COMPARATIVE ANALYSIS OF PARVOVIRUS B19 PERSISTENCE AND
DEVELOPMENT OF ANTI-NON-STRUCTURAL PROTEIN –1 ANTIBODIES IN THE
DEVELOPMENT OF PARVOVIRUS B19 ARTHROPATHY

by

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Parvovirus B19 (B19) is a non-enveloped, single-stranded DNA virus in the family Parvoviridae. The B19 capsid is composed of two structural proteins, VP1 and VP2, which contain multiple epitopes that are recognized by virus-neutralizing antibodies. The major non-structural protein, NS-1, is vital for viral replication and is also toxic to host cells. The most common clinical sequelae of B19 infection is erythema infectiosum (EI) which is characterized by fever, ‘flu’-like symptoms, and a rash on the face and torso. Fifty percent of B19-infected adults experience acute arthropathy (arthritis and/or arthralgia) lasting up to 1 month. Of these individuals, 20% will develop chronic arthropathy (lasting more than one month and persisting from months to years). This thesis examines the hypothesis that B19 persistence and the presence of anti-NS-1 antibodies in peripheral blood are correlated with the development of B19 infection-associated arthropathy, and that the development of anti-NS-1 antibodies is dependent upon B19 persistence. A study of the possible associated factors leading to B19 arthropathy may help in the diagnosis of this condition and serve to provide insights into its pathogenesis.

B19 PCR and NS-1 immunoblot were used to examine the prevalence of B19 persistence (presence of B19 DNA) and anti-NS-1 antibodies in peripheral blood samples from a cohort of individuals with recent B19 infections. Potential differences in these parameters were investigated between subjects who developed and did not develop arthropathy. Subjects with histories of past, undocumented B19 infections were also tested to determine the population incidence of peripheral blood B19 persistence and anti-NS-1 antibodies. Differences in the occurrence of B19 DNA and anti-NS-1 between recent and past-infected groups would indicate changes in these parameters over time. Finally, the effects of re-exposure to active B19 on the population frequency of peripheral blood B19 DNA and anti-NS-1 were examined in subjects with past undocumented B19 infections.
Results indicate that the presence of B19 DNA in peripheral blood was not related to the development of acute or chronic arthropathy following B19 infection. Instead, the ability to detect peripheral blood B19 DNA was correlated with the amount of time that had passed following B19 infection (an indirect indicator of the development of immunity to B19). A possible accelerated clearance of B19 from peripheral blood after infection was observed in subjects who did not develop arthropathy following B19 infection. It was hypothesized that this accelerated immune response may prevent formation of soluble immune complexes that are believed to mediate acute B19 arthropathy. The role that a delay in B19 clearance has on the development of chronic B19 arthropathy is unknown. In addition, anti-NS-1 antibodies were not found to be related to B19 persistence or the development of B19 arthropathy. Instead, anti-NS-1 antibodies were found to be indicators of past B19 infection in certain individuals. It was hypothesized that the development of anti-NS-1 was due to high initial B19 viremia related to prolonged or repeated exposure to B19. Finally, a transient boost in the incidence of peripheral blood B19 DNA and anti-NS-1 antibodies in subjects re-exposed to B19 indicate that subclinical reinfection with B19 may occur.
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Chapter 1.

Introduction

1.1. Parvovirus B19 Virus Biology.

1.1.1. Classification and Morphology.

Parvovirus B19 is a member of the genus Erythrovirus in the family Paroviridae (Brown et al., 1994). The virion is 18-26 nm in diameter, nonenveloped, and has icosahedral symmetry. Because it is non-enveloped, B19 is stable in most solvents (pH 3-9) and is resistant to temperatures of 56°C for at least 1 hour (Brown, C.S., 1994). The B19 genome is a single stranded DNA molecule, 5.6 kB in length. Both sense and antisense strands are packaged in equal proportions in the capsid. A characteristic feature of the B19 genome is the identical terminal palindromic repeats (383 bases) that fold back on themselves through complementary base pairing to form hairpin structures (Astell et al., 1997). The hairpins serve not only to maintain the integrity of the B19 genomic termini, but also function as self-primers during genome replication (Brown, C.S., 1994). The viral non-structural proteins are encoded on the 5' portion of the genome while the capsid proteins (VP1 and VP2) are encoded downstream at the 3' end. A single strong promoter is located at map unit 6 (p6) and drives the transcription of a primary transcript that is differentially processed to yield both non-structural and capsid proteins (Brown et al., 1994). A second promoter located in the middle of the B19 genome at map unit 44 (p44) was characterized as a potential promoter for capsid transcription, but was later established to be non-functional (Liu et al, 1992).
1.1.2. Structural Proteins.

The parvovirus B19 capsid is composed of two structural proteins. The major capsid protein, VP2 (58 kDa), comprises 95% of the 60 unit icosahedral structure. The minor capsid protein, VP1 (83kDa), accounts for the remaining 5% (Brown et. al, 1994; Astell et al., 1997). VP1 and VP2 have overlapping, in-frame ORFs located on the right side of the genome and the VP1 and VP2 proteins are identical to each other except for an additional 227 amino acids at the amino terminus of VP1.

Recombinant, self-assembling empty capsids have been generated that contain VP2 alone, or both VP1 and VP2. Although capsids containing VP2 alone are highly antigenic when used to immunize rabbits, they fail to elicit strong neutralizing antibody activity. Capsids consisting of both VP1 and VP2 were similarly antigenic but elicited a strong neutralizing response when used to immunize rabbits, indicating the importance of VP1 in protective immunity (Rosenfeld et al., 1994). Immunoprecipitation of infectious virions and empty recombinant capsids containing both capsid proteins with antibodies directed against the unique VP1 region has led to the suggestion that this region is exposed to the external surface of the capsid (Naides, 1998). Furthermore, the ability of antibodies against the unique VP1 region to neutralize viral replication in bone marrow cultures of the virus indicates that this region is involved in cell attachment and entry (see humoral immune response below).

As transcription of the genome is driven from a single promoter with post-transcriptional splicing, regulation of the relative proportions of capsid proteins takes place at the post-transcriptional level. Multiple AUG codons upstream of the putative translation initiation codon of VP1 greatly reduce translation efficiency of the VP1 mRNA. Splicing that removes these limiting AUG codons from the VP2 mRNA, greatly improves the overall synthesis rate of VP2 (Ozawa et al., 1988a).
1.1.3. Nonstructural Proteins.

There are two nonstructural (NS) proteins encoded in the left portion of the genome. NS-1 (71 kDa) is vital for viral function and is also toxic to host cells (Brown et al., 1994; Astell et al., 1997, Moffatt et al., 1998), while the function of NS-2 (63 kDa) is unknown. NS-1 serves as a transcriptional transactivator of the B19 promoter (p6) and also has DNA-nickase and helicase activities, vital for viral replication (Brown et al., 1994).

Recently, NS-1 expression in a human erythroleukemia cell line (K562) and an erythropoietin-dependent megakaryocytic cell line (UT-7/Epo) was shown to induce cell death by apoptosis, with characteristic cell morphological changes (cell rounding, cytoplasmic blebbing) and genomic DNA fragmentation (Moffat et al., 1998). This apoptotic mechanism was found to be mediated by activation of a pathway involving caspase 3. Furthermore, NS-1-induced apoptosis was slowed by undefined factors found in serum, a finding supported by the optimization of apoptosis when the host cells were serum-starved. The toxicity of NS-1 for erythroid and other precursors in the bone marrow provides a mechanism for the cytopenias associated with active parvovirus B19 viremia (see Section 1.2.3.3.).

Experiments using human hematopoietic cell lines transfected to express NS-1 showed an increased expression of IL-6. It was found that NS-1 functioned as a trans-acting transcriptional transactivator on the IL-6 promoter (Moffat et al., 1996). Furthermore, mutations in the nucleotide triphosphate binding domain of NS-1 were found to rescue erythroid lineage cells from apoptosis without influencing NS-1 mediated activation of IL-6 expression, indicating that there may be separate functional domains on NS-1 (Moffat et al., 1998). Both NS-1 induced apoptosis and IL-6 production have implications in B19 pathogenesis and clinical manifestations (see Section 1.4.).
1.1.4. Cellular Receptor.

B19 is highly tropic for cells of the erythroid lineage, with susceptibility to B19 infection increasing with erythroid differentiation. Thus, late erythroid progenitors, cfu-e, are more permissive for B19 infection and replication than the more primitive burst forming erythroid progenitors, bfu-e (Brown et al., 1994; Brown et al., 1993). Other studies have indicated that pronormoblasts and normoblasts, which are further differentiated from the cfu-e cells, are also permissive for B19 replication. Appearance of giant pronormoblasts is characteristic of in vitro and in vivo B19 infection (Morey et al., 1992). The ability of B19 to hemagglutinate and infect erythroid progenitors, and the blocking of these effects with both soluble surface P antigen and monoclonal antibodies against surface P antigen confirmed that P antigen is the cellular receptor for B19 (Brown et al., 1993). P antigen (globoside, a tetrahexose ceramide) is found on a variety of cell types, including erythrocytes, erythroid progenitors, megakaryocytes, synovium, fetal liver and myocardium, and placenta (Munshi et al., 1993; Shimonmura et al., 1992; Naides, 1998).

1.1.5. Viral Culture.

B19 has been propagated in bone marrow cultures, megakaryocytic leukemic cell lines (Munshi et al., 1993; Shimonmura et al., 1992), as well as in hematopoietic progenitors from fetal liver, peripheral blood, and cord blood (Brown, C.S., 1994). However, these cultures have been of short-term duration and long-term culture of the virus has not been successful (Brown, C.S., 1994). Thus, B19 cannot be effectively produced in vitro for viral studies or vaccine production. Also, isolation of virus from infected individuals has been difficult due to the narrow time frame of peripheral blood viremia (usually between days 5-8 during the prodromic period). However, as noted above, recombinant B19 empty capsids produced in cell culture systems are structurally and antigenically similar to natural virus. Expression systems have included Chinese hamster ovary (CHO) cells transfected with recombinant B19 plasmids, Spodoptera frugiperda cells infected with
B19 recombinant baculovirus, and COS-7 cells transfected with B19-SV40 hybrids (Cohen et al., 1995). These empty capsids show promise for use in diagnostic assays, but may not be suitable for use as vaccine immunogens (Brown, C.S., 1994).

Determination of the efficacy of B19 vaccine candidates to induce neutralizing antibodies has been hampered by the relative insensitivity and irreproducibility of the currently available neutralization assay for B19 (cfu-e inhibition assays). These assays measure the ability of sera or antibody preparations to protect cfu-e cells, isolated from human bone marrow aspirates and cultured in the presence of erythropoietin (Epo), from B19 infection and subsequent cell cytotoxicity (Sato et al., 1991; Sato et al., 1991a). Typically, the bone marrow cultures employed were highly variable in cell composition and the serum dilutions often added significant concentrations of cytokines that affected growth in the primary bone marrow cultures. Thus, a quantitative comparison of the cfu-e inhibition assays was extremely difficult.

Recently, a promising sensitive and reproducible reverse transcription-polymerase chain reaction (RT-PCR) based assay has been developed to assess B19 neutralizing antibody titres (Bostic et al., 1999). This method employs human megakaryoblastic cells modified to grow with erythropoietin (UT-7/Epo). UT-7/Epo cells are cultured in the presence of a predetermined fixed quantity of virus (from a viremic serum source) and sera containing the potential neutralizing antibodies. Total cellular RNA is isolated and cDNA is produced by reverse-transcription. PCR is subsequently performed to detect spliced B19-specific cDNA. Neutralizing ability of sera is defined as the highest dilution of serum that yields no virus-specific RT-PCR products.

1.1.6. Cell Tropism Differential Transcription.

*In vitro* infection and transfection experiments in both semi-permissive erythroid progenitor cells and non-permissive cell lines has led to the discovery of differential transcriptional patterns of the B19 genome. Cell tropism and non-productive and productive replicative cycles are all under
the control of both viral genomic and host cellular factors (Pallier et al, 1997). The different events of the B19 viral cycle in permissive and non-permissive cells have potential relevance with respect to viral pathology in humans (see Section 1.4.).

In erythroid progenitors that are permissive for B19 replication, capsid transcripts are the predominant mRNA species. Regulation of non-structural versus structural protein abundance is thought to be at the transcriptional level, with read-through transcription of the capsid being dependent on the secondary structures formed at the 3' end of the NS-1 transcript that affect downstream mRNA polymerization of the capsid transcript. Alternatively, 3' end processing of new transcripts may affect stability of the transcripts. Processing of transcripts in permissive cells may also be dependent on viral genomic changes during cellular entry, nuclear localization, and genomic replication, and by cell-specific factors (Liu et al., 1992). Regardless of the mechanism, cells that permit B19 replication characteristically have low concentrations of B19 NS-1 protein early in infection, which may serve to regulate replication of B19. Later in infection, concentrations of NS-1 rise to levels that likely lead to cell death and release of virus as well as NS-1 protein (Ozawa et al., 1988).

Non-permissive cells have been consistently found to overexpress NS-1 transcripts, leading to cell cytotoxicity. Transfection of HeLa cells with plasmids containing the B19 NS-1 portion of the genome prevented colony formation, with the effect being reversed with mutation of the NS-1 sequence (Ozawa et al., 1988). Removal of the 3' processing signal of NS-1 or disruption of the polyadenylation signal of the NS-1 region in HeLa cells transfected with cloned whole B19 genome resulted in increased readthrough of full-length B19 transcripts including the capsid proteins. This suggested that stem-loop structures in newly formed NS-1 transcripts may lead to premature transcription termination (Liu et al., 1992). However, recent findings have indicated that the common 3' untranslated region (UTR) of the B19 capsid proteins, along with undefined cellular
factors from non-permissive cells, inhibit capsid protein synthesis by preventing the binding of B19 capsid mRNA to 40S ribosomal subunits (Pallier et al., 1997).

1.2. Parvovirus B19 Pathology.

1.2.1. History.

Parvovirus B19 was discovered in 1975 by Yvonne Cossart in England while screening serum samples for hepatitis B virus (Brown, C.S., 1994). A particular sample, sample 19, yielded anomalous results, leading to the discovery of parvovirus-like particles by electron microscopy (Brown et al., 1994). Subsequently, the virus was associated with transient aplastic crisis in sickle-cell patients in 1981 and was formally accepted as the etiological agent of erythema infectiosum in 1984 (Brown, C.S., 1994). Characterization of the viral genome lead to its classification as a member of the family Parvoviridae in 1985, and it was arbitrarily given the name B19 after Cossart's original sample number in which it was discovered (Brown et al., 1994).

1.2.2. Epidemiology.

Transmission of the virus is normally via the upper respiratory tract. However, B19 infection may occur after transfusion with infected blood or blood products or via the placenta from an infected mother to the fetus (Brown et al., 1994). Transmission rates are high, and range from 50% in close household contacts, 20-32% in daycare centres, and 10-60% in schools experiencing B19 outbreaks. The prevalence of B19 infection world-wide is high, with 40 - 60% of young adults and 90% of the elderly showing evidence of earlier infection with the presence of serum B19-specific IgG antibodies (Brown et al., 1994). The annual seroconversion rate in women of childbearing age is 1.5% (Brown et al., 1993). Although infection rates are high, detection of B19 virus in blood is often missed due to the narrow window of viremia during the prodromal phase of infection when symptoms are relatively nonspecific. B19 outbreaks are seasonal, with the highest
infection periods occurring during the late winter and early summer. However, more severe outbreaks occur in 3-4 year cycles (Brown et al., 1994).

1.2.3. Clinical Features.

In children, B19 typically leads to a benign, self limiting disease, erythema infectiosum (EI) lasting from 1 to 2 weeks (Brown et al., 1994). B19-infected adults usually develop an acute, 'flu' -like febrile illness that may include rash on the face, trunk, and extremities. As well, 50% of cases show additional complications typically in the form of joint manifestations including arthralgias (joint pain only) or arthritis (joint pain with objective evidence of inflammation and loss of function) (Brown et al., 1994). The major manifestations of B19 infection are described below.

1.2.3.1. Asymptomatic Infection.

Serologic studies suggest that many B19 infections go unrecognized or may be asymptomatic. In both adults and children, the incidence of B19 infection without overt clinical signs is believed to be about 25% (Brown et al., 1993; Brown et al., 1994).

1.2.3.2. Erythema Infectiosum (EI).

Erythema infectiosum (EI), also known as fifth disease or “slapped cheek” disease, is initially characterized by fever, flu-like symptoms, and a lacy facial rash that may also be present on the torso. It is the most common manifestation of B19 infection in children and is usually mild and self-limiting, with uncomplicated resolution within 1-2 weeks of rash onset. Rashes may be reticular, macular, maculopapular, and sometimes, vesicular. Pruritis occurs in approximately half of the cases of EI (Naides, 1998). The rash is thought to be immune complex-mediated as it appears at the time of onset of the B19-specific antibody response between 10-15 days after initial infection (Brown et al., 1994). Although symptoms usually resolve in 1-2 weeks, recurrent rashes may occur for weeks or months after initial infection. There is no evidence for a correlation between recurrent rash and viremia or viral shedding (Naides, 1998).
1.2.3.3. Hematologic Disorders.

B19 is highly tropic for, and cytotoxic to, cells of erythroid lineage. In normal, healthy individuals, reticulocytopenia occurs at the peak of viremia (days 5-8 after infection) with recovery shortly after the development of B19-specific antibodies. Consequently, hemoglobin levels also fall, but these levels do not typically lead to clinical signs or symptoms (Naides, 1998). Conversely, B19-infected individuals with underlying chronic haemolytic anemias (hereditary spherocytosis, glucose-6-phosphate dehydrogenase deficiency) or other red cell disorders (thalassemia, sickle cell disease, anemia) are susceptible to life threatening aplastic crises that may require blood transfusion (Brown et al., 1994). Infection with B19 is believed to be the cause of 90% of transient aplastic crises in such individuals (Brown, C.S., 1994), and is characterized by appearance of giant pronormoblasts in the bone marrow. In immunocompromised individuals (e.g. AIDS patients, patients on long term immunosuppression, chemotherapy, etc.), B19 infection may become persistent, leading to chronic anaemia. Neutropenia, lymphopenia, and thrombocytopenia may occur both in B19-infected normal and immunocompromised individuals (Naides, 1998), implying that the virus also targets precursors of the myeloid, lymphoid, and megakaryocyte lineages.

1.2.3.4. Fetal Infection.

Congenital infection with B19 can lead to non-immune hydrops fetalis, characterized by aplastic crisis, reflex high output cardiac failure, and hemodynamic changes that lead to generalized edema in the fetus and possibly fetal death (Brown et al.,1994; Naides, 1998). Alternately, direct B19-mediated cardiomyopathy has been suggested as a cause of fetal hydrops (Naides, 1998). The link between B19 infection and fetal loss was discovered in 1984 with the detection of B19 DNA in fetal tissues by in situ hybridization (Brown, C.S., 1994). The risk of fetal loss is greatest if the mother is infected during the second trimester of pregnancy, with the fetal death rate being approximately 9% (Brown et al., 1994). A Japanese study of antenatal B19 infection leading to
non-immune hydrops fetalis calculated the risk of fetal death to be 10.2%. The highest risk of fetal loss was 12.4% for B19 infections occurring during the first 20 weeks of pregnancy (Yaegashi et al., 1998). This study suggested that the critical time during pregnancy for B19-infections that lead to fetal hydrops was during the hepatic period of fetal hematopoiesis. B19 affinity and toxicity for hepatic erythroid progenitors was thought to be augmented because of the rapid expansion and high turnover rate of these cell populations during the second trimester of pregnancy (Yaegashi et al., 1998).

1.2.3.5. Autoimmune Disease.

Although no direct etiological role has been confirmed so far, parvovirus B19 infection has been linked to several autoimmune diseases including systemic lupus erythematosus (SLE) (Nesher et. al, 1995; Fawaz-Estrup, 1996), Kawasaki disease (Nigro et al, 1994), and rheumatoid arthritis (RA) (see Section 1.4.3.). The rash, fever, non-erosive arthritis, and hematologic manifestations that may accompany some B19 infections, coupled with the occasional findings of anti-nuclear antibodies (ANA) and antiphospholipid antibodies has led to episodic misdiagnosis of B19 infection as SLE (Naides, 1998). B19 infections have sometimes been found to yield autoantibodies, including antinuclear antibodies, antiphospholipid antibodies, and anticollagen antibodies, that may have implications in the etiopathogenesis of B19 arthropathy and other B19-related pathologic symptoms (Kerr and Boyd, 1996; Lunardi et al., 1998) (see Section 1.4.).

1.2.3.6. Arthropathy.

B19-associated arthropathy is becoming an increasingly recognised consequence of B19 infection. Arthropathy is defined as arthralgia (subjective joint pain only) and/or arthritis (joint pain with one or more signs of swelling, heat, tenderness, or reduction of function). The incidence of acute arthropathy (lasting 1-4 weeks) following B19 infection in children is 10 - 19% (Brown et al, 1994). Incidence increases with age, with acute arthropathy occurring in approximately 50% of
adult B19 infections (Brown et al, 1994). There is a prominent sex distribution in occurrence of
arthropathy, with 60% of infected women and only 30% of infected men developing joint
symptoms (Heegard and Hornsleth, 1995). Of affected women, about 20% experience chronic
(recurrent or persistent) joint manifestations lasting from months to years (Brown et al., 1994). The
incidence of chronic arthropathy in men is much less common and has not been well documented.
Chronic joint manifestations in B19-infected children have been observed only rarely (Nocton et al.,
1993). The onset of acute joint manifestations coincides with the development of B19-specific
antibodies at 10-15 days after initial infection, suggesting that acute arthropathy is mediated by
immune complexes that lead to type III immune injury (Brown et al., 1994). The mechanism of
chronic B19 infection-associated arthropathy is presently unknown. However, several clinical
studies have associated chronic arthropathy with persistent B19 infections (Naides et al., 1992; Foto
et al., 1993; Klouda et al., 1996; Woolf et al., 1989). Several possible hypotheses concerning the
pathogenesis of B19 arthropathy are discussed in Section 1.4.

B19 infection-induced joint manifestations in adults closely resemble those of rheumatoid
arthritis. Arthropathy may occur in single or multiple joints, and is often symmetrical. Typically,
B19 arthropathy patients experience morning stiffness lasting more than one hour, and have
symmetric involvement of joints in the hand including the proximal interphalangeal,
metacarpophalangeal, and wrist joints. Shoulders, elbows, knees, ankles, and feet are also
commonly affected, with occasional involvement of the cervical and lumbosacral spine.
Rheumatoid factor has also transiently been observed in B19 arthropathy (Naides, 1998). Joint
symptoms are usually acute, lasting only 1-3 weeks, but can recur or be unremitting in
approximately 20-30% of affected individuals (Brown et al., 1994). Two reports claim that
approximately 50% of all chronic arthropathies linked to earlier B19 infection met the American
Rheumatoid Association (ARA) criteria for a diagnosis of rheumatoid arthritis (RA) or juvenile RA
(IRA) (Naides, 1993; Nocton, 1993). However, a definitive etiological link between B19 infection and rheumatoid arthritis has not been proven (see Section 1.4.3.).

Two clinical studies revealed potential associations of human leukocyte antigen (HLA) Class II immunogenetic background and risk for development of acute B19-associated arthropathy (Klouda et al., 1996; Woolf et al., 1989). These results suggested positive associations with the HLA-DR4 phenotype (analogous to RA) and negative associations with the DR1 phenotype (Klouda et al., 1996). One study of 336 individuals examined during a community outbreak of B19 infection revealed that 18 (17 females, 1 male) patients who had serologically-confirmed (B19 IgM+) infection went on to experience persistent polyarthritis (median duration of 6 months). The remainder of the study population (controls, n=318) had not experienced joint manifestations and were found to have only IgG antibodies directed to B19, indicative of past infection. Of the 18 patients who experienced arthropathy, 12 (66.7%) expressed the DR4 phenotype, while only 115 of the controls (36.2%) were DR4 positive. This difference was found to be statistically significant (p<0.02). Conversely, none of the arthropathy patients expressed the DR1 phenotype, while a significantly (p < 0.019) higher number (64 or 20.1%) of controls were DR1 positive, suggesting that DR1 genes may be associated with resistance to development of B19 infection-associated joint manifestations. No associations were observed with any of the other Class II phenotypes assessed (DR2, DR3, DR5, DR6, or DR7) or with HLA Class I (A,B, or C) genes (Klouda et al., 1996).

Another study of 54 adults with serologically confirmed recent B19 infection revealed that five patients with persistent symptoms (> 2 months duration) also did not express the DR1 phenotype (Woolf et al. 1989). The association of the DR4 phenotype with tendency to develop acute and persistent B19-associated joint manifestations is intriguing in that certain DR4 alleles have been positively associated with other chronic arthropathies such as rheumatoid arthritis. Although these early studies did not define the actual alleles involved, their results suggest that HLA Class II
influences on the presentation of parvovirus B19 antigens may lead to differential effectiveness of the immune response in viral clearance and could influence whether the virus persists in host tissues.

1.3. Immune Response to Parvovirus B19.

1.3.1. Cellular Immune Responses.

Very little is known about the role of T cells in the immune response to B19. The influence of cellular immunity in B19 infections was demonstrated in individuals suffering from AIDS who experienced chronic anemia due to persistent B19 infections (Frickhofen et al., 1990). It is presumed that a reduced CD4+ population in these individuals is responsible for a defective antibody response to B19. Furthermore, in individuals experiencing chronic B19-associated arthritis, persistent serum B19 DNA was demonstrated despite the presence of neutralizing antibodies (Kurtzman et al., 1989). These subjects also failed to class switch from B19-specific IgM to IgG antibodies, suggesting that helper T-cells are required for viral clearance. A recent study testing the T cell responses to recombinant B19 capsid proteins (rVP1, rVP2) in 10 healthy B19 seropositive (IgG+) individuals revealed that all had CD4+, HLA class II-restricted lymphoproliferative responses to at least one capsid protein (von Poblotzki et al., 1996). In contrast, 6 B19 seronegative individuals did not exhibit a B19-specific T-lymphoproliferative response. These findings suggest that B19-specific CD4+ T-cells may support a humoral mechanism for clearance of B19.

1.3.2. Humoral Immune Responses.

1.3.2.1. Antibody Response to B19 Capsid Proteins.

Viral clearance and protection against subsequent B19 infection is assumed to be antibody-mediated. The appearance of circulating anti-B19 antibody is correlated with normal recovery from infection (Brown et al., 1994). Transfer of immune globulin confers passive immunity to B19
infection and has been shown to ameliorate persistent B19 infection in immunodeficient patients (Kurtzman et al., 1989; Brown et al., 1994; Heegard and Hornsleth, 1995).

Virus may be detected in the blood 5 - 6 days after initial infection, peaking at 8 - 9 days (Brown et al., 1994). The first antibody species to appear (10 - 12 days after infection) are IgMs directed to both linear and conformational epitopes of VP2 (Soderlund et al., 1995). The appearance of B19-specific antibodies is concurrent with the reduced levels of virus in bone marrow and serum. Failure to class-switch to production of capsid-specific IgG and prolongation of the specific IgM response is considered indicative of persistent infection. However, in presumably immunocompetent individuals with self-limited courses of B19 disease, anti-B19 IgM has been found in serum for 3-6 months after infection (Naides, 1998; Cassinotti et al., 1997).

Antibodies directed to linear VP2 epitopes decline after 14 days and usually disappear by six months, while antibodies to conformational VP2 epitopes persist (Soderlund et al., 1995). The major antibody species present in convalescent sera are IgGs directed mainly to linear epitopes within the unique region of VP1 (Soderlund et al., 1995). These antibodies are detected within 14 days after infection and usually persist in the circulation for years (Brown et al., 1994). The amino terminus of VP1 has been found to be on the exterior surface of native B19 capsids and is involved in cell binding and entry. Binding of B19 to the globoside receptor on target cells has been hypothesised to lead to conformational changes in the capsid and concomitant release of energy that together may aid in cellular entry and decapsidation. Antibodies targeting the amino terminus of VP1 may mimic the conformational changes elicited from B19 binding to globoside, disabling the ability of B19 to bind host cells and effectively neutralizing the virus (Rosenfeld et al., 1994). Furthermore, immune globulin preparations used to treat serious hematologic disease in persistent B19 infections are composed mainly of antibodies targeted against VP1 (Kurtzman et al., 1989).
Failure in certain individuals to produce antibodies to the unique, amino terminal of VP1 may have implications for B19 persistence and pathogenesis (see Section 1.3.3.).

Earlier studies identified neutralization domains on VP1 and VP2 using a variety of methods. Synthetic peptides corresponding to hydrophilic, and potentially surface exposed, regions of VP1 and VP2 have been used to identify epitopes recognized by mouse monoclonal and human antibodies (Sato et al., 1991; Sato et al., 1991a). Some of these peptide-specific antibodies were effective in neutralizing B19 inhibition of erythroid colony formation (cfu-e assays) (Sato et al., 1991; Sato et al., 1991a). Further identification of neutralizing epitopes in the unique region of VP1 was accomplished by immunizing rabbits with peptides corresponding to this region (Anderson et al., 1995). Antibodies raised to these peptides were tested for their neutralization capability using cfu-e inhibition assays. Thus, these studies identified linear epitopes in the unique region of VP1, the VP1/VP2 overlapping region, and the C-terminal portion of VP2 that constitute neutralization domains of B19 virus (Anderson et al., 1995).

B19 neutralization studies in recently infected B19 subjects using the RT-PCR based assay again confirmed that antibodies reactive to recombinant capsid and VP1 had neutralizing activity (Bostic et al., 1999). Interestingly, there was no linear correlation between serum antibody titres to capsid or VP1 alone (as determined by ELISA) and neutralizing capacity. Although sera that contained high titres of anticapsid and anti-VP1 antibodies generally showed high neutralizing ability, several sera with moderate to high levels of anti-capsid IgG had low or absent neutralizing activity. These findings illustrate the importance of antibody affinity to specific neutralizing epitopes in developing protective immunity. As well, these results may challenge conventional dogma that assumes the presence of serum anticapsid IgG antibodies will confer protective immunity against B19.
1.3.2.2. Antibody Response to NS-1.

Currently, little is known about the humoral immune response to B19 nonstructural proteins. It has been suggested that the presence of NS-1 antibodies may indicate persistent B19 infection (von Poblotzki et al., 1995; von Poblotzki et al., 1995a). Antibodies recognizing recombinant NS-1 (r-NS-1) were demonstrated in the sera of three persistently infected individuals using enzyme linked immunoassay (EIA), while such antibodies were absent in sera from 18 B19-infected individuals who did not experience joint complications. The three persistently B19-infected patients (one with recurrent granulocytic aplasia, one on immunosuppressive therapy for chronic B-cell lymphatic leukemia, and one with pancytopenia and complete bone marrow aplasia) were monitored for 3-18 months. Detection of B19 genomic sequences by PCR or by dot-blot hybridization in serum confirmed viral persistence. B19-specific IgM directed to capsid proteins and NS-1 fluctuated in concordance with clinical symptoms, with NS-1-specific IgG eventually developing in all three subjects (von Poblotzki et al., 1995a). Another preliminary study found NS-1 specific IgG antibodies in three patients with severe arthralgia or arthritis associated with acute B19 infection, but not in 18 control subjects with histories of B19 infection without joint complications and 9 B19 seronegative controls (von Poblotzki et al., 1995). The same study examined the epitope specificities of NS-1 antibodies present in the sera of the three B19 arthritis patients using NS-1 fusion proteins, and identified antibody recognition domains within residues 305-403 of the carboxy terminus. The functional status of these antibodies was not addressed. These studies suggest a possible correlation between the development of anti-NS-1 antibodies and B19 persistence and/or B19 arthropathy (see Section 1.4.4.).

Anti-NS-1 antibodies may also be found in healthy, immunocompetent individuals (Venturoli et al., 1998). This study found equal prevalence of anti NS-1 antibodies in patients with clinical manifestations associated with B19 infection (arthropathy, hematologic disorders, and non-
immune fetal hydrops) and healthy, age and sex-matched controls. NS-1-specific immunoblots revealed that 33 of 119 (27.7%) patients and 15 of 69 (21.7%) controls had IgG recognizing NS-1. In both groups, identification of anti-NS-1 antibodies was predominantly related to a serologic profile consistent with remote B19 infection (anti-VP2 IgM-, IgG+). However, the prevalence of anti-NS-1 antibodies in both groups was relatively low. It was speculated that individuals with anti-NS-1 antibodies might have experienced greater antigenic stimulation/exposure to NS-1 during infection due to extended B19 viremia or persistence. Thus, anti-NS-1 antibodies may be indicative of past infection only, and may not be indicators of abnormal outcomes of B19 infection.

The sera of 15 out of 33 pregnant women with recent B19 infection were shown to have IgG antibodies reactive to NS-1 using immunoblotting (Searle et al., 1998). Of the 15 women with NS-1 antibodies, 7 were symptomatic (2 had joint manifestations while 5 presented with rash and ‘flu’-like symptoms). The remaining 8 patients were thought to have subclinical infections. Furthermore, of the 7 cases where fetal complications had occurred, only 3 mothers (43%) had evidence of NS-1 antibodies in sera. As well, anti-NS-1 antibodies were consistently detected no earlier than 6 weeks after onset of acute infection. Thus, anti-NS-1 may not represent a marker for altered, persistent, or severe B19 infections in pregnant women.

A monoclonal antibody (mAb) with specificity to NS-1 was shown to have a weak but significant neutralizing activity in cfu-e inhibition assays, albeit at a 50-fold reduced ability when compared to capsid-specific mAbs (Gigler et al., 1999). It was hypothesised that a partial or transient expression of NS-1 protein on the B19 capsid surface may account for the neutralizing activity of NS-1 antibodies and may explain the presence of this antibody species in some individuals. This unproven assumption was based on a report of transitory surface expression of the non-structural protein of murine parvovirus (Cotmore et al., 1989).
1.3.3. **B19 Persistence in Immunocompromised Individuals**

Limited studies of immunocompromised individuals failing to produce B19-specific antibodies have shown that the virus may persist leading to chronic bone marrow failure with severe anemia (Kurtzman et al., 1989; Kurtzman et al., 1989a). A study of 6 immunocompromised individuals revealed variable levels of B19-specific IgM using immunoblotting and RIA (Kurtzman et al., 1989a). IgM levels were inversely related to levels of viremia, with B19 virus detectable in the serum by dot-blot hybridization when IgM levels were low, and disappearing when IgM levels were high. Serum IgG levels were low in immunocompromised patients in relation to those found in control sera obtained from 6 subjects with normal (self-limiting) courses of infection. Furthermore, neutralizing antibodies were not detected in the sera of immunocompromised patients using the cfu-e inhibition assay. Elevated B19-specific IgM and low (but detectable) IgG directed exclusively to VP2 was observed in the serum of another persistently B19-infected patient with recurrent aplastic crisis (Kurtzman et al., 1989). In this patient, the anemia completely resolved after administration of a commercial immunoglobulin preparation containing B19 antibodies. These observations suggest that a persistent B19 infection may result from a failure to produce neutralizing antibodies to the capsid proteins.

1.3.4. **B19 Persistence in Immunocompetent Individuals.**

There is evidence that B19 may persist in healthy, immunocompetent individuals. IgG subclass 4 (IgG4) directed against VP1 was consistently detected in the serum of individuals during the convalescent phase of uncomplicated B19 infection (200-700 days after onset of symptoms) and in serum pools of remotely infected controls. IgG4 antibodies are known to be associated with longstanding or repeated exposure to antigens, suggesting that B19 may persist in infected individuals or that certain individuals may experience repeated subclinical infections to B19 (Franssila et al., 1996).
B19 DNA was detected by PCR in the bone marrow of 17/84 patients with clinical manifestations of B19 infection and in 3/28 individuals with remote B19 infection with no clinical pathology (Cassinotti et al., 1997). Synovial membranes of 8/28 (28%) children (average age 8.5) with chronic arthritis of unknown etiology had evidence of B19 DNA determined using nested PCR, with no evidence of B19 DNA in blood, bone marrow, or synovial fluid (Soderlund et al., 1997). However, an even higher proportion 13/27 (48%) of slightly older healthy controls (average age 20.2) undergoing therapeutic arthroscopy for joint trauma had detectable B19 DNA in their synovial membranes. Both groups were serologically confirmed to have patterns of remote B19 infection (IgG+IgM- for VP2 protein). Although the cellular receptor for B19 (globoside) is present in synovium, it is not known which cell type in the synovial membrane, monocyte-lineage cells or fibroblastic cells, is host to B19 DNA (Soderlund et al., 1997). Furthermore, B19 has not been successfully cultured in synoviocytes (Miki and Chantler, 1992). Thus, B19 DNA may persist in both B19 infected individuals with overt pathology and in healthy, asymptomatic individuals.

1.4. Pathogenesis of B19 Arthropathy.

The pathogenesis of B19 infection-associated arthropathy remains elusive and controversial, despite active research into the subject. Although the temporal association of the development of anti-B19 capsid antibodies with acute arthropathy suggests that short-term joint manifestations following B19 infection is immune complex mediated (Brown et al., 1994), definitive evidence for this has not yet been demonstrated. The mechanism of development of chronic B19 infection-associated arthropathy has also not been conclusively determined. Found below is a summary of current theories on the etiology and pathogenesis of B19 arthropathy.

1.4.1. B19 Strains Associated with B19 Arthropathy.

Although genetic variation among B19 isolates has been observed, associations between a particular B19 isolate and a specific clinical syndrome have been conflicting and inconclusive.
Nucleotide and amino acid sequences of the complete VP1/VP2 region were highly conserved in 19 B19 isolates representing 8 separate epidemiological outbreaks in the United States between 1984 to 1994 (Erdman et al., 1996). An additional 10 B19 isolates were obtained from 7 different countries. Although there was greater genomic and amino acid diversity in B19 isolates obtained from different countries, no particular isolate was distinctively associated with arthropathy or other clinical manifestations of B19 infection. Furthermore, there was no evidence of a predominant variant in B19 isolates obtained from chronic B19 arthritis patients (Kurtzman et al., 1989). Single strand conformational polymorphism (SSCP) analysis of a region of NS-1 (nt 1399-1682) found 6 mutations representing 5 SSCP types in 50 DNA samples (Kerr et al., 1995a). The samples were obtained from subjects originating from 6 different countries with a wide range of clinical symptoms associated with B19 disease (EI, arthropathy, hematological disease, or intrauterine fetal loss). All mutations were found to be silent. There was a correlation of SSCP type and country, but no association of SSCP type and clinical illness. SSCP types 3 and 4 comprised 92% of all B19 strains. Interestingly, SSCP type 3 predominated in females, while males had equal distribution of types 3 and 4.

In contrast, other reports have suggested that B19 persistence and B19 arthropathy may be linked to particular B19 SSCP types. Follow-up assessment of 53 patients acutely infected with B19 found persistent DNA in the sera of 7 patients (Kerr et al., 1995b). Interestingly, none of the persistently infected patients had anti-B19 IgM. SSCP analysis of a B19 genomic fragment in the NS-1 coding region (nt 1399-1682) isolated from each of the persistently infected patients revealed an identical SSCP type in 5/7 of the patients, suggesting that this SSCP type may persist in host cells or tissues. In addition, 2 SSCP types were found in one persistently infected patient with persistent arthralgia and chronic fatigue syndrome, indicating that an individual may be co-infected with more than one strain of B19.
Genomic and protein sequences of 20 different B19 isolates obtained from individuals with a variety of B19 infection-associated clinical manifestations were compared to the sequence of B19 isolate pYT103 published by Shade et al., 1986 (Hemauer et al., 1996). Four regions representing half of the B19 genome including parts of NS-1, VP1, and VP2 were sequenced and compared in each isolate. Patients with arthritis or persistent infections demonstrated significantly higher degrees of genomic and protein sequence variations than acutely infected patients in the NS-1 C-terminal region. In arthritic and persistently infected patients, amino acid substitutions were commonly shifted from polar to basic residues, potentially altering secondary and tertiary protein structures significantly. These shared amino acid motifs may have implications in the transcription, antigenicity, and cytotoxicity of NS-1 and may also explain the differential pathogenic sequelae of B19 infection in individuals. Although variability was relatively uniform in the VP1 unique region and the VP1/VP2 junctional region, a high degree of non-conservative variation was observed in the protein sequences of 2/6 persistently infected patients. Since the unique region of VP1 and the VP1/VP2 junctional region are important sites of attachment for neutralizing antibodies, non-conservative mutations within this region may play a role in persistence and pathogenesis of B19 in some infected individuals.

1.4.2. Production of Autoantibodies in B19 Infections.

B19 infection has been associated with production of autoantibodies, although the clinical significance of these autoantibodies is uncertain. A cohort of 53 patients with serologically confirmed acute B19 infection with clinical symptoms were assessed for the presence of serum autoantibodies at an average follow-up interval of 57 months (Kerr and Boyd, 1996). At follow-up, 14 of 53 (26.4%) patients demonstrated serum autoantibodies (defined as having titres > 40) as opposed to only 2 of 53 (3.8%) age and sex-matched controls who were B19 seropositive due to remote, undocumented B19 infection. Only 5 of the 14 patients with autoantibodies had chronic
arthropathy at follow-up. Autoantibodies found included: anti-nuclear antibody, anti-smooth muscle antibody, gastric parietal cell antibody, anti-reticulin antibody, anti-mitochondrial antibody, and rheumatoid factor (RF). Seven patient sera had high autoantibody titres (>160), however only one of these patients had persistent symptoms (rheumatoid factor-positive rheumatoid arthritis). Thus, although acute B19 infection may have led to the development of autoantibodies, the association of these autoantibodies and the development of autoimmune disease was not supported.

In contrast, serum autoantibodies have been observed in patients with persistent B19 infection and autoimmune disease. Antibodies reactive with a 24 amino acid synthetic peptide representing part of the VP1/VP2 overlapping region were purified from individual sera of 10 chronic arthropathy patients (Lunardi et al., 1998). The patients had symptoms of chronic symmetric arthritis resembling rheumatoid arthritis or recurrent arthritis and cutaneous vasculitis lasting between 4 months to 2 years. Persistent B19 infection was inferred by the presence of persistent serum anti-B19 VP2 IgM. Purified IgM and IgG reactive against the B19 peptide were found to cross-react with keratin, type II collagen, ssDNA, and cardiolipin in ELISA assays. Interestingly, the autoantibody specificity corresponded to the clinical manifestations experienced in the individual from which the sample was purified. Arthritis patients had anti-type II collagen as the predominant autoantibody species, and patients with cutaneous lesions had high affinity anti-keratin antibodies. Sera obtained from healthy, B19 seropositive controls had low affinity antibodies that recognised the B19 peptide but did not bind any of the autoantigens. No sequence homologies between the B19 peptide and any of the autoantigens were identified. However, the autoantibodies may recognize common conformational motifs shared by the B19 peptide and these autoantigens thereby allowing for some degree of antigenic mimicry.
1.4.3.  **B19 as a Potential Etiological Agent for Rheumatoid Arthritis (RA).**

B19 infection-associated arthropathy has many clinical similarities to early RA, including morning stiffness greater than 1 hour in duration, joint distribution, and, in some cases, the presence of rheumatoid factor (see Section 1.2.3.6.). However, there are significant differences between chronic B19 infection-associated arthropathy and classic RA. These include absence of rheumatoid nodules, synovial inflammation, and joint erosions in B19 arthropathy (Naides, 1998). Furthermore, the symptoms of B19 arthropathy have been effectively controlled using non-steroidal anti-inflammatory drugs (NSAIDs) (Naides, 1998), while effective control of RA is usually only accomplished using disease modifying anti-rheumatic drugs (DMARDs). Thus, it is important to determine if there is a causal link between B19 infection and RA.

B19 DNA was detected by PCR in synovial tissue samples from 30/39 (77%) RA patients as opposed to only 4/26 (15%) samples from osteoarthritis patients (OA) and 5/31 (16%) samples from patients experiencing acute joint trauma (Takahashi et al., 1998). In the same study, immunohistochemical analysis with a VP1-specific monoclonal antibody (mAb) found VP1 protein exclusively in synovial cells from 27/27 RA patients with active joint lesions but not in control OA or joint trauma groups. Co-cultivation of synovial or bone marrow cells from 5 RA patients with B19-negative indicator cells (bone marrow, tonsil cells, UT-7 cells, macrophage-derived U937 cells, or THP-1 cells) transferred VP1 expression and induced the inflammatory cytokines IL-6 and TNFα in the indicator cells. Addition of anti-VP1 mAb to the co-cultivation system inhibited the transfer of IL-1, TNFα, and VP1 expression to indicator cells. Synovial cells derived from OA patients did not transfer B19 markers or induce cytokine production. The synovial cells derived from the OA patients were found to be B19 negative using PCR and in-situ hybridization. Thus, infectious B19 may be present in the synovium of active RA lesions, and absent in OA patients and joint trauma patients. Furthermore, in RA patients, B19 may contribute to joint inflammation by the
induction of inflammatory cytokines. \textit{In-situ} hybridization experiments using DNA probes found B19 DNA and RNA present in macrophages, follicular dendritic cells, T cells, and B cells, but not in synovial lining cells of the RA patients (Takahashi et al., 1998). These results suggest that the presence of B19 in synovial tissue of RA patients may be due to B19 infection of inflammatory cells that migrate to joints as opposed to direct infection of synovium.

In contrast, other reports have found no increased incidence of B19 infection in RA patients. Serum anti-B19 IgG antibodies were found in statistically significant equal proportions in both RA and OA patients (20/26 and 21/26 patients, respectively) (Kerr et al., 1995). B19 capsid proteins and globoside were absent in synovial tissue samples of both patient groups. However, B19 DNA was detected by PCR in synovial tissue of 10 of the RA patients and 9 of the OA patients, supporting the hypothesis that B19 infects inflammatory cells rather than synovial tissue. Another study examined serum and synovial fluid samples from 18 RA patients and 11 controls with acute joint swelling of unknown etiology for the presence of B19 antibodies and DNA (Kerr et al., 1996). Anti-B19 IgG was detected in 14/18 (77%) RA and 8/11 (73%) control sera, and in 9/18 (50%) RA and 7/11 (64%) control synovial fluid samples. Anti-B19 IgM and B19 DNA was not found in RA or control patients. However, these studies made no attempt to isolate replicative virus or to assess induction of inflammatory cytokines in indicator cells.

Thus, evidence exists both for and against an etiologic role for B19 infection in the triggering of RA. The lack of characteristic joint erosions in chronic B19 arthropathy differs significantly from the classic picture of RA. Furthermore, the high incidence of B19 infection in the general population may make temporal occurrences of B19 infection with RA purely coincidental. However, evidence of infectious B19 in inflammatory cells of RA patients should not be dismissed. It is possible that the presence of B19 in synovial fluid inflammatory cell populations such as macrophages and lymphocytes may exacerbate the inflammation of RA by inducing
proinflammatory cytokines such as IL-1 and IL-6. The presence of RF and anti-type II collagen in some B19 infected individuals (see Section 1.4.2.) may in fact serve to mediate the autoimmune injury commonly suspected as being the initial event in the pathogenesis of RA.

1.4.4. B19 Persistence and NS-1 Overexpression.

The persistence of B19 in infected individuals may play a crucial role in the pathogenesis of B19 arthropathy. B19 DNA was detected by PCR in the bone marrow and was absent in the sera of 4 patients who developed chronic B19 arthritis after acute infection (Foto et al., 1993). Continuous, severe joint symptoms were observed in 3 of the patients, while the remaining patient had 2 years of persistent symptoms followed by a 1 year remission before relapse. All patients were serum anti-B19 capsid IgM+IgG+ at disease onset (indicating recent B19 infection) and were IgM-IgG+ at time of bone marrow aspiration. In addition, 3 patients with only acute arthropathy after B19 infection also had evidence of B19 DNA in bone marrow (taken at 2,9, and 18 months, respectively) after infection. B19 DNA was not detected in the bone marrow or sera of 6 previously infected (IgM-IgG+) and 7 B19 seronegative healthy controls.

The mechanism for acute B19 arthropathy is believed to be immune complex mediated, while the mechanism of chronic B19 arthropathy is unknown (see Section 1.2.3.6.). However, the results of the above study may indicate that B19 persistence may be a common finding in individuals who develop joint symptoms following B19 infection, and may help in the determination of the pathogenesis of B19 arthropathy. The persistence of B19 DNA in subjects developing acute and/or chronic arthropathy following B19 infection suggests a common inability to clear the virus in both these clinical groups (Foto et al., 1993).

There are many possible explanations for the establishment of a persistent B19 infection. Genomic variations may lead to altered capsid proteins that may be less immunogenic than other strains, leading to immune evasion (Hemauer et al., 1996, see Section 1.4.1.). An underlying
immune defect has also been suggested to be the cause of B19 persistence in B19 arthropathy (Foto et al., 1993; Naides et al., 1992). Associations with B19 arthropathy and HLA DR4 molecules may indicate that antigen processing and presentation play a role in immune evasion and viral persistence (Klouda et al., 1996; Woolf et al., 1989, see Section 1.2.3.6.). Alternately, a defective T cell response may prevent viral clearance (see Section 1.3.1.). The role of antibody affinity to neutralizing epitopes on B19 capsid proteins in the effective clearance of B19 is well known (see Antibody Response to B19 Capsid Proteins). As VP1 has been shown to be a primary target of antibodies present in convalescent serum because of the protein’s numerous neutralizing domains, failure to produce anti-VP1 antibodies may lead to persistence of B19 (Foto et al., 1993; Naides, 1998). A preliminary study has shown that 16 patients with B19 infection-associated arthropathy had IgG recognizing whole B19 capsid but lacked IgG recognizing short (9aa) peptides representing neutralization domains in the unique region of VP1 (Naides et al., 1992). In contrast, subjects who had uncomplicated outcomes of B19 infection had IgG antibodies reactive with both whole capsid and the VP1 peptides.

Failure to mount an effective immune response against B19 or a delay in development of neutralizing antibodies may increase viral load, which may have implications in pathogenesis. Results of RT-PCR-based B19 neutralization assays found individuals with neutralizing antibody titres <200 developed clinical symptoms following B19 infection, while neutralizing antibody titres >1000 were found to protect B19-infected subjects from developing B19 disease (Bostic et al., 1999). Prolonged B19 persistence may lead to an overexpression of NS-1. In permissive cells, the capsid proteins are the predominant B19 proteins early in infection. NS-1 levels gradually rise later in the B19 infection and contribute to host cell toxicity and release of B19 infectious virions and proteins, including NS-1 (Ozawa et al., 1988). Non-permissive cells infected with B19 have a preferential expression of NS-1 protein, which mediates cytotoxicity within these cells (Ozawa et
al., 1988; Liu et al., 1992). Co-infection of B19-infected non-permissive cells with aden-associated virus may contribute to the increased NS-1 levels (Ponnazhagen et al., 1995). Given these observations, NS-1 overexpression may be a key factor in the pathogenesis of B19 infection-associated arthropathy. Although B19 DNA was found in equal frequency in both synovial membranes of patients with chronic arthropathy of unknown etiology and patients undergoing arthroscopy for joint trauma (Soderlund et al., 1997, see Section 1.3.4.), it is conceivable that NS-1 production may by favoured in some individuals leading to synovial cytotoxicity (Naides, 1998). Alternatively, inflammatory cells (neutrophils, lymphocytes, macrophages) infected with B19 may migrate to joint spaces. NS-1 transactivation of the IL-6 promoter and expression of this potent inflammatory cytokine (Moffat et al., 1996; Moffat et al., 1998, Takehashi et al., 1998) may underlie the symptoms of arthropathy associated with earlier B19 infection.

1.5. Thesis Rationale.

Persistent B19 infection leading to NS-1 mediated joint pathology is one of the most popular yet controversial mechanistic theories of B19 arthropathy. Both acute and chronic B19 arthropathy have been associated with viral persistence and may indicate underlying immune deficit (see Section 1.4.4.). However, evidence of persistence of B19 in asymptomatic, immunocompetent individuals confounds this theory (see Section 1.3.4.). The presence of anti-NS-1 antibodies has also been associated both with B19 persistence and arthropathy following B19 infection (von Poblotzki et al., 1995; von Poblotzki et al. 1995a). However, anti-NS-1 has been demonstrated in healthy individuals and may, in fact, be a marker for past infections in which NS-1 released from dying cells was in sufficient quantity to trigger an NS-1-specific antibody response in the host (see Section 1.3.2.2.).

Most investigations of B19 persistence and its association with anti-NS-1 antibodies have been hampered by the limited number of study subjects. Furthermore, studies illustrating an equal
distribution of anti-NS-1 antibodies in B19 patients with clinical symptoms and healthy controls were potentially flawed, as the healthy controls had only serologic evidence of past undocumented B19 infection (Venteroli et al., 1998, see Section 1.3.2.2.). Since the time of occurrence of B19 infection had not been documented in the healthy controls, it is conceivable that some may have experienced NS-1-mediated arthropathy at the time of acute infection. These individuals may have initially been unable to clear B19, resulting in high viremia and subsequent increased release of NS-1 from dying cells. Eventual production of neutralizing antibodies would have cleared the virus, with immune memory developing for both capsid proteins and NS-1. Evidence in support of this theory comes from observations of a patient with recurrent granulocytic aplasia associated with recurrent B19 infection (von Poblotzki et al., 1995a). The patient was initially unable to sustain sufficient levels of anti-B19 capsid IgG and thus suffered 3 relapsing episodes of granulocytic aplasia, which were effectively treated with intravenous immunoglobulin. Eventually, the patient developed stable IgG against B19 capsid proteins and was able to clear viral DNA from her serum. The development of NS-1-specific IgG during the course of the patient's clinical episodes suggested that either an anamnestic response to NS-1 protein or a prolonged exposure to NS-1 was needed before specific antibodies were detected.

The objective of this thesis was to resolve conflicting data concerning the relationship between B19 persistence and/or anti-NS-1 antibodies and the development of parvovirus B19 infection-associated arthropathy. Previous studies failed to adequately document the course of B19 arthropathy with respect to viral persistence and development of B19-specific antibodies reactive against capsid proteins and NS-1 from the time of initial B19 infection. Furthermore, although serum anti-NS-1 antibodies have been demonstrated separately to be associated with B19 persistence and with B19 arthropathy, there has not been a comprehensive study examining the relationship of all three of these parameters with each other. This study compared B19 persistence
and anti-NS-1 antibodies in individuals who developed acute and/or chronic arthropathy with individuals that did not experience joint manifestations from the time of initial B19 infection to examine the hypothesis THAT: Individuals infected with parvovirus B19 who develop acute and/or chronic arthropathy, as opposed to individuals who do not develop joint manifestations, will have persistent B19 infection and serum anti-NS-1 antibodies and THAT:
The development of anti-NS-1 antibodies is correlated with persistent B19 infections.

A study of the association of B19 arthropathy with viral persistence and with the presence of anti-NS-1 antibodies will help in the understanding of the underlying pathogenic mechanisms of both acute and chronic B19 arthropathy. As well, detection of B19 persistence and anti-NS-1 antibodies may assist in the diagnosis and subsequent treatment of B19 arthropathy.

Specific objectives of this thesis are discussed in point form below:

1. **To recruit study subjects:** In order to study the development of B19 persistence and anti-NS-1 antibodies after a recent B19 infection, it was necessary to identify and recruit a cohort of individuals with serologically-confirmed B19 infections. A recent B19 infection was defined by the presence of serum IgM antibodies directed against the VP2 capsid protein of B19 using commercial EIA. Once recruited, the cohort of recent B19 infections would be classified according to clinical outcome of B19 infection (arthropathy versus no arthropathy).

2. **To develop assays to identify B19 persistence and presence of serum anti-NS-1 antibodies:** In order to assess B19 persistence and the presence of anti-NS-1 antibodies in peripheral blood, PCR and NS-1 immunoblot assays were adapted from previously-established techniques.

3. **To determine the incidence of B19 persistence in recent B19 infections:** The incidence of B19 genomic DNA in serum (assessed using PCR) was determined in the cohort of recent B19
infections. If a difference in the incidence of B19 persistence was observed between subjects who developed acute and/or chronic arthropathy and subjects who did not develop joint manifestations, a model for the development of arthropathy following acute B19 infection could be proposed.

4. To determine the incidence of anti-NS-1 antibodies in recent B19 infections: The incidence of anti-NS-1 antibodies was assessed (using immunoblot assays) in sera from subjects with recent B19 infections to determine if these antibodies were related to the development of arthropathy following B19 infection.

5. To assess the relationship between B19 persistence and the development of anti-NS-1 antibodies: A correlation between the persistence of B19 in infected individuals and the presence of anti-NS-1 antibodies would aid in the development of a model for both the development of this antibody species and the development of B19 arthropathy.

6. To determine the incidence of B19 persistence and anti-NS-1 antibodies in individuals with past (undocumented) B19 infections: Much of the data concerning persistent B19 infections and the incidence of anti-NS-1 antibodies have come from studies of subjects with past (undocumented) B19 infections (anti-B19 capsid IgM-IgG+). The incidence of B19 DNA and anti-NS-1 antibodies in these subjects may reflect that of the general population. Therefore, a study of B19 persistence and anti-NS-1 antibodies in a population of B19 seropositive subjects was performed. Differences between the incidence of B19 DNA and anti-NS-1 antibodies in subjects with past B19 infection compared with recent B19 infection may indicate changes in these parameters over time.

7. To investigate the effects of re-exposure to B19 on individuals with past (undocumented) B19 infections: The development of anti-NS-1 antibodies has been speculated to be the result of repeated or prolonged exposure to B19 (see Section 1.3.2.). Very little is known about the effect that re-exposure to B19 has on subjects that with past histories of B19 infection. Therefore a population of subjects with past B19 infection that had been re-exposed to cases of acute B19
infection was examined to determine if the incidence of B19 DNA and anti-NS-1 antibodies was different from the incidence found in recent or in past B19 infections. An increase in the incidence of these parameters upon re-exposure to B19 would suggest subclinical reinfection.
Chapter 2

Materials and Methods

2.1. Study Subjects.

A majority of study subjects (n=137) were identified through parvovirus B19 antibody testing rosters provided by the Virology Laboratory at the B.C. Centre for Disease control (BCCDC, Vancouver, Canada). Subjects were referred for B19 serology by their respective physicians in order to diagnose rash illness, rheumatologic disorder, or to determine B19 serologic status in pregnant women exposed to active B19 infections. A cohort of healthy volunteers (n=16) were recruited by the study team and subsequently tested for B19 serological status at the BCCDC. Antibody testing for B19-specific IgM and/or IgG was performed using commercial enzyme immunoassay (EIA) that employed recombinant baculovirus-expressed B19 VP2 antigen (Biotrin International, Mount Merrion, Eire). Potential study subjects were screened for inclusion criteria that required subjects to be over the age of 18 years and not be immunocompromised or on immunosuppressive therapy. The 153 study subjects (23 males, 130 females) ranged in age from 18-61 years, with an average age of 35 years.

Selected study subjects were contacted with the permission of referring physicians. Clinical histories were obtained from each patient following acquisition of informed consent using procedures approved by the University of British Columbia Ethics committee. Additional patient clinical history was obtained from referring physicians when possible. Each subject’s history included details of the circumstances leading to the request for B19 serology, signs and symptoms of current illness, personal and family histories of connective tissue or other autoimmune diseases, medications, and pregnancy status.
2.2. Sample Acquisition.

Original serum specimens obtained after physician referral were retrieved from the BCCDC frozen specimen archives and used for B19-specific PCR and immunoblot assays (see sections 2.3 and 2.4). Follow-up EDTA and clotted blood samples were collected by venipuncture. Peripheral blood mononuclear cells (PBMNC) were isolated from EDTA blood samples for use in B19-specific PCR assays (see section 2.3). Sera obtained from clotted blood samples were used in both B19-specific PCR and immunoblot assays (see sections 2.3 and 2.4, respectively). After informed consent and clinical history documentation, blood specimens (as above) were also obtained from the healthy volunteer controls. Sera from these subjects were sent to the BCCDC for determination of B19 serological status.

2.3. PCR Detection of B19 DNA in Study Subject Sera and PBMNC.

2.3.1. Preparation of Sera for PCR.

Sera obtained from BCCDC archives or clotted blood was stored at −70°C until needed. Preparation of serum samples for B19-specific PCR involved heating sera at 95°C for 5 minutes to denature serum proteins. The sera was then centrifuged at 2000 rpm for 5 minutes and the supernatant collected and stored at 4°C for use in PCR assays.

2.3.2. Isolation of B19 DNA from Peripheral Blood Mononuclear Cells (PBMNC) for PCR.

For isolation of PBMNC DNA, the following buffers were used:

Red blood cell lysis buffer: 0.144 M NH₄Cl, 0.001 M NaHCO₃

Cell lysis buffer: 10 mM Tris (pH 7.8), 5 mM EDTA, 0.5% SDS

Saturated ammonium acetate buffer

Tris-EDTA: 10 mM Tris (pH 8), 1 mM EDTA

EDTA blood samples were centrifuged at 2000 rpm for 15 minutes. The resultant buffy coat was collected and 8 ml of cold red blood cell (RBC) lysis buffer added. RBC lysis was allowed to proceed at room temperature for 5 minutes, then the resultant solution was centrifuged at 1500 rpm...
rpm for 5 minutes. The cellular pellet was subjected to lysis and protein digestion by adding 2 ml of cell lysis buffer and 5 ul of proteinase K (20 mg/ml) (Canadian Life Technologies, Burlington, Ontario) and incubating for 2 hours at 65° C. After proteinase K digestion, 0.66 ml saturated ammonium acetate was added and the mixture was centrifuged at 2000 rpm for 20 minutes. DNA was precipitated from the supernatant with 2 volumes of 95% ethanol, washed in 70% ethanol, and allowed to air-dry. The DNA was dissolved in 100 µl Tris-EDTA and stored at 4°C until use in PCR assays.

2.3.3. B19-Specific PCR.

A nested PCR procedure was used to detect B19 DNA within sera samples or DNA isolated from PBMNC. Primer pairs that were used for amplification were designed from a description of RNA transcripts that were found to be abundant in permissive cell lines infected with B19 (Ozawa et al., 1987). Primer sequences represent those reported by Shade et al. (Shade et al., 1986) (Table 1).

Table 1. Parvovirus B19-Specific Primer Sequences for Nested PCR

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Tm</th>
</tr>
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<tbody>
<tr>
<td>PAR-A (2312 – 2297): 5’ - TTA TAC CAA GCC CCT A - 3’</td>
<td>Tm = 40 – 45 °C</td>
</tr>
<tr>
<td>PAR-B (2077 – 2092): 5’ - ACC AGT TCA GGA GAA T - 3’</td>
<td></td>
</tr>
<tr>
<td>PAR-1 (2286 – 2263): 5’ - ATG GGG ACA AAG TCC CAA CCC TCC - 3’</td>
<td>Tm = 65 – 70 °C</td>
</tr>
<tr>
<td>PAR-2 (2095 – 2119): 5’ - TTT GTC GGA AGC CCA GTT TCC TCC - 3’</td>
<td></td>
</tr>
</tbody>
</table>
Each 50 μl reaction mixture contained 2 μg of DNA, 10 pmol of each primer, 200 μM of unlabelled dNTPs (Boehringer-Manheim, Laval, Quebec), and 1 unit of Taq polymerase (Bio/Can, Mississauga, On). Primers PAR-A and PAR-B were used in the first stage amplifications. A 3-step thermal cycle of 94 °C for 20 sec, 45 °C for 30 sec, and 72 °C for 30 sec was performed for 25 cycles after an initial 2 min denaturation at 94 °C. Second stage amplifications employed primers PAR-1 and PAR-2 in a 2-step thermal cycle of 94 °C for 20 sec and 65 °C for 1 min for 25 cycles following an initial 2 min denaturation at 94 °C. All amplifications were performed on a Perkin-Elmer-Cetus thermal cycler. PCR products were electrophoresed on 2% agarose-ethidium bromide gels in Tris-borate EDTA buffer, which were subsequently photographed under u.v. light. Positive controls included an acute serum specimen obtained from a patient with serologically confirmed B19 infection and a plasmid construct (pSVOdΔ170) containing approximately 5000 bp of B19 DNA (provided by Dr. C. Astell, Department of Biochemistry and Molecular Biology, University of British Columbia).

2.4. NS-1 Immunoblot.

2.4.1. Parvovirus B19 Recombinant Nonstructural Protein (rNS-1).

Recombinant NS-1 protein (rNS-1) used as the antigen in immunoblot assays was the generous gift of Dr. C. Astell (Department of Biochemistry and Molecular Biology, University of British Columbia). A (His)_6-NS-1 fusion protein was cloned by PCR into an expression plasmid, pQE40 and was expressed in E. coli (Dr. C. Astell, unpublished results). rNS-1 was solubilized in 6 M guanidine hydrochloride and purified on a Ni²⁺-Sepharose column (Invitrogen, San Diego, Ca) in the presence of 8 M urea. Purified rNS-1 was renatured by dialysis against a buffer containing 1% NP-40, 50 mM Tris, 150 mM NaCl (pH 7.9). Polyclonal sera (A13RD), obtained by immunizing rabbits with a synthetic peptide representing a C-terminal sequence of NS-1, was used to characterize the rNS-1 protein using immunoblot.
2.4.2. Immunoblot Procedure.

Buffers used in the immunoblot protocol were as follows:

Sample buffer (reducing): 0.1 M Tris-HCl (pH 6.8), 10% glycerol, 0.25% SDS, 1.5% mercaptoethanol, 0.005% bromphenol blue (BPB)

Stacking gel: 4% Acrylamide, 0.1% Bis, 0.125 M Tris-HCl (pH 6.8), 0.05% TEMED and ammonium persulfate (APS)

Separating gel: 9% Acrylamide, 0.2% Bis, 0.375 M Tris-HCl (pH 8.8), 0.05% TEMED and APS

Running buffer: 200 mM glycine, 25 mM Tris, 0.1% SDS

Transfer buffer: 200 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol

Blocking buffer: TBS (pH 7.4), 4% skim milk powder

Washing buffer: 0.05% Tween-20 in TBS

Substrate buffer: 50 mM Tris-HCl (pH 9.6), 140 mM NaCl, 5 mM MgCl₂, 0.3 mg/ml 5-bromo-4-chloroindolyl phosphate (BCIP), 0.3 mg/ml nitroblue tetrazolium (NBT)

SDS-PAGE was performed using a Mini-Protean II apparatus. rNS-1 was diluted in sample buffer to a final concentration of 2 μg/ml and heated at 60 °C for 3 minutes. A total of 65 μl of rNS-1 in sample buffer was loaded onto a single long well formed by a 0.75 mm comb inserted into the stacking gel. Separation was performed at 300 mA constant current and ran until the BPB dye front reached the bottom of the gel. The rNS-1 preparation was electrophoretically transferred to nitrocellulose for 30 minutes at 300 mA constant current in transfer buffer. The nitrocellulose blot was blocked for 1 hour in blocking buffer and then cut into 4 mm strips. Individual strips were incubated overnight in blocking buffer at 4 °C with individual subject sera or controls at a 1:25 dilution. To remove antibodies which might have
reacted with *E. coli* antigens remaining in the rNS-1 preparation, subject sera were pre-absorbed by incubation with an *E. coli* lysate for 4 hours at 4 °C, then cleared by centrifugation at 10 000 rpm for 10 minutes prior to immunoblot. Positive controls included a rabbit polyclonal antibody raised against an NS-1 peptide (A13RD, see section 2.4.1. above) and a mouse anti-(His)$_6$ monoclonal antibody (Qiagen Inc., Santa Clarita, CA). Negative controls included pre-immunization serum from the rabbit that was immunized to obtain the positive control antibody, A13RD, and pooled sera from 5 healthy human donors that were seronegative for B19 VP2 antibodies. After overnight incubation in the primary antibody solutions, the strips were washed on a shaker for 30 minutes in washing buffer. The strips were then added to blocking buffer containing a 1:500 dilution of alkaline phosphatase (AP) conjugated goat anti-human IgG (in the case of human sera), AP conjugated mouse anti-rabbit IgG (in the case of A13RD), or AP conjugated goat anti-mouse IgG (in the case of anti-(His)$_6$) (all obtained from Kirkegaard & Perry Laboratories, Gaithersburg, MD). After a 2 hour incubation at room temperature and a 30 minute wash (as above), the strips were developed in substrate buffer with constant shaking until specific bands (see section 3.3.) appeared on the positive control strips and the positive patient serum specimens (approximately 30 minutes). The strips were rinsed with distilled water, allowed to air dry, and then interpreted independently by two investigators (R.L. and L.M.) for concordance of results.
Chapter 3.

Results and Discussion.

3.1. Classification of Study Subjects.

For data analysis, study subjects were classified according to B19 serological status (as determined by commercial EIA). Subjects were further sub-categorized by clinical signs and symptoms. Definitions of each serological and clinical group are found in Table 2. Detailed clinical information for individual subjects within the recent B19 infection group and the past (undocumented) B19 infection group is listed in Tables 3 and 5, respectively (see below).

3.2. Detection of B19-Specific DNA by PCR

A representative agarose-ethidium bromide gel of the products of nested PCR to detect B19 sequences is shown in Figure 1. A single 192 bp band was amplified in a positive control consisting of an acute serum specimen obtained from a patient with serologically confirmed B19 infection (see Materials and Methods), and was the standard by which all sample sera were compared. The sensitivity of the assay was estimated to be approximately 3 fg, or 1 molecule per 50 μl reaction, using a plasma construct (pSVOdA170) containing approximately 5000 bp of B19 DNA (provided by Dr. C. Astell). The specificity of the assay was determined using 29 controls that were identified as seronegative for IgM and IgG antibodies specific for the VP2 capsid protein of B19. This seronegative control group consisted of 7 subjects undergoing rheumatologic assessment for acute or chronic arthropathy, 3 subjects with undiagnosed rash, 2 subjects with undiagnosed ‘flu’-like illness, and 17 healthy subjects. B19 DNA was not found in the sera or PBMNC of any of the seronegative controls (data not shown).
Table 2. Serologic and clinical category definitions for B19 study subjects.

<table>
<thead>
<tr>
<th>SEROLOGIC CATEGORY</th>
<th>CLINICAL CATEGORY</th>
<th>DEFINITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>RECENT B19 INFECTION</td>
<td>Acute Arthropathy</td>
<td>Possesses IgM specific for B19 VP2 protein, detected in acute phase serum. Clinical confirmation of B19 infection.¹</td>
</tr>
<tr>
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<td>Chronic Arthropathy</td>
<td>Arthropathy (arthralgia² and/or arthritis³) developing within 31 days of B19 infection and lasting less than 4 weeks.</td>
</tr>
<tr>
<td></td>
<td>No Arthropathy</td>
<td>Arthropathy developing within 31 days of B19 infection and lasting for more than 4 weeks.</td>
</tr>
<tr>
<td></td>
<td>Rash Illness</td>
<td>Rash of chicken pox, rubeola (red measles), or undiagnosed facial rash.</td>
</tr>
<tr>
<td></td>
<td>Healthy</td>
<td>Healthy B19 IgG positive volunteers. No clinical signs or symptoms of recent B19 disease¹. No personal or family history of joint or connective tissue disease.</td>
</tr>
<tr>
<td>PAST (UNDOCUMENTED) B19 INFECTION, EXPOSED TO B19</td>
<td>Pregnant, Healthy</td>
<td>Possesses serum IgG antibodies specific for VP2 protein. Absence of serum anti-VP2 IgM antibodies. Exposed to a confirmed case of acute B19 disease during pregnancy.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No signs or symptoms of B19 disease. No adverse effects to pregnancy.</td>
</tr>
</tbody>
</table>

¹ History of ‘flu'-like illness with one or more of the following signs: fever, facial rash progressing to maculopapular rash on trunk and limbs, with or without joint manifestations.  
² Arthralgia: subjective joint pain only.  
³ Arthritis: joint pain with one or more of the following signs: swelling, heat, tenderness, loss of function.
3.3. Detection of NS-1-specific Antibody in Immunoblot Assays.

IgG antibodies directed to recombinant B19 NS-1 protein (rNS-1) were detected using immunoblot assays (see Materials and Methods). NS-1 immunoblot performed with B19 positive controls or patient sera detected an array of 8 bands ranging from approximately 77 kDa to 25 kDa (Fig.2). A triplet of bands (bands 1-3) ranging from 71-77 kDa was detected by the binding of NS-1-specific rabbit polyclonal antiserum (A13RD) and of monoclonal anti-His6 antibody. This triplet of bands corresponded to previously identified major bands of the rNS-1
antigen and represents full-length rNS-1 protein (Astell et al., 1997). The five additional bands at approximately 68, 55, 48, 34, and 25 kDa (bands 4-8, respectively) were recognized by A13RD and by some study subject sera. Since bands 5-8 were recognized by A13RD (specific for a C-terminal peptide of NS-1) but not by anti-His$_6$ (a monoclonal antibody specific for the N-terminus of NS-1), they were assumed to represent post-translational cleavage products present in the rNS-1 preparation or possible truncated synthetic products. Therefore, as the NS-1 immunoblot was performed under reducing conditions, presence of one or more bands within the 71-77 kDa triplet was used as the criteria for the determination of NS-1-specific IgG in the sera of study subjects. No particular relationship between recognition of bands 4-8 and clinical symptoms was observed (data not shown).

**Figure 2. Immunoblot assay for parvovirus B19 nonstructural protein-1 (NS-1).** Recombinant NS-1 was transferred to nitrocellulose membranes after SDS-PAGE under reducing conditions and probed with immune sera (see Methods). The positions of stained antibody bands (numbered arrowheads) were compared to molecular weight markers transferred onto the same nitrocellulose membrane (not shown). A: Mouse monoclonal anti-(His)$_6$ showing staining of bands 1-3 (77-71 kDa). B: Rabbit anti-NS-1 C-terminal peptide (A13RD) showing staining of bands 1-3, 5 (55 kDa), 7 (34 kDa), and 8 (28 kDa). C: Acute phase serum from a subject who developed acute arthropathy following B19 infection (subject 077) showing staining of bands 1-3, 4 (68 kDa), and 6.
The specificity of the NS-1 immunoblot was determined using sera from the 29 subjects that were seronegative for VP2 protein (see Section 3.2). No bands representing anti-NS-1 antibodies were detected with NS-1 immunoblot in any of these control sera (data not shown).

3.4. B19 Persistence and Anti-NS-1 IgG Antibodies in Recent B19 Infections.

A comparative analysis of B19 persistence (determined by PCR for viral DNA) and of the presence of anti-NS-1 IgG was performed on a study population that was determined to have been recently infected with B19 on the basis of detection of IgM against VP2 capsid protein in acute serum specimens. The aim of this study was to determine if B19 persistence (as demonstrated by a positive PCR result) and/or presence of anti-NS-1 antibodies was related to the development of acute and chronic joint manifestations following B19 infection. The study population was separated into three clinical categories: acute arthropathy (n=27), chronic arthropathy (n=13), and no arthropathy (n=7) (Table 1). Study subjects were followed for up to one year after confirmation of B19 infection with serial blood samples. Acute frozen serum samples (taken within 1 month of onset of clinical symptoms) were obtained from BCCDC. Follow-up late convalescent blood samples were collected 6-12 months following onset of clinical symptoms to obtain sera and PBMNC. A summary of laboratory findings and clinical observations is shown in Table 3, and are detailed below.

3.4.1. B19 DNA and Joint Manifestations.

The mechanism of acute B19 arthropathy remains unknown, although it has been speculated to be mediated by the formation of immune complexes (Brown et al., 1994). Development of chronic arthropathy following B19 infection has been associated with B19 persistence (Naides et al., 1992; Foto et al., 1993; Klouda et al., 1996; Woolf et al., 1989). However, other studies have shown no relationship between B19 persistence and arthropathy (Cassinotti et al., 1997; Soderlund et al., 1997). The objective of this experiment was to determine if the presence of B19 DNA was correlated with the development of acute and/or
Table 3. B19 DNA and Anti-NS-1 IgG in Sera of Subjects With Recent B19 Infections.

<table>
<thead>
<tr>
<th>Study No.</th>
<th>SEX</th>
<th>AGE</th>
<th>Acute Sample</th>
<th>Late Convalescent Sample</th>
<th>Clinical Findings and Duration</th>
</tr>
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1 Sample obtained within 30 days of onset of clinical signs of B19 infection (fever, rash, and/or joint manifestations)
2 Sample obtained between 6-12 months after onset of clinical signs of B19 infection
3 Time after onset of clinical signs of B19 infection
4 Presence of B19 DNA in serum as determined by nested PCR (see Materials and Methods)
5 Presence of NS-1 specific IgG as determined by immunoblot (see Materials and Methods)

ND = Not determined
<table>
<thead>
<tr>
<th>Study No.</th>
<th>SEX</th>
<th>AGE</th>
<th>Acute Sample</th>
<th>Late Convalescent Sample</th>
<th>CLINICAL FINDINGS AND DURATION</th>
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**Chronic Arthropathy (n=13)**

**No Arthropathy (n=7)**
chronic arthropathy following recently documented B19 infection. Nested PCR was performed with acute and late convalescent sera, as described in Materials and Methods. DNA extracted from peripheral blood mononuclear cells (PBMNC) isolated from late convalescent blood samples was also tested for B19 sequence by PCR. Presence of B19 DNA in these samples at various times after infection was related to clinical outcome (development of joint manifestations).

Of 27 acute phase serum samples (sample 1) obtained from subjects who experienced acute (short-term) arthropathy following B19 infection (Table 2), 19 (70%) had B19 DNA detectable by PCR. Of the 15 subjects from whom sequential (acute and late convalescent) serum samples were taken, 12 (80%) acute phase samples and 2 (13%) late convalescent samples (sample 2) tested positive for B19 DNA, demonstrating a decline in B19 PCR positivity with time after onset of disease (Fig. 3A). Thirteen acute phase serum samples were obtained from the 13 subjects who experienced chronic arthropathy (persistent or recurrent arthropathy lasting more than 1 month) following B19 infection. B19 DNA was detected in 4 (31%) of the acute phase serum samples. Of the 7 subjects from which convalescent samples were obtained, 2 (29%) acute phase samples and 1 (14%) late convalescent phase samples tested positive for B19 genomic DNA (Fig. 3B). There were 7 subjects comprising a third clinical group that did not experience any joint symptoms with recent B19 infection. B19 DNA was detected in 3 (43%) acute phase serum samples. Of the 3 individuals from whom sequential samples were obtained, 1 (33%) acute phase sample and none of the convalescent phase samples were positive for B19 DNA (Fig. 3C). B19 DNA was not found in PBMNC from any of the study groups.

3.4.1.1. B19 DNA in Acute Phase Sera and Acute Arthropathy.

Prospective studies of B19 viremia in volunteers revealed that B19 may be detected in
Figure 3. **B19 DNA in Sera of Subjects with Documented Recent B19 Infection.** The presence or absence of B19 DNA in the sera is expressed for each clinical group (A. acute arthropathy only, B. chronic arthropathy, C. No Arthropathy). Total acute samples represent the total number of acute samples obtained for the clinical group (one per subject) and were collected within 31 days of onset of clinical symptoms of serologically confirmed B19 infection. Follow-up subjects had sequential acute and late convalescent (obtained between 6-12 months after onset of clinical symptoms or B19 infection) samples taken for B19 PCR.

Blood samples 5-6 days after initial infection, with peak viremia occurring 8-9 days after infection (Brown et al., 1994). Reduction in viral load is correlated with the development of specific antibodies to B19 10-12 days after initial infection (Soderlund et al., 1995). By
compiling the presence of B19 DNA in acute serum samples and plotting them against their respective acquisition times, a similar trend in the incidence of B19 DNA during the acute and early convalescent phases of B19 infection was observed in this study (Fig. 4). The peak prevalence of detectable B19 DNA was between 5 – 10 days after onset of clinical symptoms of B19 infection, although B19 DNA was detected in sera of a few subjects for up to 20 days after onset.

![Figure 4. Incidence of Serum B19 DNA Relative to Time of Infection.](image)

The prevalence of B19 genomic DNA within acute phase samples of subjects experiencing acute arthropathy after B19 infection (70%) was higher than the prevalence within the subject groups that experienced chronic arthropathy (31%) and no arthropathy (43%). These results may be attributed to the relative difference in acquisition time of the acute samples between groups. In the acute arthropathy group, acute samples were obtained earlier (median time after onset = 10.5 days) than in the groups experiencing chronic joint manifestations (median time after onset = 23 days) or no joint problems (median time after onset = 14 days).
The earlier sampling of the acute arthropathy group may have allowed for a bias towards PCR positivity by being closer to the peak of B19 viremia (Fig. 4). Sampling of the chronic arthropathy and no arthropathy groups occurred at a later stage in the course of B19 infection, where there was likely to be a lower prevalence of B19 DNA. This would also explain the difference in prevalence of B19 DNA between chronic arthropathy and no arthropathy groups, with the former group having the latest average timing of first samples and the fewest sera with detectable B19 DNA.

Results of clinical studies suggest a link between failure to produce B19 virus-neutralizing antibodies with B19 persistence and with the development of arthropathy (Foto et al., 1993; Naides, 1998; Bostic et al., 1999). Interestingly, in this study B19 DNA was not observed in any samples obtained 13-16 days after estimated time of infection in four subjects who did not develop joint manifestations. In contrast, B19 DNA was detected in samples obtained 11-20 days after onset from 6/9 acute arthropathy subjects and from 1/2 subjects who developed chronic joint manifestations. As the number of subjects were low, and the time of sampling was not uniform for the clinical groups, definitive conclusions concerning the significance of this finding cannot be made. Although speculative, it may be that individuals who do not develop acute arthropathy following B19 infection develop neutralizing antibodies at a faster rate than individuals who experience joint manifestations. Earlier development of neutralizing immunity may control B19 viremia and thus reduce the amount of time that soluble immune complexes, thought to be involved in acute B19 arthropathy, remain in the body.

3.4.1.2. Persistent B19 DNA in Late Convalescent Samples and Chronic Arthropathy

Although B19 persistence has been linked with the development of chronic arthropathy following infection, results of this study do not support this correlation. Of the 7 late convalescent serum samples obtained from the 13 individuals experiencing chronic arthropathy following B19 infection, only 1/7 (14%) had B19 DNA (Fig. 3B). Moreover, B19 DNA was
found in 2/15 (13%) late convalescent serum samples from subjects experiencing only acute arthropathy (Fig. 3A). B19 DNA was not found in any of the convalescent sample PBMNC in any of the clinical groups. Therefore, it is likely that the differences in frequency of detection of B19 DNA in serum samples among clinical groups reflects only the timing of the samples relative to disease onset and hence, the onset of viremia-controlling immune responses.

B19 DNA was found in similar proportions in late convalescent samples of acute and chronic arthropathy groups. However, none of the second samples of the no arthropathy group demonstrated detectable B19 DNA. The average interval from disease onset to late convalescent sampling was similar for the acute arthropathy, chronic arthropathy, and no arthropathy groups (7.8, 9.0, and 8.6 months, respectively). Failure to detect B19 DNA in second samples of the latter group may have been due to the small number samples (three) obtained for this group.

An alternate explanation for the lack of detectable B19 DNA in convalescent sera of the no arthropathy group may reflect an early effective immune response capable of producing adequate levels of neutralizing antibodies that completely clear B19 from the infected subjects. In contrast, the incidence (albeit low) of B19 in convalescent sera of acute arthropathy and chronic arthropathy groups indicates persistence of the B19 in these individuals. This finding may reflect an extended viremic phase of B19, possibly due to a lack of, or delay in, development of neutralizing antibodies to B19 (as discussed above). It is possible that the individuals within the arthropathy groups may have experienced this delay in development of immunity, with the majority eventually developing neutralizing antibodies leading to clearing of B19 (shown by the temporal decline of B19 DNA in sequential serum samples). Conversely, the healthy group may have quickly developed effective immunity to B19, thereby avoiding development of acute and chronic arthropathy. However, this alternative explanation is purely speculative due to the small number of convalescent samples obtained for the no arthropathy group. In addition, the presence of persistent B19 DNA was studied in peripheral blood only.
As B19 DNA has also been demonstrated to persist in bone marrow (Foto et al., 1993; Kurtzman et al., 1989a) and synovial membranes (Soderlund et al., 1997; Takahashi et al., 1998), it is possible that B19 DNA may have been found in these tissues in the study subjects.

3.4.2. Relationship of Anti-NS-1 IgG to the Development of B19 Arthropathy

Results of previous studies involving small numbers of subjects suggested that NS-1 specific IgG antibodies may be found more frequently in individuals experiencing arthropathy following B19 infection than in those who have uncomplicated outcomes (von Poblotzki et al., 1995, von Poblotzki et al., 1995a). In contrast, other studies found NS-1-specific IgG equally in symptomatic and subclinical B19 infections (Venturoli et al., 1998; Searle et al., 1998) suggesting that anti-NS-1 antibodies are markers for past infection only. Furthermore, it has been suggested that anti-NS-1 antibodies are a reflection of greater antigenic stimulation/exposure to NS-1 protein or re-exposure to B19 (Venturoli et al., 1998). This study was designed to determine if the incidence of serum anti-NS-1 antibodies was linked with clinical outcome following recent B19 infection. Using immunoblot assays (see Materials and Methods), anti-NS-1 IgG was detected in acute and late convalescent sera following documented B19 infection. Results are listed in Table 3.

Anti-NS-1 antibodies were found in serum samples from all 3 clinical study groups that were recently infected with B19 (Fig. 5). In the acute arthropathy group, 9/27 (33%) acute samples collected tested positive for anti-NS-1 antibodies (Fig. 5A). Of the 15 individuals within this group from which sequential serum samples were obtained, 7 (47%) acute samples and 6 (40%) late convalescent samples had NS-1-specific IgG. The group experiencing chronic arthropathy had NS-1-specific IgG in 2/13 (15%) acute samples (Fig. 5B). In the seven individuals followed sequentially, none of the acute samples had NS-1-specific IgG and only two (29%) convalescent samples were positive for anti-NS-1. Interestingly, of the subjects that did not develop joint manifestations, 2/7 (29%) acute samples demonstrated anti-NS-1 antibodies.
(Fig. 5C). Of the 3 subjects followed sequentially, none were positive for NS-1 IgG acutely, while one (33%) convalescent sample contained anti-NS-1 IgG.

**Figure 5. Anti-NS-1 IgG in Sera of Subjects with Documented Recent B19 Infection.** The presence or absence of anti-NS-1 IgG in the sera of subjects with recent B19 infections was assessed using immunoblot assays. Results are expressed for each clinical group (A, acute arthropathy only, B, chronic arthropathy, and C, no arthropathy). Total acute samples represent the total number of acute samples obtained for the clinical group (one per subject) and were collected within 31 days of onset of clinical symptoms or documented B19 infection. Follow-up subjects had sequential acute and late convalescent (obtained between 6-12 months after onset of clinical symptoms or B19 infection) samples.
Variation among the clinical groups with respect to proportions of individuals with serum NS-1 antibodies may have been due to the smaller number of acute and convalescent serum samples obtained for the chronic arthropathy and no arthropathy groups relative to the acute arthropathy group. However, the data suggest anti-NS-1 antibodies persist into the late convalescent phase in certain individuals regardless of clinical outcome. No correlation was found between the presence or persistence of anti-NS-1 antibodies and the development of acute or chronic arthropathy. These results suggest that anti-NS-1 antibodies are more common than previously noted, and may only be a marker for previous infection rather than indicators of arthropathy.

3.4.3. B19 Persistence and Anti-NS-1 Antibodies

The development of anti-NS-1 antibodies has been reported to be linked with persistent B19 infections and B19 arthropathy (von Poblotzki et al., 1995; von Poblotzki et al., 1995a). However, other studies have found anti-NS-1 antibodies to be indicators of past infection only and not with B19 persistence (Venturoli et al., 1998; Searle et al., 1998). Because of the possible link between anti-NS-1 antibodies and arthropathy, associations between persistent B19 infections and anti-NS-1 antibodies were explored to provide insight into the mechanism of development of this antibody species. The prevalence of NS-1-specific IgG antibodies and B19 DNA were compared in acute and late convalescent sera collected from patients with serologically confirmed recent B19 infection (Table 4).

Twenty-seven acute samples were collected from the clinical group experiencing acute arthropathy only. B19 DNA was detected in 8/9 (89%) sera testing positive for anti-NS-1 antibodies and 11/18 (61%) sera testing negative for anti-NS-1 antibodies. Sequential serum samples were obtained for 15 of the acute arthropathy subjects. B19 DNA was found in 6/7 (86%) anti-NS-1 positive acute sera and 6/8 (75%) anti-NS-1 negative acute sera. B19 DNA was detected less often in the convalescent samples, with 1/6 (17%) anti-NS-1 positive and 1/9 (11%)
Table 4. Relationship Between Incidence of B19 DNA and Anti-NS-1 IgG Antibodies in Sera from Subjects with Recent B19 Infections.

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<th>CHRONIC ARTHROPATHY:</th>
<th>NO ARTHROPATHY:</th>
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</table>

1 Total number of acute samples for each clinical group (acute arthropathy only, chronic arthropathy, no arthropathy). One sample was collected for each subject.

2 Subjects from which both acute and late convalescent samples were obtained.

3 Acute samples were obtained within 31 days of B19 infection or of onset of B19-related symptoms.

4 Late convalescent samples were obtained between 6-12 months after B19 infection or after onset of B19-related symptoms.
anti-NS-1 negative sera testing positive for B19 DNA. Similar observations were made in the group that developed chronic arthropathy. Of the 13 acute samples taken from this group, B19 DNA was detected in 1/2 (50%) anti-NS-1 positive sera and 3/11 (27%) anti-NS-1 negative sera. Sequential serum samples were obtained for 7 of the chronic arthropathy subjects. B19 DNA was detected only in acute samples, both of which were negative for anti-NS-1 antibodies. In late convalescent phase, only one sample (which was negative for NS-1 IgG) was positive for B19 DNA. There were 7 acute samples obtained from the group that did not experience arthropathy following B19 infection. B19 DNA was found in both anti-NS-1 positive sera samples and 1/5 anti-NS-1 negative sera. Sequential serum samples were obtained from 3 of these subjects. B19 DNA was found in only one of the acute serum samples (all NS-1 IgG negative). No B19 DNA was found in either the single anti-NS-1 positive or the two anti-NS-1 negative late convalescent samples in this clinical group.

No correlation was found between viral persistence in sera or PBMNC and the production of anti-NS-1 antibodies. Although B19 DNA was found in a number of acute serum samples from each clinical group, there was a sharp decline in the number of late convalescent samples testing positive for B19 DNA. Furthermore, this decline in the number of samples with detectable B19 DNA was not related to NS-1 seropositivity. Instead, the presence of serum B19 DNA was related to the time of acquisition of the sample, which is a reflection of the stage of convalescence of B19 infection (see Section 3.4.1.).

3.4.4. Summary.

The results of this study revealed no correlation between presence of serum B19 DNA or NS-1-specific IgG and the development of joint manifestations. The presence of serum B19 DNA, a reflection of B19 persistence, was not found to be linked to the development of acute or chronic post-B19 infection associated arthropathy. Instead, B19 DNA in sera appears to be a function of time relative to infection and the stage of immune response to B19. In addition, B19
DNA was not found in peripheral blood mononuclear cells obtained at late convalescence. There was no relationship between persistence of B19 DNA and the presence of serum anti-NS-1 IgG. Therefore, persistence of anti-NS-1 IgG is not related to clinical outcome and may only be an indicator of past B19 infection in a small proportion of B19-infected individuals.

3.5. B19 Persistence and Anti-NS-1 Antibodies in Past (Undocumented) B19 Infections.

Anti-NS-1 antibodies have been reported to be correlated with persistent infection and with B19 arthropathy (von Poblotzki et al., 1995; von Poblotzki et al., 1995a). However, anti-NS-1 antibodies were found in equal proportions of subjects experiencing clinical symptoms (arthropathy, hematologic disorders, non-immune fetal hydrops) of B19 infection (27.7%) and healthy B19 seropositive controls (21.7%) (Venturoli et al., 1998). These subjects were determined to have past (undocumented) B19 infections by the presence of VP2-specific IgG and absence of VP2-specific IgM. Therefore, anti-NS-1 antibodies may reflect a history of past B19 infection only, and the prevalence of anti-NS-1 antibodies in remote B19 infections may simply demonstrate the population incidence of this antibody species. Thus, the objective of this study was to determine the prevalence of anti-NS-1 antibodies in a group of individuals with past (undocumented) B19 infections as defined in Table 2. The relationship between the presence of serum NS-1 IgG and B19 persistence (assessed by presence of serum DNA) was also studied. PCR for B19 DNA and NS-1 IgG immunoblot assays were performed on single serum specimens from 41 B19 seropositive individuals (as determined by detection of VP2-specific IgG in commercial EIA, see Materials and Methods). As these study subjects reported no recent illness resembling B19 infection, it was assumed that they were seropositive as a result of a past, undocumented B19 infection. These subjects fell into two clinical categories according to the reasons underlying investigative B19 serology: “chronic arthropathy of unknown etiology”, and “undefined rash illness” (Table 2). Healthy B19 VP2 IgG positive volunteers were included as controls. Results are listed in Table 5. By performing B19-specific PCR and NS-1 immunoblots
Table 5. B19 DNA and Anti-NS-1 IgG in Sera of Past (Undocumented) B19 Infections

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1 Date of acquisition of serum sample
2 Presence of B19 DNA in serum as determined by nested PCR (see Materials and Methods)
3 Presence of NS-1-specific IgG as determined by immunoblot (see Materials and Methods)
on these samples, it was possible to examine B19 persistence and prevalence of anti-NS-1 antibodies in a study population assumed to be well past the late convalescent phase of B19 infection.

3.5.1. B19 Persistence in Individuals with Past (Undocumented) B19 Infections.

None of the sera from 16 subjects with chronic arthropathy of unknown etiology, 10 subjects with rash illnesses or 15 healthy controls had B19 DNA detectable by PCR. Failure to detect B19 virus by PCR in these individuals was likely due to the remoteness of their original infections and was as expected in the general population in relation to the biology of B19. Thus, in the group experiencing chronic arthropathy, there was no evidence of an etiologic role for parvovirus B19.

Persistence of B19 DNA has been previously demonstrated in bone marrow and synovial membranes of B19 seropositive individuals with no recent history of B19 infection (Cassinotti et al., 1997; Soderlund et al., 1997). Although it is possible that in a larger study group some individuals with persistent B19 DNA in serum may have been identified, serum may not be the ideal tissue in which to detect B19 DNA in individuals past the late convalescent stage of B19 infection.

3.5.2. Frequency of Anti-NS-1 IgG Antibodies in Remote B19 Infections.

NS-1 immunoblot was performed on serum samples taken from subjects with past B19 infections (Fig. 6). Anti-NS-1 IgG was found in 5/16 (31%) of subjects with chronic arthropathy of unknown etiology and in 3/10 (30%) of subjects with undefined rash illness. These proportions were similar those found in the late convalescent (6-12 month post-infection) samples obtained from the recent B19 infection group (see Section 3.4.2.). Thus, a certain proportion of B19-infected individuals appears to develop humoral immunity to NS-1. Anti-NS-1 antibodies were absent in all 15 healthy subjects with remote B19 infections. The reason for this difference in prevalence of anti-NS-1 antibodies is unknown, since the B19 infection history,
including time of infection and clinical course of B19 infection, was also unknown. However, it is likely that this result is due to the small number of subjects studied in the group.

![Figure 6. Incidence of Serum Anti-NS-1 IgG in Remote (Undocumented) B19 Infections.](image)

**Figure 6. Incidence of Serum Anti-NS-1 IgG in Remote (Undocumented) B19 Infections.** NS-1 immunoblot was performed using single serum samples obtained from subjects with remote (undocumented) B19 infections. Results were expressed as number of subjects in each clinical category (chronic arthropathy of unknown etiology, undefined rash illness, and healthy) with or without anti-NS-1 IgG in sera.

### 3.5.3. Relationship Between B19 Persistence and Serum Anti-NS-1 Antibodies.

As there were no subjects with detectable B19 DNA in any of the clinical groups that were remotely infected with B19, no relationship between B19 persistence and the presence of serum anti-NS-1 antibodies could be determined.

### 3.5.4. Summary.

B19 PCR and NS-1 immunoblots were performed in order to examine the frequency of B19 DNA and anti-NS-1 IgG in the sera of subjects serologically defined to have past, undocumented B19 infection. B19 DNA was not detected in the sera of these B19 seropositive subjects, regardless of the presence or absence of clinical symptoms during the time of sample acquisition. Anti-NS-1 IgG was present in a small number of individuals that were experiencing chronic arthropathy or rash illnesses not known to be temporally or directly related to B19.
infection. The proportion of individuals with anti-NS-1 IgG within the chronic arthropathy or rash illness groups were similar to the proportions found for all late convalescent samples obtained after recent B19 infection. The frequency of anti-NS-1 IgG antibodies within these study groups may be representative of the frequency of this antibody species in the general population. There was no correlation between the presence of serum anti-NS-1 antibodies and B19 persistence in sera.

3.6. Response of Previously Immune Individuals to Re-exposure to Parvovirus B19.

Due to concerns about the effects of maternal B19 infection on the development of the fetus, pregnant women who have been exposed to confirmed cases of erythema infectiosum (EI) constitute the second most frequently investigated group (after patients with exanthematous illness) in serological testing programs at public health laboratories. Very little is known about the effects of re-exposure to B19 in previously immune individuals, although it is assumed that pre-existing immunity to B19 should protect against reinfection. Therefore, a cohort of 36 pregnant women referred for B19 serology after contact with confirmed cases of EI was identified from the BCCDC testing rosters. All these women were found to have undocumented histories of past B19 infection (possessing IgG antibodies to VP2 protein without VP2-specific IgM using commercial EIA), and none reported any signs of B19-related illness or B19-related complications to pregnancy (Table 2). Frozen serum samples (sample 1) drawn within 3 weeks of initial exposure to B19 were obtained from BCCDC collections, while follow-up blood samples (sample 2) were obtained at 6-12 months following B19 exposure to obtain sera and PBMNC. None of the women were pregnant at the time of follow-up. B19-specific PCR and NS-1 immunoblot assays were performed on each serum sample. DNA was extracted from PBMNC from follow-up specimens and tested for B19 genome by nested PCR.

Although B19 DNA was detected in a small number of late convalescent samples following recent B19 infection (see Section 3.4.1.2.), no B19 DNA was found in the population of study subjects serologically defined as having past (undocumented) B19 infections (see section 3.5.1.). These results suggest that the likelihood of detecting B19 DNA in sera is reduced as individuals are temporally further removed from the time of acute B19 infection. To determine if B19 virus could be found in B19 “immune” (anti-VP2 IgG seropositive) individuals re-exposed to B19, we performed PCR on sera and PBMNC samples from 36 women accidentally exposed to B19 in pregnancy. PCR revealed B19 DNA in 2/36 (6%) of the first serum samples. Follow-up peripheral blood samples were obtained for 23 of the women, with 1/23 (4%) first samples and none of the second samples having detectable B19 DNA. B19 DNA was not detected in any of the PBMNC samples collected at follow-up (data not shown). It is possible that the few subjects that had serum B19 DNA had first samples taken at a late convalescent stage after recent B19 infection. Alternately, a sub-clinical re-infection with B19 may have occurred in these women.

3.6.2. Frequency of NS-1 Antibodies Following Re-Exposure to B19

NS-1 immunoblot was performed on a cohort of healthy, pregnant women re-exposed to active B19 infections to determine if re-exposure to B19 might be reflected in increased incidence of detection of NS-1 in serum. Since pregnancy is associated with depression of cell-mediated immunity but not humoral immunity, there may be a risk of subclinical B19 reinfection. As no pre-exposure serum samples were available, the immune response to re-exposure to B19 could only be examined in the context of the post-exposure response.

Anti-NS-1 IgG was detected in 14/36 (39%) first serum samples by immunoblot. A temporal decline in anti-NS-1 IgG was observed in 23 follow-up samples, with 9/23 (39%) first samples and 5/23 (22%) second samples testing positive for anti-NS-1 IgG (Fig. 7). Although
the infection history from this group is unknown, it is speculated that the difference in anti-NS-1 IgG frequencies over time may be due to subclinical re-infection with B19 leading to anamnestic immune response to NS-1. Furthermore, the temporal decline in the prevalence of anti-NS-1 antibodies observed in serial serum samples indicate that re-exposure to B19 may only cause a transient elevation in anti-NS-1 antibodies for certain individuals within this re-exposed group. If the above findings are proven to be correct, increases in the levels of serum anti-NS-1 antibodies may be a useful tool in assessing whether re-infections with B19 have occurred, especially in pregnant females who are at risk for B19-related fetal complications.

Figure 7. Incidence of Anti-NS-1 IgG in Sera of Previously Immune Subjects Exposed to B19. Serum samples from a population of pregnant women with past (undocumented) B19 infections were obtained after accidental exposure to a known case of erythema infectiosum (EI). Immunoblot assays were used to determine the presence or absence of anti-NS-1 IgG in serum samples. Acute samples represent the total number of acute samples obtained (one per subject) and were collected within 30 days of exposure to EI. Follow-up subjects had sequential acute and late convalescent serum samples (obtained between 6-12 months after exposure to EI) taken for use in NS-1 immunoblot.

3.6.3. B19 Persistence and Anti-NS-1 Antibodies.

As with the other study groups, there was no correlation found between the persistence of B19 DNA and the presence of anti-NS-1 IgG in pregnant, B19-exposed women. B19 DNA was found in only one of 14 individuals with anti-NS-1 IgG in their first serum sample and none of
the 5 individuals with anti-NS-1 in their second serum sample. These results indicate that the presence of serum anti-NS-1 antibodies is independent of B19 persistence in this re-exposed group.

3.6.4. Summary.

Exposure of healthy, pregnant females to individuals with active B19 infections did not result in any clinically significant sequelae to the women or to the outcome of their pregnancies. However, B19 PCR and NS-1 immunoblot indicated increases within this group in the frequency of subjects with serum B19 DNA and NS-1 IgG when compared to non-exposed, non-pregnant, counterparts. B19 DNA was observed in a small number of first samples following exposure to B19. However, none of these subjects had B19 DNA in follow-up second sera and PBMNC samples. Anti-NS-1 antibodies were found in both first and second samples of the B19-exposed women in similar proportions to the samples obtained from other groups of remote B19 infections with chronic arthropathy or rash illness (see section 3.5.2.). However, there was a temporal decline in the number of subjects with serum anti-NS-1 antibodies during the 6-12 months between exposure to B19 and the acquisition of follow-up second samples. There was no correlation between the persistence of B19 and the presence of serum anti-NS-1 antibodies. It may be speculated that re-exposure to B19 may cause transient subclinical re-infections and/or anamnestic immune responses that may account for the short term presence of serum B19 DNA and the increase in number of individuals with anti-NS-1 antibodies.
Summary Discussion and Future Directions.


B19-specific PCR was performed on serum samples taken within one month of onset of clinical symptoms following B19 infection. The goal was to examine the relationship between the presence of B19 virus in serum and the development of acute arthropathy following B19 infection. No such correlation was found, as results showed that B19 DNA was found in a high proportion of all serum samples obtained, regardless of the presence or absence of acute joint manifestations (see section 3.4.1.1). Instead, detectable B19 in sera appeared to correlate with the amount of time that had passed following recent B19 infection, and was an indirect indicator of the development of immunity to B19.

It is conceivable that the immune response to B19 was accelerated in subjects that did not develop joint manifestations following recent B19 infection (see section 3.4.1.1). It has been suggested that delays in the development of neutralizing immunity lead to viral persistence and the development of clinical symptoms following B19 infection (Bostic et al., 1999; Foto et al., 1993, Naides et al., 1992; Naides, 1998). Therefore, the present results lead to the speculation that individuals that did not develop acute B19 arthropathy may have rapidly developed high-affinity antibodies to B19, causing the formation of large, insoluble immune complexes that are readily eliminated by the reticulo-endothelial system. In contrast, B19 arthropathy patients may
have delayed affinity-maturation of antibodies to B19. The formation of low-affinity antibodies would result in small, soluble immune complexes that are thought to migrate to joints and mediate B19 arthropathy (Brown et al., 1994). However, this premise is based on a small cohort of individuals recently infected with B19. Confirmation of this observation will require a larger number of study subjects as well as quantitative and qualitative assessments of viral load and the specific antibody response. Increasing the number of serial serum samples obtained in the acute and early convalescent stages of B19 infection, with standardized times of sampling among subjects, will further enhance the understanding of the temporal development of antibody responses to B19. The use of B19 neutralization assays and quantitative PCR will determine the efficacy of the immune responses that develop in subjects with different clinical symptoms following recent B19 infection. As well, the determination of the influence of cellular immunity on both direct viral clearance (via cytotoxic T-cells) and regulation of the humoral immune response against B19 (via T-helper cells) (see Section 1.3.1.) will help in the understanding of the pathogenesis of B19 arthropathy. Furthermore, the influence of immunogenetic background on B19 antigen presentation and subsequent immune response (see Section 1.2.3.6.) will aid in the characterization of epitopes recognized by neutralizing antibodies and the elucidation of the mechanism of B19 arthropathy.

4.2. B19 DNA Persistence in Late Convalescent Stages of B19 Infection: Perspectives on Chronic Arthropathy.

A possible correlation between persistent B19 infection and chronic B19 infection-associated arthropathy (Naides et al., 1992; Foto et al., 1993) was not supported by the results of this study. PCR assays revealed that B19 DNA could be found only rarely in late convalescent serum samples after documented B19 infection, and was not related to development of arthropathy (see section 3.4.1.2.). Furthermore, B19 DNA was not detected in any PBMNC samples during the late convalescent period. Differences in the incidence of detection of B19
DNA in late convalescent sera appeared to be dependent on the time of sampling relative to onset of clinical symptoms, and thus on the development of viremia-controlling immune responses. Similarly, B19 DNA was not detected in samples from subjects who were B19 seropositive due to past, undocumented B19 infections (see section 3.5.1).

Discrepancies between my findings and previous published reports linking B19 persistence to the development of chronic arthropathy may be due to several factors. Previous investigations of B19 persistence used healthy B19 seropositive controls that had undocumented histories of B19 infection (Foto et al., 1993). Therefore, a true comparative analysis of B19 persistence between symptomatic and asymptomatic B19 infections was not performed in these studies. The present study utilized a cohort of subjects with serologically confirmed recent B19 infection and employed stricter sampling intervals. Hence, a more accurate representation of the relationships between presence of B19 in serum over time and clinical symptoms following B19 infection was obtained. Secondly, the choice of tissues in which to examine B19 persistence was limited to peripheral blood constituents. Sera and PBMNC were used to assess B19 persistence in the present study because of their availability. Furthermore, serum is an effective medium in which to assess B19 viremia, and it has been previously used by other studies to determine B19 persistence (Foto et al., 1993; Kurtzman et al., 1989a). However, previous investigations have found persistent B19 DNA in bone marrow (Foto et al., 1993; Cassinotti et al., 1997) and synovial membranes (Soderlund et al., 1997, Takahashi et al., 1998). Localization of B19 in the joints may contribute to pathogenesis of chronic B19 arthropathy by direct damage to synovial tissue or by induction of inflammatory cytokines in inflammatory cells recruited to the site of persistent virus (Moffat et al., 1996; Moffat et al., 1998; Takahashi et al., 1998). Therefore, it would be desirable to expand the types of tissues examined in order to obtain a complete representation of B19 persistence and its possible relation to the etiology of chronic B19 arthropathy.
The absence of B19 DNA in late convalescent serum samples from subjects that did not develop arthropathy following recent B19 infection (see section 3.4.1.2.) may simply reflect the small number of samples obtained (three). Alternately, it may be speculated that the absence of serum B19 DNA may reflect an accelerated development of neutralizing immunity to B19 in this group, and an extended viremia in the groups that developed arthropathy following B19 infection (see section 4.1). To resolve these two possibilities, future experiments will have to employ larger cohorts of recent B19-infected subjects and be more prospective in sampling protocols (a difficult task to achieve in wild-type infections). Also, such studies should employ quantitative PCR and B19 neutralization assays to determine if there are differences in development of neutralizing immunity to B19. Investigations into antibody recognition of neutralization domains on B19 capsid proteins and determination of the role of cellular immunity in clearance of B19 infections will also provide insight into the immune response to B19. If differences in immunity maturation between clinical groups are found, the mechanism by which extended viremia leads to chronic arthropathy will have to be determined.

4.3. Anti-NS-1 IgG Antibodies, Viral Persistence, and B19 Arthropathy.

Previous studies have found the presence of serum anti-NS-1 antibodies to be linked with both B19 persistence and arthropathy following B19 infection (von Poblotzki et al., 1995; von Poblotzki et al., 1995a; Naides, 1998). However, the results of the present study do not support these findings. Instead, the data reported herein support other evidence that anti-NS-1 antibodies are not exclusively correlated with arthropathy and that they are markers for past B19 infection only (Venturoli et al., 1998; Searle et al., 1989). Anti-NS-1 IgG was detected in acute serum samples after B19 infection, indicating that this antibody species may develop shortly after infection (see section 3.4.2.). Furthermore, anti-NS-1 IgG antibodies were observed in relatively constant proportions (29-40%) of subjects during the late convalescent stages of documented B19 infection (see section 3.4.2), in subjects with past (undocumented) B19 infections (see
section 3.5.2) and in B19 seropositive pregnant women who were accidentally exposed to B19
(see Section 3.6.2). Therefore, it is speculated that a certain proportion of all B19-infected
individuals develops anti-NS-1 antibodies regardless of clinical outcome.

The reason for the development of anti-NS-1 antibodies in only 29-40% of the general
population is unknown. It is conceivable that individuals that develop anti-NS-1 antibodies may
have high levels of initial B19 viremia due to prolonged exposure to B19 or repeated exposure to
B19 (for example, home contacts with an infected child, or daycare workers). B19 infection of
permissive erythroid progenitor cells normally results in release of low levels of NS-1 protein
(Ozawa et al., 1988) that may not be sufficient to stimulate antibody production. It is possible to
suggest that high initial B19 viremia may result in release of NS-1 protein from infected cells at
the time of cell death in concentrations sufficient to be immunogenic. Alternatively, high initial
B19 viremia may result in a higher incidence of B19 infection in non-permissive cells which
favour NS-1 production and subsequent release (Ozawa et al., 1988; Liu et al., 1992). This
hypothesis is supported by previous suggestions that anti-NS-1 antibodies are a reflection of
greater antigenic stimulation/exposure to NS-1 protein (Venturoli et al., 1998). In individuals
who develop anti-NS-1 but not develop arthropathy, a high initial B19 viremia may be cleared at
a sufficient rate to prevent the development of joint manifestations (see Sections 4.1. and 4.2.).
To test these hypotheses, prospective studies will need to be performed using quantitative PCR
(to determine initial viremia levels) and NS-1 antibody studies (to relate B19 titre to the
development of anti-NS-1 antibodies). Furthermore, demonstration of NS-1 transcripts within
tissue samples other than peripheral blood would support the hypothesis that B19 may reside in
these cells and produce NS-1 protein capable of stimulating humoral immunity.

Interestingly, a possible boost in the incidence of anti-NS-1 antibodies was found in
healthy, pregnant women previously immune to B19 who were exposed to active B19 infections
(see section 3.6.2.). These women demonstrated a decline in prevalence of anti-NS-1 between
first and follow-up second samples, leading to the speculation that subclinical re-infections with temporary anamnestic responses to B19 antigens may have occurred. The possibility of repeated subclinical infections with B19 was previously suggested by demonstration of VP-1-specific IgG4 antibodies (Franssila et al., 1996, see Section 1.3.4.). The prevalence of anti-NS-1 antibodies in the second samples for these women (22%) was similar to the prevalence found in late convalescent samples following recent B19 infection and in remote B19 infections, emphasizing the transience of the B19 "booster" response (see above). However, putative changes in immune regulation during pregnancy may also account for the higher prevalence of anti-NS-1 antibodies in these pregnant women. Thus, prospective studies on the effect of exposure of previously immune pregnant (and non-pregnant) individuals to active B19 infections will be required to determine if increased prevalence of anti-NS-1 antibodies is a normal finding in pregnancy or is an indicator of subclinical reinfection with B19. Quantitative B19 PCR, EIA, and anti-NS-1 antibody assays would determine if reinfection with B19 had occurred, and whether a booster response to NS-1 had developed. If subclinical reinfection with B19 were demonstrated, the length of time that “booster” responses to B19 last will also have to be defined in order to assess the utility of anti-NS-1 antibodies in diagnosing subclinical B19 reinfection in populations at risk for B19-related complications (pregnant women, chronic hematological disorders, etc.).

4.4. The Role of NS-1 Protein in Chronic B19 Arthropathy

The present results indicate that the presence of serum anti-NS-1 antibodies is not an exclusive predictor of B19 infection-associated arthropathy. However, this finding does not rule out the possible pathogenic role of NS-1 protein in the development of chronic arthropathy following B19 infection. B19 may persist in tissues at sites not examined in this study, including bone marrow and synovial tissue. NS-1 protein was found to be preferentially expressed in non-permissive cells (Ozawa et al., 1988; Liu et al., 1992). The possibility remains that chronic B19
arthropathy patients may have B19 in non-permissive synovial tissue resulting in NS-1-mediated synovial cytotoxicity and subsequent joint manifestations. Alternately, B19 may be sequestered within inflammatory cells that localize to joints. NS-1 may transactivate the IL-6 promoter (Moffat et al., 1996; Moffat et al., 1998; Takahashi et al., 1998) and mediate inflammation within joints following B19 infection (see section 1.4.4.). Future investigations will require prospective studies regarding the localization of B19 following infection and the possible induction of inflammation within joint tissue.
Chapter 5.

References.


