serum B19 DNA negative, IgG-positive, and IgM-negative), 7 female and 7 male (mean age 40.4, range 27–59 years), and nine persis-tently infected individuals (IgG-positive and B19 IgM-negative), 7 female and 2 male (mean age 49.3 years, range 31–65 years), were included in the study. The latter group had been referred to the Department of Infectious Diseases in Borås, Sweden, and suffered from fatigue and symptoms including fever, recurrent rash, chronic arthralgia, and other inflammatory manifestations that were unexplained despite thorough clinical and laboratory investigation. They all had detectable B19 DNA in their bone marrow, during the entire follow-up, which ranged from 2 to 8 years. In addition, three B19 IgG-negative and IgM-negative healthy persons were included as controls for assay specificity.

Serology, PCR, and HLA-Typing

B19 IgG and IgM were tested by a commercial enzyme immunoassay (Biotrin, Inc., Dublin, Ireland). A nested PCR was used for B19 DNA detection, with a sensitivity of 10^3 copies/ml [Tolfvenstam et al., 2001]. HLA class I tissue typing was performed by multiplex PCR (SSP Unitray HLA-ABC Kit, ABC SSP Unitray Biotech, Oslo, Norway).

IFN-γ ELISpot

The ex-vivo cellular immune responses were measured in terms of antigen-specific interferon-γ (IFNγ) secretion by an enzyme-linked immunospot assay, as previously described [Larsson et al., 1999], with some modification. Briefly, a panel of 210 overlapping peptides were synthesized by Fmoc chemistry, representing the entire B19 NS1 and VP1 and two proteins, representing 92% of the total viral protein sequence,

TABLE I. Peptide Specifications

Peptide pool	Protein	No. of peptides	Peptide length[a] (aa)	Overlap (aa)	Coverage[b] (aa)
NS-1	NS1	10	15	10	1–65
NS-2	NS1	10	15	10	56–115
NS-3	NS1	10	15	10	106–165
NS-4	NS1	10	15	10	156–215
NS-5	NS1	10	15	10	206–265
NS-6	NS1	10	15	10	256–315
NS-7	NS1	10	15	10	306–365
NS-8	NS1	10	15	10	356–415
NS-9	NS1	10	15	10	406–465
NS-10	NS1	10	15	10	456–515
NS-11	NS1	10	15	10	506–565
NS-12	NS1	10	15	10	556–615
NS-13	NS1	10	15	10	606–665
NS-14	NS1	3	15 (11)	10	656–671
VP-1	VP1	10	20	10	1–110
VP-2	VP1	10	20	10	101–210
VP-3	VP1	2	20 (17)	10	201–227
VP-4	VP2	10	20	10	1–110
VP-5	VP2	10	20	10	101–210
VP-6	VP2	10	20	10	201–310
VP-7	VP2	10	20	10	301–410
VP-8	VP2	10	20	10	401–510
VP-9	VP2	5	20 (14)	10	501–554

[a]Numbers in parenthesis refer to the lengths of the last peptide in the respective protein products. aa, amino acid.
[b]Numbering according to Shade et al. [1986].

as published by Shade et al. [1986] (Table I). Peripheral blood mononuclear cells (PBMCs) were separated from lithium-heparinized whole blood by Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden) and used in the assay within 8 hr after venesection. Ninety-six-well IPS-lined microtiter plates (Millipore) were coated with 5 µg/ml of anti-IFNγ monoclonal antibody (mAb) (MAB 1-D1K, Mabtech, Stockholm, Sweden) and subsequently blocked with 5% pooled human serum (BioWhittaker, Maryland). Triplicates of 2×10^5 viable PBMCs were resuspended in 50 µl of media (RPMI-1640 supplemented by 15% fetal calf serum, streptomycin/penicillin, and L-glutamine) and incubated with 10 µg/ml of peptide for 18 hr at 37°C in 5% CO_2. Instead of peptide, 10 µg/ml phytohemagglutinin (Sigma-Aldrich, Munich, Germany) was used as a positive control, and media only was added to a negative control. An irrelevant peptide (HIV-1, SLYNTVATL) was used in to rule out non-specific IFNγ-responses. One µg/ml of biotin-linked anti-IFNγ mAb (MAB 7-B6-1-Biotin, Mabtech) was used as secondary antibody, which was conjugated subsequently with streptavidin alkaline phosphatase (AP) (Bio-Rad, Hercules, CA), and visualized by an AP conjugate substrate Kit (Bio-Rad). Defined spots with fuzzy border and blue color were counted in a stereomicroscope. IFNγ-responses were reported as mean spot-forming cells (SFC) per million PBMCs, after subtracting the IFNγ-release from non-stimulated cells. Specific IFNγ-responses were defined as values of at least 20 SFC/10^6 PBMCs exceeding the plate baseline calculated as the mean plus two standard deviations of all triplicate responses in an experiment, excluding the values from non-stimulated cells and the three highest and the three lowest responses.

Ethical approval was obtained for the study from the local ethical committee at the Karolinska University Hospital, Karolinska Institutet, Stockholm, Sweden.

RESULTS

IFNγ-Responses Are Mainly Detected Against the NS1 Protein in Healthy Seropositive Individuals

When testing healthy seropositive individuals for reactivity against NS1-peptides, specific IFNγ-responses appeared in 24 of 196 possible combinations of PBMCs and peptide pools, tested in triplicate, representing responses to 12–14 pools of NS1-peptides (Fig. 1). The responses derived from eight individuals that reacted to 1-4 NS1-peptide pools each. Corresponding figures for VP-peptides were 5 of 126 combinations, representing responses to 4 of 9 VP-peptides pools from three individuals reacting to 1–2 VP-peptide pools each. Two persons responded to both NS1 and VP-peptide pools. A significantly larger number of responses were thus shown to NS1- than to VP-peptides in healthy individuals ($P = 0.015$, Fisher's exact test). Also, the mean level of specific IFNγ-response to the NS1-peptide pools greatly exceeded that to the VP pools in healthy
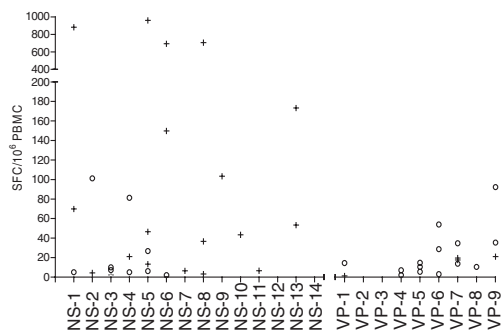

Fig. 1. B19 NS1- and VP-specific IFNγ-responses. The IFNγ-responses of the healthy individuals (+) and persistently infected individuals (O), expressed as SFC/106 PBMCs, are plotted for each peptide pool derived from the B19 NS1 and VP proteins. No specific IFNγ-responses were detected against the B19 antigens in the seronegative individuals.

individuals (99 and 5 SFC/10^6 PBMCs, respectively; $P = 0.013$, Mann–Whitney).

IFNγ-Responses Are Detected Mainly Against the VP Proteins in Persistently Infected Individuals

In the persistently infected, specific IFNγ-responses were shown in 9 of 126 possible combinations of PBMCs with NS1-peptides, and 14 of 81 combinations with VP-peptides, representing 6 of the 14 NS1- and 7 of the 9 VP-peptide pools, respectively (Fig. 1). The responses derived from five individuals that reacted to 1–3 NS1-peptide pools each, five that reacted to 1–5 VP-peptide pools each, and three individuals showing reactivity to both NS and VP-peptide pools. Thus, the number of responses to VP-peptides was significantly larger than that to NS1-peptides in persistently infected individuals ($P = 0.039$, Fisher's exact test). However, there was no significant difference in mean level of specific IFNγ-responses to NS1- and VP-peptide pools in this group (13 and 17 SFC/10^6 PBMCs, respectively; $P = 0.14$, Mann–Whitney).

Comparison of IFNγ-Responses, B19 IgG Titers, and HLA Types Between B19 Persistently Infected and Healthy Seropositive Individuals

Comparison between the groups revealed a significantly higher number of specific IFNγ-responses directed to VP-peptides among the persistently infected compared to the healthy seropositive individuals, whereas no such difference emerged for NS1-peptides ($P = 0.0022$ and 0.19, respectively, Fisher's exact test). The mean level of specific IFNγ-response to NS1-peptide pools was significantly higher among healthy seropositive individuals than among persistently infected, but responses to VP-peptide pools were virtually the same ($P = 0.012$ and 0.092, respectively, Mann–Whitney). The mean B19 IgG level in persistently infected was 97 IU/ml, which was significantly higher than in the

healthy seropositive individuals with a mean of 61 IU/ml ($P = 0.012$, unpaired t-test). Taking age into account in a multiple regression analysis provided a p-value for B19 IgG difference of 0.011. With respect to HLA class I genotype distribution, any overrepresentation of alleles were assessed by multiple χ^2 tests. No significant differences between the groups or clustering of a single HLA allele were thus noted that would indicate HLA non-responsiveness as a possible explanation for the observed differences in responses. No specific controls were detected in the three seronegative control, and no deficiency in intrinsic IFNγ-secretion was found, since all individuals mounted strong responses to phytohe-magglutinin (above 1,500 SFC/10^6 PBMCs).

DISCUSSION

Neutralizing antibodies are believed to be the most important mode of clearance of B19. The VP1 unique region is a well-characterized immunodominant site for the humoral immune response and corresponds to the sequence with known neutralizing activity able to elicit long lasting immune responses [Musiani et al., 2000; Zuffi et al., 2001]. However, in-spite of the presence of functional neutralizing antibodies, a proportion of apparently immunocompetent individuals retain B19 DNA in their bone marrow for years [Lundqvist et al., 1999a]. It was found that the distribution of cellular immune responses is discordant in healthy seropositive individuals and individuals with B19 persistence. Host IFNγ-responses were elicited primarily to the virus NS1 protein in the first group, in concordance to a recent assessment in acutely infected individuals [Norbeck et al., 2005]. In B19 persistently infected, these responses were skewed towards a VP-directed response with a lower magnitude of NS1 response. Bone marrow samples could not be obtained from the healthy individuals to assess B19 DNA, but there is substantial support for the assumption that these persons did not have persistent B19 infections. Firstly, lower B19 IgG levels in the healthy individuals may reflect a lack of continuous antigen stimulation responsible for the elevated B19 IgG levels in the persistently infected individuals. Secondly, the frequency of B19 DNA in bone marrow was previously estimated at 4% using the same PCR assay in patients with unselected hematological disorders and at 2.1% in healthy bone marrow donors [Lundqvist et al., 1999b; Heegaard et al., 2002]. With that said, it can be speculated that absent B19 clearance could be the result of an insufficient NS1 or a pre-dominating VP host cellular immune response. However, exhaustion of the response because of continuous antigenic stimulation should be considered as an explanation for the weak NS1 response, a phenomenon speculated to occur during persistent infections. Indeed, overexpression of NS1 is a speculative feature of B19 persistence [von Poblotzki et al., 1995; Probst et al., 2003]. The larger number of VP-responses is more difficult to explain, but could be a secondary effect after an initial failure of the infected host respond effectively

to the NS1 protein. Hepatitis B virus (HBV) establishes persistence in a proportion of infected individuals whose very low cytotoxic T-lymphocyte levels may stem from immune tolerance [Penna et al., 1991; Nayersina et al., 1993; Rehermann et al., 1995; Rehermann, 2000]. Proposed tolerogenic mechanisms include anergy, immunological exhaustion, imbalance in lymphokine production, and defective antigen presentation [Kakimi et al., 2002]. This parallel between B19 and HBV infection is interesting, since these two viruses may have an immunopathological resemblance. Moreover, the clear dominance of NS1 responses in distribution and magnitude in healthy individuals warrants further study considering that the current B19 candidate vaccine consists only of structural proteins, to which a large distribution of cellular immune responses and neutralizing antibodies appears compatible with B19 persistence.

REFERENCES

Cassinotti P, Schultze D, Schlageter P, Chevili S, Siegl G. 1993. Persistent human parvovirus B19 infection following an acute infection with meningitis in an immunocompetent patient. Eur J Clin Microbiol Infect Dis 12:701−704.

Faden H, Gary GW, Jr., Anderson LJ. 1992. Chronic parvovirus infection in a presumably immunologically healthy woman. Clin Infect Dis 15:595−597.

Franssila R, Hokynar K, Hedman K. 2001. T helper cell-mediated in vitro responses of recently and remotely infected subjects to a candidate recombinant vaccine for human parvovirus b19. J Infect Dis 183:805−809.

Heegaard ED, Brown KE. 2002. Human parvovirus B19. Clin Microbiol Rev 15:485−505.

Heegaard ED, Petersen BL, Heilmann CJ, Hornsleth A. 2002. Prevalence of parvovirus B19 and parvovirus V9 DNA and antibodies in paired bone marrow and serum samples from healthy individuals. J Clin Microbiol 40:933−936.

Kakimi K, Isogawa M, Chung J, Sette A, Chisari FV. 2002. Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: Implications for immunotherapy of persistent viral infections. J Virol 76:8609−8620.

Klenerman P, Tolfvenstam T, Price DA, Nixon DF, Broliden K, Oxenius A. 2002. T lymphocyte responses against human parvovirus B19: Small virus, big response. Pathol Biol (Paris) 50:317−325.

Larsson M, Jin X, Ramratnam B, Ogg GS, Engelmayer J, Demoitie MA, McMichael AJ, Cox WI, Steinman RM, Nixon D, Bhardwaj N. 1999. A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1-positive individuals. Aids 13:767−777.

Lindblom A, Isa A, Norbeck O, Wolf S, Johansson B, Broliden K, Tolfvenstam T. 2005. Slow clearance of human parvovirus B19 viremia following acute infection. Clin Infect Dis 41:1201−1203.

Lundqvist A, Tolfvenstam T, Bostic J, Soderlund M, Broliden K. 1999a. Clinical and laboratory findings in immunocompetent patients with persistent parvovirus B19 DNA in bone marrow. Scand J Infect Dis 31:11−16.

Lundqvist A, Tolfvenstam T, Brytting M, Stolt CM, Hedman K, Broliden K. 1999b. Prevalence of parvovirus B19 DNA in bone marrow of patients with haematological disorders. Scand J Infect Dis 31:119−122.

Musiani M, Manaresi E, Gallinella G, Venturoli S, Zuffi E, Zerbini M. 2000. Immunoreactivity against linear epitopes of parvovirus B19 structural proteins. Immunodominance of the amino-terminal half of the unique region of VP1. J Med Virol 60:347−352.

Nayersina R, Fowler P, Guilhot S, Missale G, Cerny A, Schlicht HJ, Vitiello A, Chesnut R, Person JL, Redeker AG, et al. 1993. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. J Immunol 150:4659−4671.

Norbeck O, Isa A, Pohlmann C, Broliden K, Kasprowicz V, Bowness P, Klenerman P, Tolfvenstam T. 2005. Sustained CD8+ T-cell responses induced after acute parvovirus B19 infection in humans. J Virol 79:12117–12121.

Penna A, Chisari FV, Bertoletti A, Missale G, Fowler P, Giuberti T, Fiaccadori F, Ferrari C. 1991. Cytotoxic T lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. J Exp Med 174:1565–1570.

Probst HC, Tschannen K, Gallimore A, Martinic M, Basler M, Dumrese T, Jones E, van den Broek MF. 2003. Immunodominance of an antiviral cytotoxic T cell response is shaped by the kinetics of viral protein expression. J Immunol 171:5415–5422.

Rehermann B. 2000. Intrahepatic T cells in hepatitis B: Viral control versus liver cell injury. J Exp Med 191:1263–1268.

Rehermann B, Fowler P, Sidney J, Person J, Redeker A, Brown M, Moss B, Sette A, Chisari FV. 1995. The cytotoxic T lymphocyte response to multiple hepatitis B virus polymerase epitopes during and after acute viral hepatitis. J Exp Med 181:1047–1058.

Sasaki T, Murai C, Muryoi T, Takahashi Y, Munakata Y, Sugamura K, Abe K. 1995. Persistent infection of human parvovirus B19 in a normal subject. Lancet 346:851.

Servant A, Laperche S, Lallemand F, Marinho V, De Saint Maur G, Meritet JF, Garbarg-Chenon A. 2002. Genetic diversity within human erythroviruses: Identification of three genotypes. J Virol 76:9124–9134.

Shade RO, Blundell MC, Cotmore SF, Tattersall P, Astell CR. 1986. Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. J Virol 58:921–936.

Tolfvenstam T, Lundqvist A, Levi M, Wahren B, Broliden K. 2000. Mapping of B-cell epitopes on human parvovirus B19 nonstructural and structural proteins. Vaccine 19:758–763.

Tolfvenstam T, Oxenius A, Price DA, Shacklett BL, Spiegel HM, Hedman K, Norbeck O, Levi M, Olsen K, Kantzanou M, Nixon DF, Broliden K, Klenerman P. 2001. Direct ex vivo measurement of CD8(+) T-lymphocyte responses to human parvovirus B19. J Virol 75:540–543.

von Poblotzki A, Hemauer A, Gigler A, Puchhammer-Stockl E, Heinz FX, Pont J, Laczika K, Wolf H, Modrow S. 1995. Antibodies to the nonstructural protein of parvovirus B19 in persistently infected patients: Implications for pathogenesis. J Infect Dis 172:1356–1359.

Weigel-Kelley KA, Yoder MC, Srivastava A. 2003. Alpha5beta1 integrin as a cellular coreceptor for human parvovirus B19: Requirement of functional activation of beta1 integrin for viral entry. Blood 102:3927–3933.

Zuffi E, Manaresi E, Gallinella G, Gentilomi GA, Venturoli S, Zerbini M, Musiani M. 2001. Identification of an immunodominant peptide in the parvovirus B19 VP1 unique region able to elicit a long-lasting immune response in humans. Viral Immunol 14:151–158.