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Human Parvovirus B19: Studies on the pathogenesis of infection

by

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Summary

Human parvovirus B19 (B19) causes the common childhood disease erythema infectiosum. The virus is transmitted by the respiratory route, infects erythroid progenitor cells, and thereby inhibits erythropoiesis. The seroprevalence in the adult population is 50-70%, rising to over 80% among the elderly. Primary infection in adults may cause arthritis of long duration and lead to aplastic crisis patients with underlying haemolytic disorder. In immunocompromised individuals, the infection can establish persistency in the bone marrow resulting in severe anaemia. Persistent B19 infection has also been observed in immunocompetent individuals, even though B19-neutralising antibodies can be detected, giving rise to a wide array of symptoms. Primary B19 infection in pregnant women has been linked to the development of hydrops fetalis, spontaneous abortion and intrauterine fetal death (IUID), most commonly during the second trimester of pregnancy.

We prospectively collected placental and fetal tissue from cases of late second and third trimester IUID, during the years 1992-99, and found 7.5% and 15%, respectively, to be B19 DNA positive. These findings indicate that B19 may be more commonly associated to IUID in the late stages of pregnancy than earlier appreciated. The majority of cases did not exhibit hydrops fetalis, lacked clinical signs of acute infection, and may thus have been infected long before clinical presentation. We therefore suggest that sensitive methods for detection of B19 DNA should be included in the routine investigation of IUID.

Previous reports have indicated that the viral genotype may determine the course of B19 infection with regard to development of persistent infection. When comparing full-length sequences from B19 viral isolates derived from immunocompromised and immunocompetent persistently infected individuals with previously published sequences, no significant difference was found. This indicates that variations in the B19 genotype may not be important in the development of persistent infection.

It has been suggested that antibodies to the B19 non-structural (NS) dominate in patients with persistent B19 infection, reflecting an altered humoral response in these individuals. However, by linear epitope mapping the anti-B19 NS-specific antibody reactivity was found to be conserved among healthy subjects and patients suffering from various B19 related complications. Neither was any specific region commonly recognised by all individuals.

Deficient cytotoxic cellular immune response against B19 may contribute to the development of viral persistence. This entity of the immune system has not previously been investigated in B19 infection. By screening overlapping peptides, an HLA B35 restricted B19-specific cytotoxic T cell epitope was found contained within the NS protein. Using interferon- γ detection and tetrameric complex binding we found that approximately 65% of all HLA-matched individuals possess functional epitope-specific cytotoxic T cells to this epitope, however in immunocompromised individuals these cells exhibit lower cytolytic function *ex vivo*.

We have thus investigated factors involved in the pathogenesis of persistent B19 infection in immunocompetent individuals, immunocompromised patients and pregnant women.

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Original papers

The work presented in this thesis is based on the following articles, referred to by their roman numerals.

- I. Skjöldebrand-Sparre L, Tolfvenstam T, Papadogiannakis N, Wahren B, Broliden K, Nyman M. *Parvovirus B19 infection: association with third-trimester intrauterine fetal death*. Br J Ob Gyn. 2000 Apr;107(4):476-80.
- II. Tolfvenstam T, Papadogiannakis N, Norbeck O, Petersson K, Broliden K. *Human parvovirus B19 in intrauterine fetal death*. Lancet 2001 May; 357: In press.
- III. Tolfvenstam T, Norbeck O, Brytting M, Modrow S, Broliden K. *Limited sequence variability of parvovirus B19 strains in persistently infected individuals*. Submitted.
- IV. Tolfvenstam T, Lundqvist A, Levi M, Wahren B, Broliden K. *Mapping of B-cell epitopes on human parvovirus B19 non-structural and structural proteins*. Vaccine. 2000 Nov 22;19(7-8):758-63.
- V. Tolfvenstam T, Oxenius A, Price DA, Shacklett BL, Spiegel HM, Hedman K, Norbeck O, Levi M, Olsen K, Kantzanou M, Nixon DF, Broliden K, Klenerman P. *Direct Ex vivo measurement of CD8(+) T-lymphocyte responses to human parvovirus B19*. J Virol. 2001 Jan;75(1):540-3.
- VI. Tolfvenstam T, Nixon DF, Shacklett BL, Spiegel HM, Hedman K, Borkowsky W, Oxenius A, Price DA, Klenerman P, Broliden K. *Parvovirus B19-specific cellular immune responses in healthy and HIV-1 infected individuals*. Submitted.

Abbreviations

aa	Amino acid
B19	Human parvovirus B19
BCL	B cell line
CD	Cluster of differentiation
CMV	Cytomegalovirus
CPV	Canine parvovirus
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
EI	Erythema infectiosum
ELISA	Enzyme linked immunosorbent assay
ELISpot	Enzyme linked immunospot assay
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
IFA	Immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IH	Immunohistochemistry
IL	Interleukin
IUFD	Intrauterine fetal death
IVIG	Intravenous immunoglobulin
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NIHF	Non-immune hydrops fetalis
NSP	Non-structural protein
nt	Nucleotide
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PRCA	Pure red cell aplasia
SPV	Simian parvovirus
TAC	Transient aplastic crisis
Th	T helper cell
VP	Viral protein

Background

HUMAN PARVOVIRUS B19 (B19) is the only parvovirus clearly linked to human disease. The virus was discovered a little more than 25 years ago in London by Yvonne Cossart and colleagues when evaluating different assays for hepatitis B virus in blood donor samples (Cossart, 1975a, Cossart *et al.*, 1975b). Specimen 19 from panel B gave an unexpected band in a counterimmunoelectrophoresis test and the novel virus was characterised. In 1982, the virus was linked to erythema infectiosum (Anderson *et al.*, 1983), a common childhood disease first described in 1799. Since then many disease manifestations have been associated with B19 infection subsequent to the development of more sensitive techniques for antibody and DNA detection.

The scope of this thesis cover the pathogenesis of B19 infection, with focus on persistent infection, clinical consequences of infection in pregnancy, the role of virus genotype and the interplay between host humoral and cellular immune responses. A deeper knowledge of these topics is important for future recommendations regarding preventive measures, enhanced therapeutic strategies, and the development of a vaccine for high-risk patient groups.

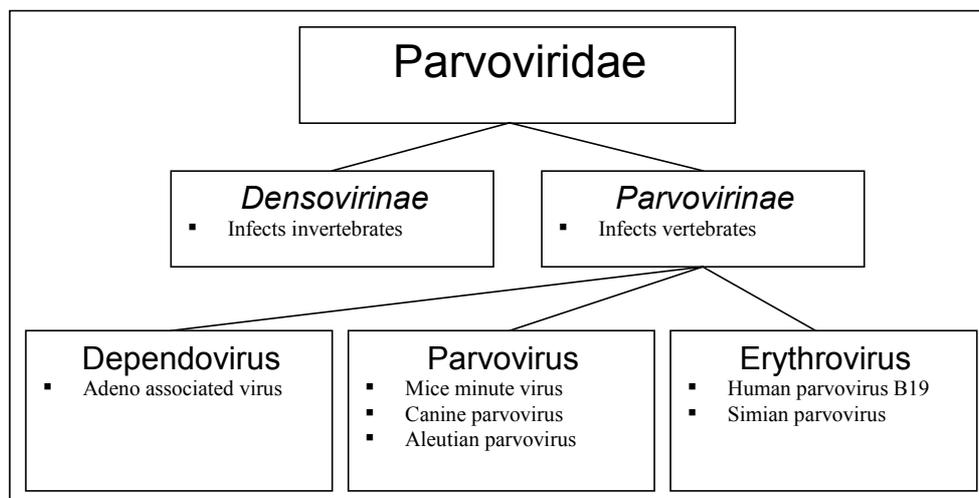


Figure 1 Parvovirus taxonomy. B19 is classified to the erythrovirus genus.

– TAXONOMY

The family Parvoviridae includes small, non-enveloped single stranded DNA viruses, which infect a wide range of animals. The subfamily Parvovirinae contains three genera: *Dependovirus* which requires helper viruses for replication, *Parvovirus* which members replicate autonomously, and the third genus to which B19 is classified, *Erythrovirus* which members replicate autonomously in erythroid progenitor cells (Pringle, 1993). (Figure 1, 2)

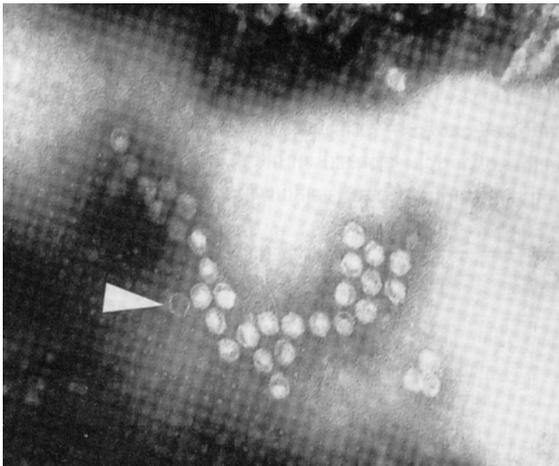


Figure 2 Electron microscopy picture of B19 virions

– VIRAL CHARACTERISTICS

All parvoviruses, including B19 have icosahedral symmetry and measure 20-25 nm in diameter (Clewley, 1984). Two major genes compose the B19 genome which has a total length of approximately 5 kilo base pairs, one encoding the non-

structural protein (NSP) which is believed to exert regulatory function on transcription, and one encoding two structural proteins (Cotmore & Tattersall, 1984, Mori *et al.*, 1987, Shade *et al.*, 1986). The two structural proteins are formed by alternative start codons from the same gene, and the products, viral protein (VP) 1 and 2 compose the viral capsid. VP2 that makes up 95% of the viral capsid, lacks the first 226 residues compared to VP1. Furthermore, two regions encode two small non-structural proteins of unknown function, 7,5 and 11,5 kilo dalton respectively (Luo & Astell, 1993, St Amand *et al.*, 1991) (Table 1, Figure 3). The genome is flanked on each end by inverted terminal repeat palindromic termini of 383 nucleotide (nt) length (Deiss *et al.*, 1990, Shade *et al.*, 1986).

Protein	Size (kDa)	No. aa	Function
NS	77	671	Replication
VP1	83	781	Virus capsid
VP2	58	554	Virus capsid
7.5 kDa	7.5	72	Unknown
11.5 kDa	11.5	94	Unknown

Figure 1 B19 translational products

B19 has strict tropism and infects only humans. The cellular receptor for B19 is the blood group P antigen, a glycolipid also known as globoside (Brown *et al.*, 1993). P antigen is expressed on erythroid progenitor cells in the stages from burst-forming units to mature erythrocytes (Rouger *et al.*, 1987, von dem Borne *et al.*, 1986). P antigen is also expressed to some extent on megacaryocytes, hematopoietic cells in the fetal liver, endothelium, kidney cortex, placenta and in the fetal myocardium (Jordan & DeLoia, 1999). The P blood group system, discovered in 1927, contains two common antigens, P₁ and P and the much rarer antigen P^k (Landstienier & Levine, 1927) (Table 2.).

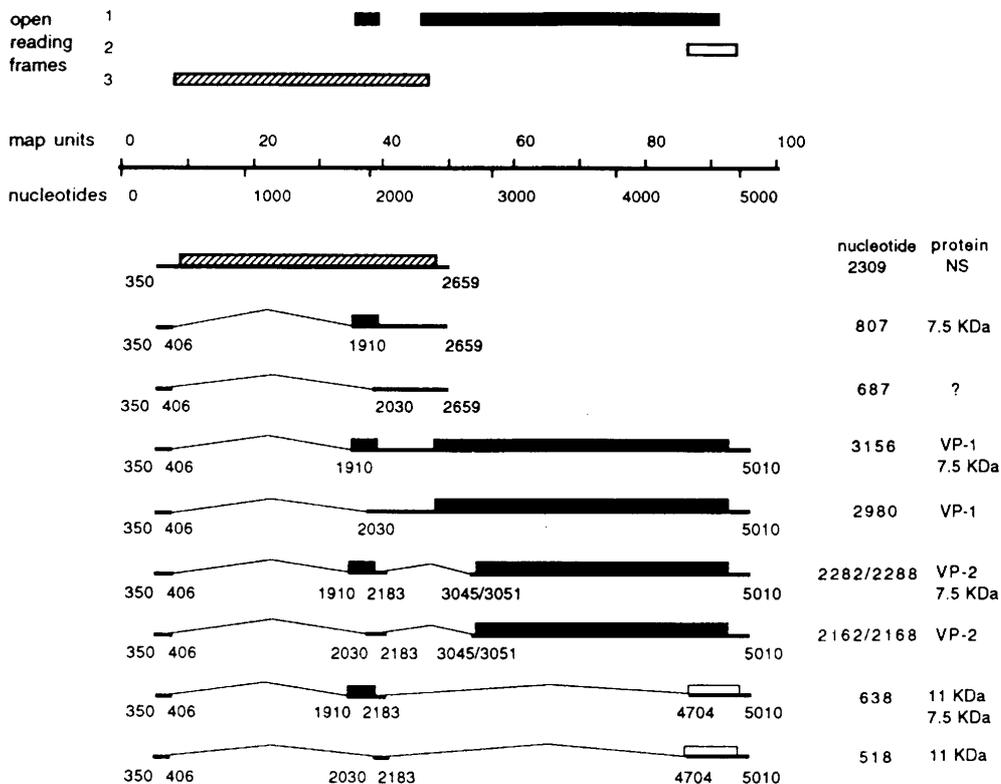


Figure 3 Transcription and coding map of B19 (Luo *et al.* 1993).

Phenotype	Antigens on cells	Frequency
P ₁	P ₁ , P	80%
P ₂	P	20%
P ₁ ^k	P ₁ , P ^k	Rare
P ₂ ^k	P ^k	Rare
p	None	1:200,000

Table 1 Overview of the P blood group system.

The prevalence of p phenotype that lacks any expression of P antigen is approximately 1:200,000 in the general population but is more common in Sweden, Japan and among the Amish people in North America (Race & Sanger, 1975). Individuals of p phenotype are not susceptible to B19 infection (Brown *et al.*, 1994).

New findings imply that P antigen by itself is not sufficient for B19 entry into cells and that the level of P antigen expression does not correlate with the efficiency of viral binding (Weigel-Kelley *et al.*, 2001). These findings suggest the existence of a cellular co-receptor for the virus.

B19 primarily infects and replicates in erythroid progenitor cells which in turn causes arrest of the erythropoiesis (Mortimer *et al.*, 1983) (Figure 4). The main target cells are erythroid colony-forming units and erythroblasts whereas the pluripotent progenitor cell is spared (Takahashi *et al.*, 1990).

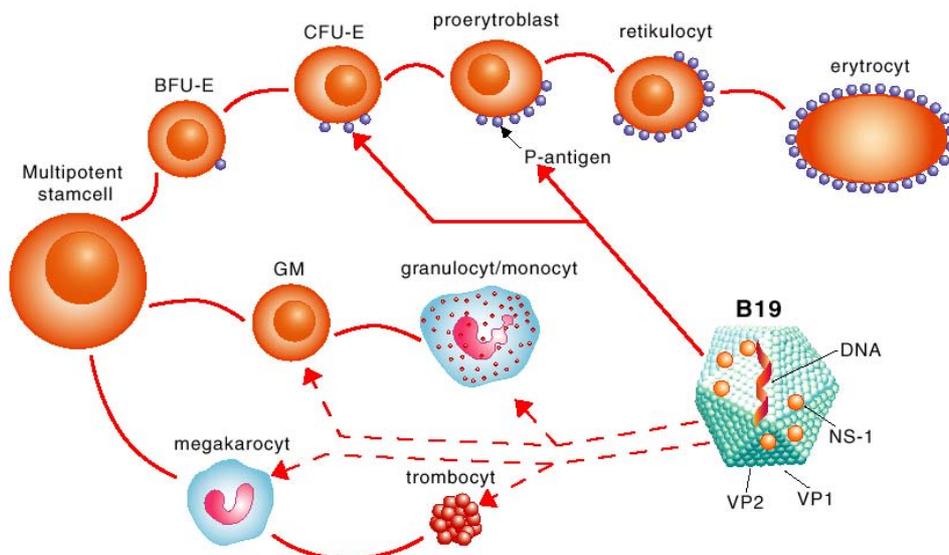


Figure 4 P antigen expression in erythroid progenitors and cells susceptible to B19 infection

B19 susceptible cells have been found to express human leukocyte antigen (HLA) ABC, cluster of differentiation (CD) 43, CD36, glycophorin A and C (Morey & Fleming, 1992). Since B19 is dependent on mitotically active cells for replication, the presence of erythropoietin is essential for viral infection. Successful short time ex vivo cultivation has been performed in human bone marrow, fetal liver and erythroleukemic blast cells (Brown *et al.*, 1991, Ozawa *et al.*, 1986, Srivastava & Lu, 1988, Takahashi *et al.*, 1989, Yaegashi *et al.*, 1989b).

Although P antigen is expressed on several cell types apart from erythroid progenitor cells, none of these cell types have shown to be permissive for B19 replication in vivo. However, B19 antigens have been demonstrated in myocardial cells of infected fetuses (Morey *et al.*, 1992b, Porter *et al.*, 1988). Attempts of long-term in vitro cultivation of B19 have been unsuccessful in erythroleukemic cell lines such as K562, HEL, KMOE-2 and KG1a, probably due to immature erythroid differentiation of the cells. Nevertheless, limited propagation have been performed in megakaryocytoblastoid cell lines UT-7, MB-02 and JK-01, but the level of B19 expression is low in all these cell lines compared with bone marrow culture, and none is suitable for long-term viral propagation (Munshi *et al.*, 1993, Shimomura *et al.*, 1992, Takahashi *et al.*, 1993).

An animal model for B19 infection would provide an opportunity to study, for example, the route of transmission and to evaluate vaccine effects. Although B19 has been shown to inhibit simian erythroid colony formation in vitro, attempts to infect Cynomolgus and Saimiri monkeys in vivo have been unsuccessful. The recently identified erythrovirus simian parvovirus (SPV) which is similar to B19, infects bone marrow in cynomolgus monkeys and causes anaemia in the infected host (Brown *et al.*, 1995, O'Sullivan *et al.*, 1994, O'Sullivan *et al.*, 1997, O'Sullivan *et al.*, 1996). Furthermore, SPV infected immunocompromised animals, due to simian immunodeficiency virus infection, develop severe and prolonged anaemia when infected by SPV; analogous to the effect of B19 infection in human immunodeficiency virus (HIV) infected humans. SPV infection in monkeys

could prove to be a valuable animal model for studies on B19 host cell interactions.

Cells infected with B19 have the typical appearance of cells undergoing apoptosis with margined chromatin, cytoplasmic vacuolisation, and nuclear blebbing (Figure 5). At the ultrastructural level, typical affected cells show presence of viral arrays in both the nucleus and the cytoplasm (Morey *et al.*, 1993). Apoptosis mediated viral cytotoxicity has been confirmed in several studies (Moffatt *et al.*, 1998, Sol *et al.*, 1999, Yaegashi, 2000, Yaegashi *et al.*, 1999). Immunogold electron microscopy has detected B19 NSP in association with both nuclear and cytoplasmic clusters of capsids and the viral cytotoxicity is believed to be a direct function of expression of NSP (Morey *et al.*, 1995). However, we have recently found that empty B19 recombinant VP2 capsids, lacking the NSP inhibit hematopoietic differentiation in vitro as well (Lindton *et al.*, 2001).

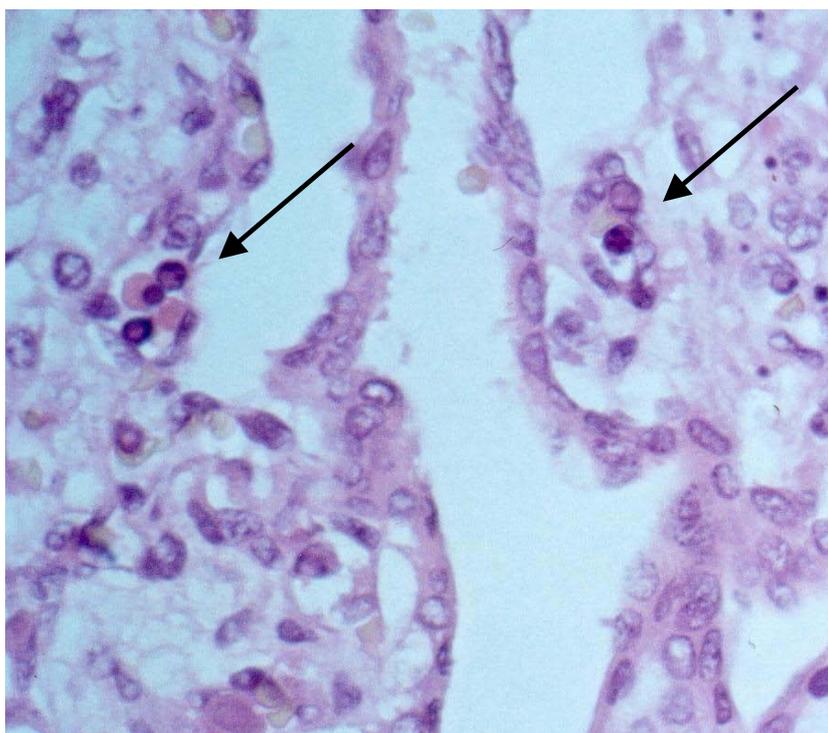


Figure 5 Placenta containing B19 infected erythroid progenitor cells localised in capillaries (arrow), exhibiting marginalised chromatin and cytoplasmic vacuolisation. (N Papadogiannakis)

– I N F E C T I O N

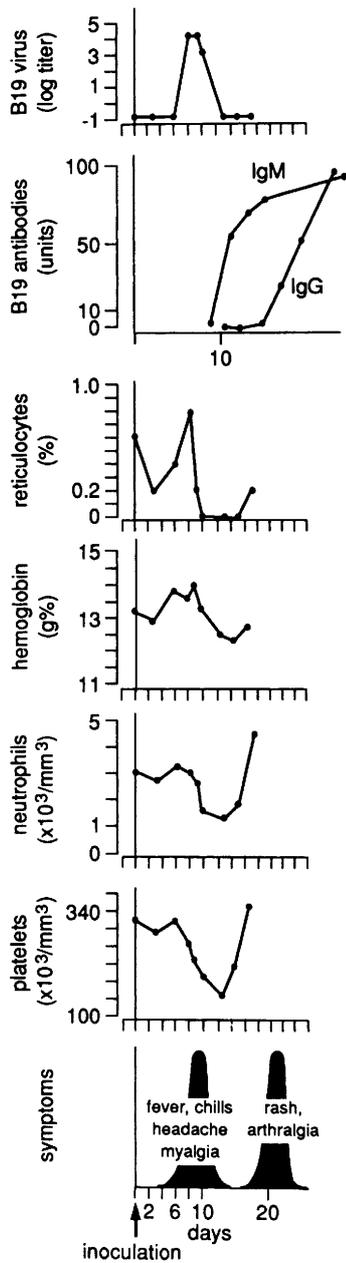


Figure 6 Clinical and laboratory parameters after experimental B19 infection in a seronegative volunteer (Anderson *et al.* 1985).

B19 is empirically known to spread by respiratory route (Chorba *et al.*, 1986, Plummer *et al.*, 1985). The mechanisms of virus transmission from the oropharyngeal mucosa to the target cells are unknown, as is the mechanism of virus release from the respiratory tract. The kinetics of B19 infection have been studied by intranasal inoculation of virus in adult healthy seronegative volunteers (Anderson *et al.*, 1985, Potter *et al.*, 1987). Subjects were inoculated with 10^8 virus particles resulting in peripheral viremia after six days then reaching a peak of 10^{11} virus particles/mL after 8-9 days (Figure 6). B19 DNA was detected in throat swabs only during viremia and was not found in urine or faeces.

The bone marrow examination was normal six days post infection but with an almost complete loss of erythroid precursors at day 10. On days 6-8, volunteers showed onset of cytokine release-related clinical symptoms such as headache, myalgia, chills and pyrexia. However, α - interferon (IFN) could not be

detected in serum. A second phase of clinical illness began on days 15-17 as the virus immunoglobulin (Ig) M peaked and IgG became detectable, with symptoms typical of

B19 infection including maculopapular rash, arthralgia and in some cases arthritis. At the height of viremia a marked drop of reticulocyte count could be noted, indicating cessation of red cell production in the bone marrow,

together with a modest drop in haemoglobin concentration, neutropenia, lymphopenia and thrombocytopenia. Seropositive individuals did not become infected and individuals with previously low titres of IgG boosted their IgG levels and only developed a mild viremia.

– I M M U N E R E S P O N S E

To date, most studies have focused on the humoral response to B19 infection. B19 specific IgM is usually present in the late viremic stage of primary infection, 10-12 days post infection. M class antibodies usually persist for about two months but can be still be detectable as late as 3-4 months post infection (Brown *et al.*, 1989, Cohen *et al.*, 1983, Schwarz *et al.*, 1988a, Yaegashi *et al.*, 1989a). G class antibodies are usually readily detectable 15-17 days post infection and persist although in slowly decreasing titres life long and are boosted when reexposed to the virus. (Anderson *et al.*, 1985, Potter *et al.*, 1987). IgA antibodies against B19 virus is present in the majority of cases within one week after the onset of clinical symptoms though the level of antibodies decline rapidly during the succeeding weeks (Erdman *et al.*, 1991). Detectable levels of B cells producing B19 antibodies can usually only be found during the acute phase of the infection, however patients in late stages of HIV infection have been shown to have IgG producing B cells in the absence of signs of viral replication, presumably indicating a non-specific activation of B cells (Arakelov *et al.*, 1993). Development of antibody correlates to clearance of viremia and protects from later symptomatic infection (Anderson *et al.*, 1985). The initial humoral response to B19 is directed to linear and conformational regions in the structural proteins, and several antigenic and neutralising regions have been mapped on the B19 capsid proteins (Figure 7, figure references: (Anderson *et al.*, 1995, Saikawa *et al.*, 1993, Sato *et al.*, 1991a, Sato *et al.*, 1991b)) (Fridell, 1993, Saikawa *et al.*, 1993, Sato *et al.*, 1991b). Several investigators have observed a gradual loss in reactivity to linear epitopes in both VP1 and VP2 following infection, whereas antibody responses against conformational epitopes persist (Corcoran *et al.*, 2000, Kaikkonen *et al.*, 1999, Kerr *et al.*, 1999, Soderlund *et al.*, 1995).

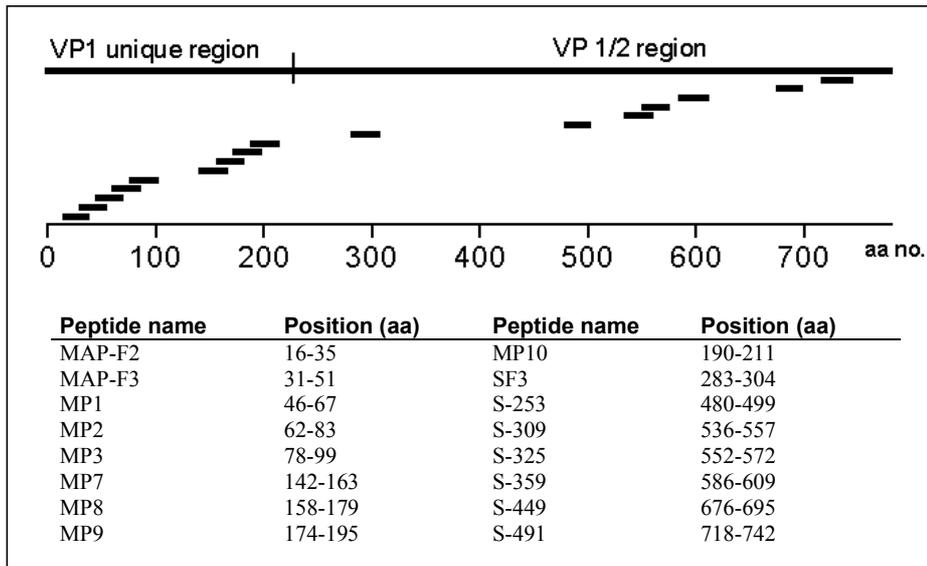


Figure 7 Antigenic and neutralising regions localised on the B19 capsid proteins.

The antibody response is dominated by production of IgG subclass 1 (Franssila *et al.*, 1996).

Antibodies are also, to a variable extent, produced against the NSP and it has been suggested that these antibodies are most often found in patients with chronic infection (Hemauer *et al.*, 2000, von Poblitzki *et al.*, 1995a, von Poblitzki *et al.*, 1995b), although this has not been confirmed in other studies (Jones *et al.*, 1999, Searle *et al.*, 1998, Venturoli *et al.*, 1998).

The cellular immune response to B19 infection has not been extensively studied. Attempts to demonstrate a proliferative immune response to native virus have been unsuccessful (Kurtzman *et al.*, 1989b). One group has identified a CD4+ response directed to recombinant VP1 and VP2 proteins, although not in the form of empty capsids (von Poblitzki *et al.*, 1996). Recently, T helper cell (Th) responses have also been demonstrated with recombinant VP1/ VP2 capsids among B19 seropositive individuals, even among remotely infected patients (Franssila *et al.*, 2001). Th 1 cellular

immune responses with high levels of interleukin (IL) 2 and IFN- γ secretion in adults have been demonstrated as well to purified viral VP1 / VP2 (Corcoran *et al.*, 2000). In this study, a lower capacity of IFN- γ production was noted in cells from children.

The placental cellular immune response which has been studied by Jordan *et al* gives evidence of an inflammation-mediated immune response in placentae from women whose pregnancies are complicated by B19 infection (Jordan *et al.*, 2001). Experimental work has also been conducted measuring T cell responses to animal parvoviruses. Canine parvovirus (CPV)-specific T cell clones isolated from immunised mice responded to stimulation with CPV antigens with production of IL2 in vitro (Rimmelzwaan *et al.*, 1990). Furthermore, lymphocytes from minks in the latter stage of Aleutian mink disease had a specific proliferative response to Aleutian disease parvovirus antigens (Race *et al.*, 1983).

– E P I D E M I O L O G Y

Parvovirus is an endemic infection all over the world. By age 15, approximately 50% of children have detectable IgG antibodies and since infection occurs throughout life more than 80% of the elderly are seropositive (Figure 8.) (Cohen & Buckley, 1988).

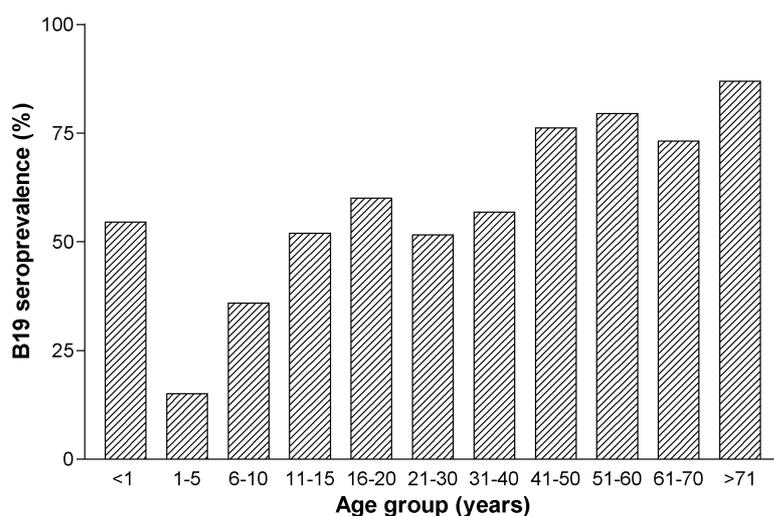


Figure 8 Prevalence of B19 specific IgG in different age groups (Cohen *et al.* 1988).

Studies from different countries; USA (Anderson, 1987), United Kingdom (Cohen & Buckley, 1988), France (Courouce *et al.*, 1984), Germany (Wiersbitzky *et al.*, 1990), Denmark (Valeur-Jensen *et al.*, 1999), Japan (Nunoue *et al.*, 1985) show similar rates of seropositivity.

However, among developing countries in South America and Africa the seroprevalence has been found to be slightly higher, possibly indicating higher transmission of the virus in poor and crowded living conditions (Nascimento *et al.*, 1990, Schwarz *et al.*, 1989, Tolfvenstam *et al.*, 2000). Furthermore, small isolated tribal communities have been found to have much lower seroprevalence of around 2-10% on Rodriguez Island, Africa and in Brazil (de Freitas *et al.*, 1990, Schwarz *et al.*, 1989). The average annual seroconversion rate among women of childbearing age during non-epidemic years has been estimated to 1.5% (Koch & Adler, 1989, Valeur-Jensen *et al.*, 1999).

The prevalence of asymptomatic, viremic individuals in the community seems to be low, as indicated by the fact that only 1:3,000 samples from blood donors have been found to contain detectable B19 DNA during non-epidemic season (McOmish *et al.*, 1993). Infections in temperate climates more commonly occur during late winter, spring and early summer months (Anderson & Cohen, 1987, Harger *et al.*, 1998). Rates of infection also increase every 3-4 years, reflected in corresponding increases in the major manifestations of B19 infection (Serjeant *et al.*, 1993). The virus is readily transmitted by close contact. The secondary seroconversion rate from symptomatic patient to susceptible household members were found to be approximately 50% (Chorba *et al.*, 1986). During outbreaks in schools and day-care centers, the secondary seroconversion rate was estimated to 20-30% among the personnel (Gillespie *et al.*, 1990). Virus can also be iatrogenically transmitted by transfusions of blood and clotting factors. Due to the lack of lipid membranes, B19 is very heat-resistant. Evidence by B19 transmission of steam-, dry heated- and solvent-detergent treated coagulation factors has been found (Azzi *et al.*, 1992, Bartolomei Corsi *et al.*, 1988, Lyon *et al.*, 1989, Williams *et al.*, 1990).

– C L I N I C A L M A N I F E S T A T I O N S

Asymptomatic infection is common after infection with B19. Studies have estimated that 25-32% of infections pass unnoticed, with an even higher figure among individuals with dark skin where rash is particularly difficult to see (Chorba *et al.*, 1986, Woolf *et al.*, 1989).

Erythema infectiosum (EI), also known as fifth disease or slapped cheek disease, is the major manifestation of B19 infection. As mentioned the manifestation was first described by Robert Willan in 1799 (van Elsacker-Niele & Anderson, 1987). It was rediscovered and named a hundred years later and in 1905 classified as the fifth exanthematous disease of childhood (Cheinisse, 1905, Shaw, 1905, Sticker, 1899). It was recognised that the disease was contagious but the single etiological agent remained unknown until 1983 (Anderson *et al.*, 1983, Anderson *et al.*, 1984, Naides, 1988, Okabe *et al.*, 1984). The symptoms include a non-specific prodromal illness with pyrexia, coryza, headache, nausea and diarrhoea (Ager *et al.*, 1966, Balfour, 1969, Brass *et al.*, 1982). The classic slapped cheek rash and relative circumoral pallor appears 2-5 days later. This is often followed by a second stage eruption, an erythematous maculopapular exanthema on the trunk and limbs, which while fading produces a lacy appearance. Adults seldom develop apparent dermatological eruptions.

Arthritis with pain, swelling, and stiffness symmetrically distributed is a common complication to B19 infection in adults, especially in women (Woolf *et al.*, 1989). The symptoms usually persist 1-3 weeks, but in 20% of the affected women the arthropathy may persist longer, maybe for years and may even meet the American Rheumatism Association's criteria for rheumatoid arthritis (Kirchesch, 1990). It has been suggested that HLA DR4-positive patients may be more susceptible to joint complications following B19 infection (Klouda *et al.*, 1986). B19 associated arthritis has

been suggested to be immune complex mediated, but some groups have observed presence of B19 DNA in synovial fluid and cells (Dijkmans *et al.*, 1988, Kandolf *et al.*, 1989).

Transient aplastic crisis (TAC) may be the result of B19 infection in patients with underlying haemolytic disorders, such as hereditary spherocytosis, thalassemia, autoimmune haemolytic anaemia and red cell enzymopathies (Owren, 1948, Young, 1988). TAC was the first clinical illness associated with B19 infection and retrospective studies have shown that 86% of TAC episodes were associated with recent B19 infection in patients with sickle-cell anaemia (Serjeant *et al.*, 1981). Typically, TAC presents by anaemia, erythroid hypoplasia in bone marrow, dyspnoea, lassitude, confusion, congestive heart failure, and in the most severe cases even bone marrow necrosis. Other hematopoietic lineages may also be affected by B19 infection, such as varying degrees of neutropenia and thrombocytopenia which have been reported to follow the infection (Doran & Teall, 1988, Inoue *et al.*, 1991, Nagai *et al.*, 1992, Saunders *et al.*, 1986). B19 infection has also been associated with hemophagocytic syndrome, however in many cases there is an underlying immunosuppression making it difficult to discriminate the pathogen as the ethiological agent from a coincidental opportunistic infection (Boruchoff *et al.*, 1990, Henter *et al.*, 1993, Koch *et al.*, 1990, Komp *et al.*, 1989, Muir *et al.*, 1992, Shirono & Tsuda, 1995, Uike *et al.*, 1993). B19 infection additionally causes pure red cell aplasia (PRCA) in patients with congenital immunodeficiency syndromes such as common variable immunodeficiency and Nezelof's syndrome (Davidson *et al.*, 1989, Gahr *et al.*, 1991, Kurtzman *et al.*, 1987).

Immunocompromised patients are particularly at risk for B19 related complications, and both congenital and acquired immunosuppression predisposes to the development of persistent B19 infection (Frickhofen *et al.*, 1990). Immunosuppressed patients often present their B19 infection with PRCA and may have a delayed or absent IgG response. Furthermore, they seldom exhibit immune-mediated symptoms like rash and arthropathy.

B19 induced anaemia in HIV-seropositive patients may be more common than earlier estimated. One study found B19 DNA in peripheral blood in 17% of transfusion dependent HIV-seropositive individuals (Abkowitz *et al.*, 1997), but resolution of the chronic, proposedly B19 induced anaemia has been observed after the initiation of highly active antiretroviral therapy (Arribas *et al.*, 2000, Ware & Moore, 2001). Another group with impaired immunologic capacity prone to B19 infections are patients with lymphoproliferative disorders on maintenance chemotherapy protocols (Broliden *et al.*, 1998, Kurtzman *et al.*, 1988). Patients may present with acute severe anaemia, low-grade persistent anaemia or in some cases where there is also an affection of other blood lineages, pancytopenia. Typically B19-specific antibody levels are initially low or absent during the course of infection in these patients. Similar risk also exists for patients undergoing immunosuppressive treatment after transplantation or patients on steroid therapy due to i.e. systemic lupus erythematosus and other autoimmune diseases (Corral *et al.*, 1993, Niitsu *et al.*, 1990, Uemura *et al.*, 1995, Weiland *et al.*, 1989).

Several case reports have shown an association between B19 infection and congestive heart failure, myocarditis and pericarditis in children and adults (Chundu *et al.*, 1995, Malm *et al.*, 1993, Nakazawa *et al.*, 1995, Saint-Martin *et al.*, 1990, Shishiba & Matsunaga, 1993, Zanella *et al.*, 1995). However, infection of myocytes has not been documented and usually only a few B19 antigen containing cells can be visualised. In many cases, heart failure may be secondary to the consequences of severe anaemia (Morey *et al.*, 1992b, Naides & Weiner, 1989, Porter *et al.*, 1988). Acute myocarditis is commonly caused by some animal parvoviruses e.g. CPV, but infection is restricted to fetal and neonatal stages when mitotic myocytes are still present (Parrish, 1995, Robinson *et al.*, 1980). Parvovirus B19 has also been associated with several other clinical manifestations mainly of neurological and rheumatological derivation (Denning *et al.*, 1987, Faden *et al.*, 1990, Finkel *et al.*, 1994, Walsh *et al.*, 1988). Nevertheless, these conditions need to be studied further as no evidence of causal connection has been established.

Gray *et al.* reported presence of B19 DNA in tissue from testicular carcinoma, a finding that was confirmed by another group (Diss *et al.*, 1999, Gray *et al.*, 1998). It currently seems unlikely that B19 would cause testicular tumour development as a prospective serological case-control study on testicular seminoma showed no association between B19 seropositivity and risk for testicular cancer (own unpublished results). This presence of B19 DNA in testicular carcinoma may instead reflect virus capacity to replicate in the immature tumour cells, which demonstrate high expression of surface P antigen (Olie *et al.*, 1996).

– B 1 9 I N F E C T I O N D U R I N G P R E G N A N C Y

B19 infection during pregnancy can lead to adverse outcomes, either miscarriage or non-immune hydrops fetalis (NIHF) (Kinney *et al.*, 1988). Transmission of the virus over the placenta and subsequent fetal infection may cause an abrupt inhibition of the erythropoiesis, which may lead to severe anaemia and fetal death. NIHF is caused by functional failure of the liver, the major fetal hematopoietic organ and is characterised by anaemia, hypoalbuminemia, peripheral oedema, and congestive heart failure, which may be fatal. Parvoviral DNA and antigen have been detected in fetal myocardial cells, suggesting that myocarditis may contribute to the pathology (Morey *et al.*, 1992a, Naides & Weiner, 1989). Variable with geographical areas, approximately 60% of pregnant women are B19 seropositive. The annual seroconversion rate has been estimated to 1.5% and 15% during non-epidemic and epidemic years, respectively (Valeur-Jensen *et al.*, 1999). The transplacental transmission rate during maternal infection has been estimated to 33% and 51% (PHLS, 1990, Yaegashi, 2000). The placental trophoblast expression of P antigen is abundant in early pregnancy, but decreases with gestational age (Jordan & DeLoia, 1999). The estimated excess risk for fetal loss attributed to B19 infection varies in different studies between 1.6% and 16% (PHLS, 1990, Warsof *et al.*, 1986, Yaegashi *et al.*, 1994), with a median of 10% (Yaegashi, 2000).

Fetal B19 infection may also be asymptomatic, and there are several observations of infants born healthy despite evidence of intrauterine infection diagnosed by the presence of IgM in umbilical cord blood (Kinney *et al.*, 1988). Studies of fetal loss in the first trimester of pregnancy have shown low association to B19 infection both by serological and virologic analysis (Rogers *et al.*, 1993). During the second trimester of pregnancy, there is a dramatic increment of hematopoiesis, and this is the gestational stage when the fetus is believed to be the most vulnerable to low hematocrite due to high tissue oxygen demand. The peak incidence of fetal loss associated to B19 infection has been reported to occur during gestational week 23-24 (Yaegashi, 2000). Furthermore, clinical presentation with NIHF is most common in the second trimester. There are few cases published describing B19 associated fetal loss in the third trimester, and a lower incidence of B19 associated fetal loss at that stage of pregnancy has been assumed to be due to greater haematological reserves in older fetuses. Some authors have even proposed B19 related fetal demise to be confined to the first and second trimester of pregnancy (Miller *et al.*, 1998). Available data suggests that B19 uncommonly, if ever, cause birth defects (Weiland *et al.*, 1987), and no long term adverse effects have been recorded in liveborn infected children (Miller *et al.*, 1998).

– P E R S I S T E N T B 1 9 I N F E C T I O N

As described earlier, B19 infection in immunocompromised individuals may cause persistent infection, usually leading to severe chronic anaemia (Frickhofen *et al.*, 1990, Graeve *et al.*, 1989, Kurtzman *et al.*, 1988, Kurtzman *et al.*, 1987, Weiland *et al.*, 1989). However, B19 DNA can persist in bone marrow and synovia even without signs of peripheral viremia or anaemia (Söderlund *et al.*, 1997). Several studies have identified a group of apparently immunocompetent individuals with prolonged (> 6 months) presence of B19 DNA in bone marrow (Baurmann *et al.*, 1992, Faden *et al.*, 1992, Kurtzman *et al.*, 1989a, Lundqvist *et al.*, 1999a, Pont *et al.*, 1992). These subjects generally exhibit unspecific symptoms such as low-grade fever, arthropathy, neuropathy, fatigue, myalgia and leucopenia, and may

possess neutralising antibodies directed against the virus (Cassinotti *et al.*, 1993, Faden *et al.*, 1992, Foto *et al.*, 1993, Kurtzman *et al.*, 1989b, Lundqvist *et al.*, 1999a, Pont *et al.*, 1992).

The prevalence of B19 positivity in bone marrow in healthy individuals has been estimated to 9% by PCR (Cassinotti *et al.*, 1997). Comparisons are complicated by different sensitivities in different assays, but we have previously found 4% of adult individuals investigated at a haematology clinic, and 10% of children receiving chemotherapy for different malignancies, to be B19 PCR positive in bone marrow (Lundqvist *et al.*, 1999b, Broliden *et al.*, 1998). The percentage of symptomatic individuals with prolonged B19 infection is not known.

– T R E A T M E N T A N D P R O P H Y L A X I S

Treatment of parvovirus B19 infection is, in most instances, directed toward symptoms. EI does not usually require therapy, however non-steroid anti-inflammatory medications can be helpful in the management of patients with postinfectious arthritis and arthralgia. On the other hand, immunosuppressive steroid therapy should be avoided, as it is yet unclear whether the arthritis symptoms are only immune mediated or reflect direct viral affection of the synovia.

Patients suffering from B19 associated TAC usually require hospitalisation and transfusion therapy until red cell production resumes, usually 1-10 days after presentation. In one study of 62 sickle cell patients with B19 associated TAC, 87% developed transfusion dependent (haemoglobin concentration transfusion limit < 6g/dL) anaemia, 63% were hospitalised and one patient died before transfusion therapy could be initiated (Goldstein *et al.*, 1987).

Management of B19 infected pregnant women may be problematic since fetal death can occur as early as a few days post infection up to several months later (Sheikh *et al.*, 1992). Fetal affection can only be suspected during ultrasonographic examination if the fetus exhibits hydropic appearance.

Assessment of maternal serum α -fetoprotein levels, which has been used to diagnose B19 associated hydrops fetalis, is neither specific nor reliable in screening for fetal disease in B19 infected women (Carrington *et al.*, 1987, Saller *et al.*, 1993, Selbing *et al.*, 1995). Intrauterine blood transfusions have been used to treat severe fetal anaemia (Fairley *et al.*, 1995, Sahakian *et al.*, 1991, Schwarz *et al.*, 1988b, Smoleniec & Pillai, 1994, Soothill, 1990). So far, no large studies have been performed to determine if intrauterine blood transfusion is associated with increased survival rate in fetal B19 infection. However, several series of cases have been reported suggesting that it may enhance the rate of fetal survival (Schwarz *et al.*, 1988b, Smoleniec & Pillai, 1994). The most comprehensive investigation retrospectively studied 38 cases of B19 infection and NIHF. Nine of 12 fetuses treated with blood transfusion survived, compared to 13 of 26 of the untreated cases (Fairley *et al.*, 1995). Considering a direct viral effect myocardium in the pathology of B19 related intrauterine fetal death, maternal intravenous immunoglobulin (IVIg) treatment has been suggested. This regime has been applied in some isolated cases but a possible benefit of the treatment has yet to be evaluated (Selbing *et al.*, 1995).

In iatrogenically immunosuppressed individuals, the infection usually resolves spontaneously upon cessation of immunosuppression (Carstensen *et al.*, 1989, Smith *et al.*, 1988). Persistent B19 infection with anaemia in immunocompromised individuals due to HIV-infection or congenital immunodeficiency has successfully been treated with IVIg (400 mg/kg x 5 days or 1g/kg x 3 days have given good results) (Corral *et al.*, 1993, Frickhofen *et al.*, 1990, Koch *et al.*, 1990, Kurtzman *et al.*, 1989a, Kurtzman *et al.*, 1987, Naides *et al.*, 1993, Nigro *et al.*, 1994, Nour *et al.*, 1993). Reticulocytosis is usually detected within one week and an increment in haemoglobin concentration within 2-3 weeks (Kurtzman *et al.*, 1989a). Some patients have recurrence of viremia with only a transient clinical response.

The role of IVIG treatment in patients with persistent B19 infection without anaemia or in immunocompetent individuals with persistent B19 infection is still unclear (Finkel *et al.*, 1994, Murray *et al.*, 1993, Oeda *et al.*, 1994). In a study of six immunocompetent individuals with persistent B19 infection diagnosed by the presence of B19 DNA in bone marrow, the effect of immunoglobulin treatment was only transient (Lundqvist *et al.*, 1999a). Interestingly, all these patients possessed neutralising B19 IgG antibodies with high avidity prior to immunisation.

Aims of the study

The aim of this study was to examine the pathogenesis of human parvovirus B19 infection, and in particular:

- To examine the role of parvovirus B19 in intrauterine fetal death and non-immune hydrops fetalis in pregnancy.
- To study the viral genotype immunocompetent and immunocompromised individuals with persistent parvovirus B19 infection.
- To define B-cell epitopes in the parvovirus B19 non-structural proteins in healthy individuals as well as in immunocompetent and immunocompromised individuals with persistent parvovirus B19 infection.
- To identify parvovirus B19 specific cytotoxic T lymphocyte epitopes and to examine the cellular immune response in parallel to the humoral response in immunocompetent and immunocompromised individuals.

Methods

– P A P E R I

Placental tissue and maternal serum were prospectively collected from all cases of third trimester intrauterine fetal death (IUFD) occurring 1992 – 1998 at Danderyd Hospital (n=93), a hospital which serves the north eastern part of the greater Stockholm area. The total number of deliveries during the seven-year study period was 33,759. Nested polymerase chain reactions (PCR) amplifying the NSP and VP1 genes were used to detect B19 DNA in the supernatant of homogenised and heat-treated freshly frozen placental tissue (Broliden *et al.*, 1998). Serological analysis of B19 specific IgG and IgM was performed using an indirect immunofluorescence assay (IFA) using as antigen recombinant VP1 in insect cells. B19 NSP PCR on autopsy material from the fetus further evaluated cases with B19 DNA positive placentas. DNA was extracted from tissues after deparaffination with Xylene/Ethanol. These formalin fixed, paraffin embedded tissue sections also underwent histopathological examination and immunohistochemical (IH) staining for viral capsid proteins utilising two monoclonal antibodies (mAb) directed to VP1 and VP2 by streptavidin-biotin technique. Other possible aetiological explanations to the fetal demise were sought by analysis of fetal nucleated red blood cells in maternal peripheral blood and toxoplasma-, rubella-, cytomegalovirus- (CMV), herpes simplex virus-, and listeria serology was performed. Amniocentesis was performed for chromosomal examination, viral and bacterial cultures. Bacterial cultures were also done on cervical swabs and fetal heart blood. For comparison, placental tissue from 50 unselected first trimester spontaneous abortions were collected (June 1994 – December 1998) and analysed for the presence of B19 DNA in the same manner as the IUFD cases.

Placental and fetal tissues were prospectively collected from all cases (n=47) of intrauterine fetal death (≥ 22 weeks of gestation) that occurred between January 1998 and May 1999 at Huddinge University Hospital, Stockholm South Hospital and Södertälje Hospital which serves the southern part of the greater Stockholm area. The total number of deliveries during the study period was 14,147. In addition to formalin fixed biopsies taken for routine examination, sections from placenta, fetal heart, lung, and liver were collected and freshly frozen. Tissues were analysed by B19 NSP PCR as described earlier after DNA extraction. The extraction was confirmed by amplification of the chromosomal HLA class II gene (Ehrlich & Bugawan, 1989). Formalin-fixed paraffin embedded tissues were examined by IH staining using a mAb directed to capsid antigen. B19 PCR positive fetal tissues and placenta were thoroughly examined for presence of histopathological indicators for B19 infection by a senior study pathologist. Other possible causes of the fetal demise were examined using karyotyping, bacterial isolation and PCR of amniotic fluid and peripheral blood for CMV and enterovirus. A faeces sample from the mother was also examined for enterovirus. Maternal peripheral blood was assessed for haemoglobin concentration, activated partial thromboplastin, and serology for anti-cardiolipin, CMV, B19, toxoplasma and rubella antibodies. Fetal length, weight and sex were noted at autopsy and an X-ray performed together with the routine pathological examination. For comparison, we also included 37 referred cases of spontaneous abortion (< 22 weeks of gestation) of unknown aetiology and 29 referred cases of fetal loss of known aetiology (< 22 weeks of gestation) derived from legal abortions. Placental tissues from 53 consecutively collected normal pregnancies at term were included in the study in order to estimate the frequency of asymptomatic carriers of B19. All samples were processed in the same manner and coded until the end of the study.

– P A P E R I I I

In this study we performed full length direct sequencing of the B19 genome from viral DNA isolates derived from six persistently B19 infected individuals (B19 DNA positive in bone marrow >6 months). Four patients were immunocompetent and two were immunocompromised. Isolates were collected from two time points during the course of infection. DNA was extracted from cryopreserved bone marrow samples and nine overlapping regions were amplified by nested PCR. Amplified DNA fragments were purified and sequenced using the cycle sequencing method. Two independent PCR products from each isolate were sequenced and the consensus was aligned to three published full-length B19 sequences for the evaluation of mutations in the coding regions and translation products. Furthermore, the within-patient variability of the viral DNA sequence was assessed by comparing paired isolates from the same patient.

– P A P E R I V

To determine B cell epitopes in the B19 NSP, linear 16-mer peptides, overlapping by six amino acids (aa), representing the whole B19 NSP were synthesised. Sera from healthy B19 IgG positive / IgM negative and seronegative individuals were tested against the peptides in an enzyme linked immunosorbent assay (ELISA). Three regions exhibited specific reactivity and were further mapped by B19 IgG positive / IgM negative samples from healthy volunteers in an ELISA using peptides containing systematic deletions in the immunodominant regions. Serum from B19 IgM positive individuals were tested but could not be evaluated by the method due to high background reactivity, not even after rheumatoid-factor adsorption and blocking procedures. B19 VP derived peptides were synthesised based on previously published neutralising regions. These regions had been defined either by immunisation of animals or by purification of human seropositive sera by affinity columns coupled with peptides (Figure 7). Sera from the group of healthy B19 IgG positive / IgM negative individuals were tested against these VP derived peptides, and

reactive regions were fine-mapped by the insertion of systematic aa deletions. Finally, 31 sera from seven immunocompetent and immunocompromised persistently B19 infected patients as well as 40 HIV-1 infected individuals were tested against the B19 specific NSP and VP epitopes defined.

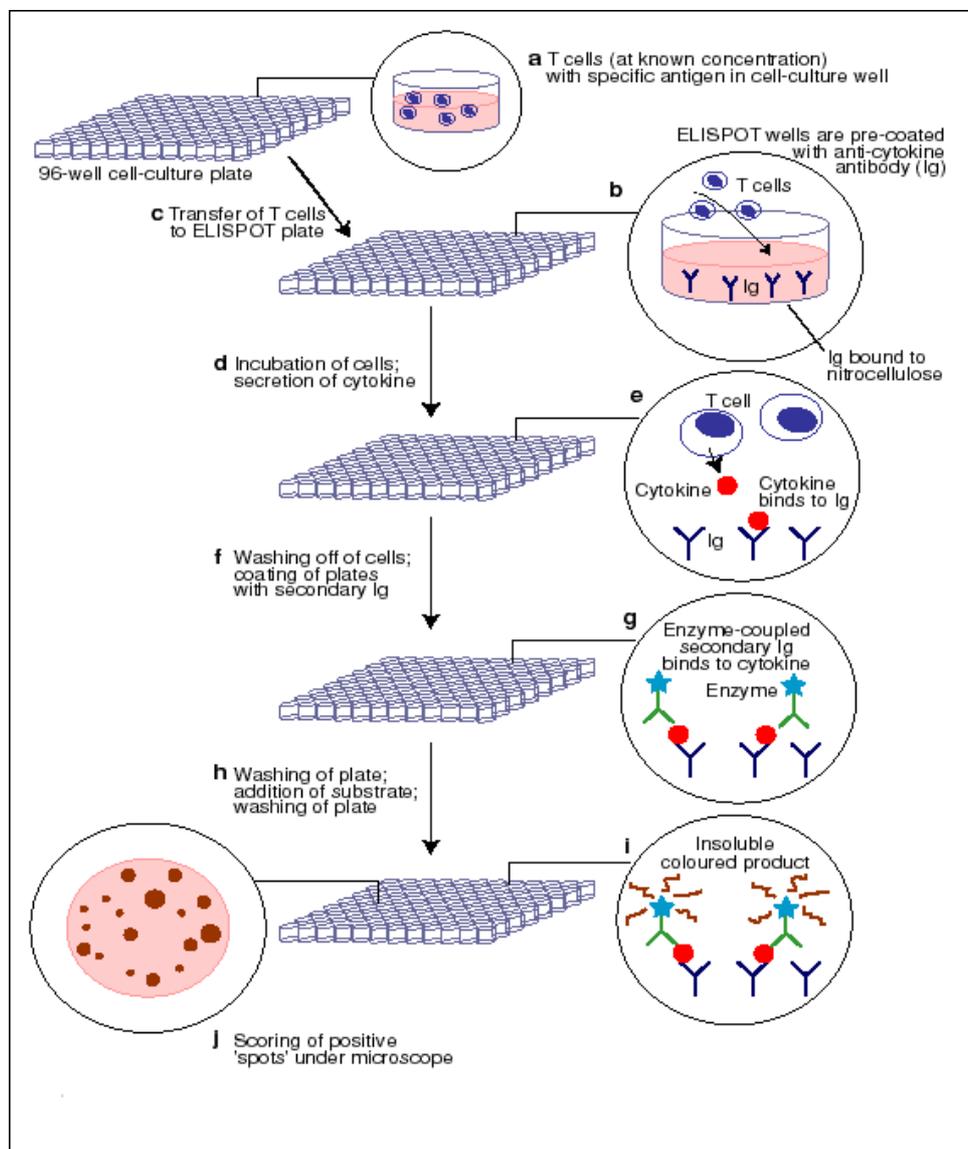


Figure 9 Schematic presentation of the ELISpot technique.

– P A P E R V

In order to screen the B19 NSP for cytotoxic T lymphocyte (CTL) epitopes, 146 15-mer linear peptides overlapping by ten aa covering the entire length of the NSP were synthesised. Pools of ten peptides were used to restimulate peripheral blood mononuclear cells (PBMC) from a normal B19

seropositive volunteer (HLA A2, A26, B35 and B62). A conventional ⁵¹chromium release assay was performed to detect cytolysis using autologous and matched B-cell lines (BCL) prepulsed with the same peptide pool as targets. The individual peptides of one pool that exhibited reactivity were assessed using the same method. The reactive peptides exhibited capacity to induce cytolysis to B35, but not A2 matched BCL and they contained a sequence with a clear B35 motif (QPTRVVDKQM). This motif, when tested alone, gave a very strong response of >80% specific lysis. T-lymphocyte responses were confirmed by prepulsing PBMC from the same donor and measuring IFN- γ secretion by enzyme linked immunospot (ELISpot) (Figure 9). An HLA-B35 restricted soluble major histocompatibility complex (MHC)-peptide tetrameric complexes conjugated to phycoerythrin was constructed using the reactive peptide (Figure 10). Samples were examined for presence of B19 specific CD8+ T-lymphocytes by co-staining with anti- CD3, CD4, and CD8 mAb conjugated to fluorescein isothiocyanate, peridinin chlorophyll protein, allophycocyanin, and analysed by four-colour flow cytometry. The phenotype of the B19 specific cells were evaluated using additional fluorochrome conjugated anti-human mAb specific to HLA-DR, CD28, CD38, CD56, CD57, CD69, CD62L, and CD45RA. The specificity of the optimised peptide was verified by unlimited BLAST homology search to all available GeneBank sequences.

– P A P E R V I

In this study, the B19 specific CTL epitope identified in paper V was used to evaluate the B19 cellular immune response in parallel to the humoral response in healthy volunteers and immunocompromised individuals. A total of 21 individuals were included in the study, 16 matched and five unmatched to the epitope HLA restriction, and longitudinal samples of cryopreserved PBMC and serum were retrospectively collected. Fourteen subjects were immunocompromised due to HIV-1 infection and the remaining seven were healthy volunteers. The study participants' HLA class I type was determined and B19 DNA in serum was detected by nested NSP

PCR. Furthermore B19 IgG was quantified and B19 IgM detected by ELISA. The G class antibodies, if present, were analysed regarding avidity and epitope type specificity. Presence of IFN- γ secreting cells in the patients' longitudinal samples of PBMC was analysed by ELISpot after stimulation with the synthetic NSP derived peptide (QPTRVDQKM) and with baculovirus produced B19 native empty capsids containing both VP1 and VP2. Enumeration of the frequency of B19 specific CD8⁺ T cells and assessment of the cell phenotype were performed by staining PBMC with soluble MHC peptide tetrameric complexes. The cytolytic capacity of antigen specific T cells were analysed by ⁵¹chromium release assay in samples demonstrating high levels of B19 specific CD8⁺ T cells.

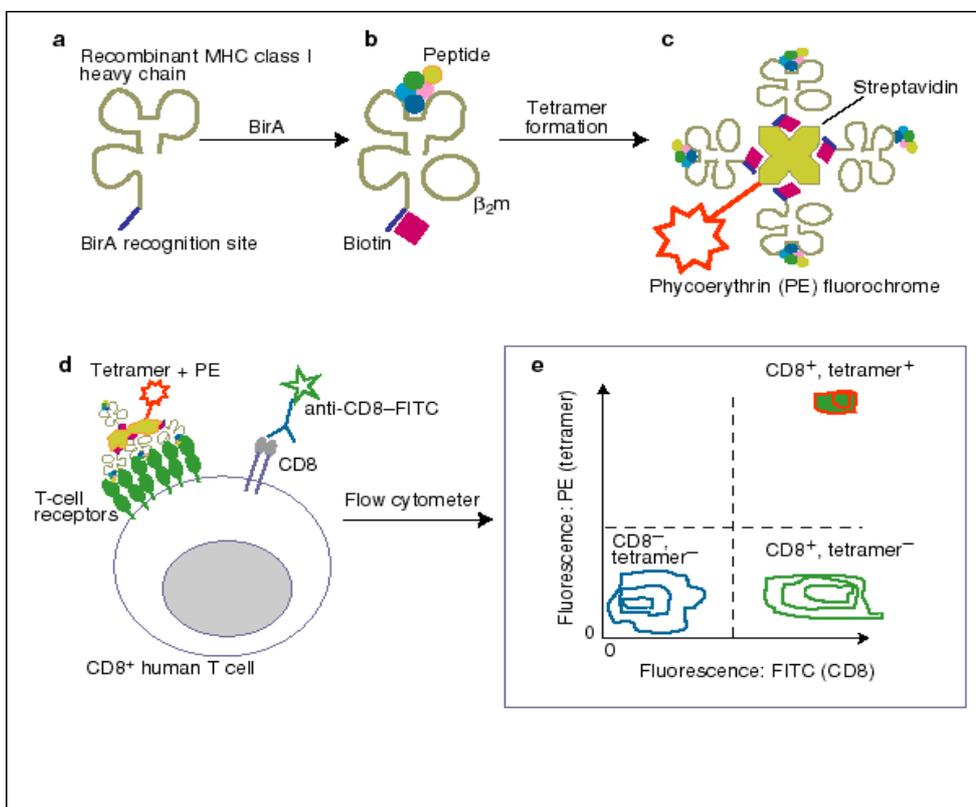


Figure 10 Schematic presentation of the tetramer technique.

Results and discussion

– P A P E R I A N D I I

Neonatal mortality rates have significantly decreased during recent years due to improved perinatal care, but no comparable reduction in antenatal mortality has been observed (Ahlenius & Thomassen, 1999, Morrison & Olsen, 1985). This could in part be explained by the fact that the aetiology in a large proportion of the cases of IUFD is unknown, and different studies show that no identifiable cause could be determined in 12-50% of stillbirths (Ahlenius *et al.*, 1995, Brans *et al.*, 1984, Fretts *et al.*, 1992, Huang *et al.*, 2000, Morrison & Olsen, 1985). To examine association between parvovirus B19 and fetal loss, we performed two studies during the years 1992-1999 in Stockholm. IUFD is classified differently in the two studies, according to Swedish legislation (paper I) and World Health Organization (WHO) guidelines (paper II). In this text, IUFD is defined according to current WHO decree, during, or after gestational week 22. Accordingly, spontaneous abortion is defined as fetal loss before gestational week 22. By detection of B19 DNA, 7/93 (7.3%) placentas (gestational week >28, paper I) and 7/47 (15%) placental or fetal tissues (gestational week >22, paper II), respectively, were found positive in cases of IUFD. The cases of IUFD constituted 0.3% of the total numbers of deliveries in both studies. For comparison placental tissues from unselected spontaneous abortions of unknown aetiology (gestational week <13) were analysed (1/50 (2%) B19 DNA positive), fetal tissues from referred cases of spontaneous abortions of unknown aetiology (gestational week 12-21) (2/37 (5%) B19 DNA positive) and legal abortions (week 12-22) (0/29 B19 DNA positive). Additionally, in order to assess the rate of asymptomatic B19 carriage in pregnancy placentas from normal pregnancies at term were collected at delivery (n=53), and all of these were found B19 DNA negative.

Case no.	Gestational week	Fetal weight (g)	B19 IgG* at partus	B19 IgM* at partus	B19 specific histo-pathology	Fetal hydrops	IH
Paper I							
1	37	2880	Neg	Neg	Neg	No	Neg
2	32	1870	Neg	Neg	Neg	No	Neg
3	41	4010	Neg	Neg	Neg	No	Neg
4	41	3375	Neg	Neg	Neg	No	Neg
5	27	970	Neg	Neg	Neg	No	Neg
6	40	2444	Pos	Neg	Neg	No	Neg
7	34	1815	Pos	Pos	Neg	No	Neg
Paper II							
1	32	1420	Pos	Neg	Neg	No	Neg
2	32	320	Pos	Neg	Neg	No	Neg
3	22	315	Pos	Pos	Pos	No	Pos
4	28	1275	Pos	Neg	Neg	No	Neg
5	40	3505	Pos	Neg	Neg	No	Neg
6	25	575	Nd	Neg	Neg	No	Neg
7	25	980	Pos	Pos	Pos	Yes	Pos

Table 2 Summary of B19 DNA positive cases of IUFD in paper I and II. (*in maternal serum.)

Although no causality can be proven between the presence of B19 DNA and the IUFD, there were no other conceivable aetiology - genetic, infectious or immunological explanation that could be found. None of the mothers who suffered IUFD with the subsequent finding of B19 DNA had experienced rash, fever, arthralgia, or other clinical signs of B19 infection during pregnancy. One of the 14 cases of B19 associated IUFD exhibited a hydropic appearance (Case 7, paper II). B19 DNA positive cases are shown in Table 3. In tissues from two of the 14 cases B19 associated viral inclusions could be observed and viral protein detected in fetal nucleated capillary erythroid progenitor cells, and IgM was present in maternal blood at time of delivery. Both these cases of fetal demise occurred in the second trimester. Seven out of 13 (one case was not tested) cases were B19 IgG positive at time of parturition. Moreover, one mother had seroconverted six months after delivery. A total of three mothers had detectable B19 IgM at delivery. Reviewing the literature, the incidence of fetal loss that could be

attributed to B19 infection (B19 DNA positive) irrespective of gestational age, has been reported to be 57/557 (10.2%) cases summarising 24 prospective studies (Yaegashi, 2000). The majority of these cases presented clinically by NIHF. Spontaneous abortions associated with B19 infection is reported to be rare in the first trimester (Rogers *et al.*, 1993). This is supported by a study performed in the same setting as paper I, where only 3% of placentas from spontaneous abortions (gestational age < 13 weeks) were found to contain B19 DNA (own unpublished results). The peak incidence of B19 DNA positive findings has been reported during the second trimester, with the median of cases presented clinically in gestational week 22.8 ± 5.1 (Enders & Biber, 1990, PHLS, 1990, Torok, 1990). It was reported that the infection, interpreted by the onset of maternal infection occurred by median 6.2 ± 3.7 weeks before the fetal demise, correlating to the period of hepatic haematopoietic activity (Yaegashi, 2000). The literature concerning third trimester B19 associated IUFD is limited to a few case reports, and B19 infection as a cause of IUFD has been reported to be rare event in the third trimester of pregnancy (Forouzan, 1997, Jordan, 1996, Knott *et al.*, 1984, Morey *et al.*, 1992a, Sanghi *et al.*, 1997). Furthermore, in this stage of pregnancy, only cases presenting with NIHF have been described. The “typical case” according to the majority of reports is characterised by a fetal loss, presented in the second trimester with fetal hydropic appearance, maternal seronegativity followed by development of B19 specific IgM antibodies and a subsequent shift to IgG. In our studies a different picture was made apparent in cases of IUFD in third trimester, indicating that third trimester IUFDs are often non-hydropic and are not readily diagnosed by maternal serology and histopathological examination.

We can only speculate on the pathogenesis of these observations, such as that a persistent B19 infection may be established early or even before pregnancy, eventually leading to fetal death many months later (Nonue T. Fetal infection with parvovirus B19: Infection time in gestation and prognosis. Poster 11.3 8th Parvovirus Workshop Mt Tremblant, Canada). B19 IgM would then not be detectable any longer as IgM usually only

persists two to four months, making a serological evaluation difficult. Possibly, a more mature immune response in third trimester fetuses could delay B19 pathogenic consequences with reduced hydrops as a result, complicating ultrasonographic diagnostics. Another possibility could be that the fetal demise is immune-mediated due to upregulated maternal Th1 response after acute B19 infection, although the fetus initially survived the viral infection itself earlier in gestation (Corcoran *et al.*, 2000). Based on our results we recommend the use of sensitive methods, such as PCR for detection of B19 DNA in amniotic fluid in cases of NIHF or in placenta and fetal tissues for diagnosis of IUFD, as diagnosis by serology only is not reliable in all cases.

– P A P E R I I I

Factors responsible for the different manifestations of B19 infection can hypothetically be divided into viral and host properties. Hemauer *et al.* has reported differences in B19 viral genotype as a plausible explanation for clinical outcomes such as persistent infection and arthritis in some individuals, whereas the virus is rapidly cleared in other patients (Hemauer *et al.*, 1996). Presentations of other partial sequences have not confirmed this discrepancy (Erdman *et al.*, 1996, Haseyama *et al.*, 1998, Hokynar *et al.*, 2000, Mori *et al.*, 1987). In order to assess the B19 genotype correlation to viral persistence, we sequenced almost the full B19 genomes from a) two B19 persistently infected immunocompromised individuals, b) four B19 persistently immunocompetent individuals. The four immunocompetent subjects were unique since they had persistent B19 infection although they possessed neutralising antibodies of high avidity (Lundqvist *et al.*, 1999a). Two samples from each patient were analysed, one sample from the earliest possible time point after diagnosis and one at a later time point. It was important to include samples from different time points since recombination of B19 strains from later superinfections of different phylogenetic origin has been proposed to occur in some cases (Johansen *et al.*, 1998). Persistent infection was defined as B19 DNA positivity in bone marrow for more than six months. In this material, all patients had B19 DNA present for an even

longer period of time. There was generally limited sequence variability between the patient isolates and previously published B19 sequences from non-persistently infected individuals (Blundell *et al.*, 1987, Gallinella *et al.*, 1995, Shade *et al.*, 1986). The only consistent protein-level mutation among the patients was a nucleotide exchange at nt 2107 in the NSP coding region leading to an exchange from serine to proline (numbering according to Shade *et al.*, 1986). However, one of the three reference sequences exhibited this sequence as well. The VP2 coding region was shown to be highly conserved, containing no amino acid exchanges at all, and the NSP coding region demonstrated most variability (0.38%). A clustering of non-consistent amino acid exchanges could however be noted in the NSP and VP1 unique coding regions, respectively, in isolates from immunocompetent persistently B19 infected patients. Paired isolates from the same patients showed high consistency (mean 99.9%). In conclusion, we found no obvious support for B19 viral genotype to be of importance in the development of persistent infection in this material, as there was generally low variability in all isolates and no consistent translated mutations could be detected differing consequently from previously published sequences. Furthermore, viral isolates from individuals showed little temporal variability. We can not rule out however that the individual mutations have significance for genome translation or viral antigenic properties, although it is unlikely that the heterogeneous structural changes observed would alone induce the course of the infection in these individuals.

Many different host factors have been proposed that may contribute to the development of persistent B19 infection in the patients exhibiting an apparently intact humoral immune capacity to B19. For example, individual differences in distribution of surface glycosphingolipids has been considered, which could lead to B19 infection of non-permissive cells (Cooling *et al.*, 1995), and a subsequent overexpression of cytotoxic NSP transcripts (Leruez *et al.*, 1994, Liu *et al.*, 1992). Some authors have indeed reported increased prevalence of NSP antibodies in persistently infected individuals, and proposed it to be due to a defective humoral immune

response in these individuals (Hemauer *et al.*, 2000, von Poblitzki *et al.*, 1995a, von Poblitzki *et al.*, 1995b). However, the cellular immune responses directed to B19 has not been investigated in these patients.

– P A P E R I V

As described above, antibodies directed to the B19 NSP have been reported to dominate in patients with severe B19-associated arthritis and persistent B19 infection. Consequently, these antibodies were present to a lesser extent in healthy individuals with past infection, and detection of NSP antibodies has been proposed useful to diagnose a severe or altered course of B19 infection. This is however, controversial (Jones *et al.*, 1999, Searle *et al.*, 1998, Venturoli *et al.*, 1998). Antigenic regions in the NSP have however not been defined in detail. In paper III, we therefore mapped linear NSP immunodominant regions by using overlapping synthetic peptides. Three antigenic regions were identified, (aa 191-206, aa 271-286, aa 371-386) when examining antibody reactivity to the B19 NSP in healthy immunocompetent individuals. Fine mapping of these regions by using sera from the same group of individuals did not reveal any particular region representing the strongest binding site, but seroreactivity was seen against all peptides in varying frequencies. The antigenic NSP regions were further tested against consecutive sera from immunocompetent persistently B19 infected individuals and immunocompromised individuals (HIV-1 infected). The frequencies of seroreactivity in these groups were found comparable to the healthy immunocompetent group. Seroreactivity in the consecutively collected samples from the persistently infected individuals varied over time, with no apparent distinct pattern.

Since no aberrant antibody reactivity pattern was found against the NSP, we compared whether reactivity to neutralising regions in the VPs could discriminate between persistently B19 infected patients and healthy controls. Among the previously published structural antigenic regions (Fridell, 1993, Saikawa *et al.*, 1993, Sato *et al.*, 1991b), three major antigenic sites were identified by screening sera from healthy

immunocompetent individuals: aa16-35 in the B19 VP1, and aa 536-571, aa 718-742 in the B19 VP2. Fine mapping of these regions using sera from the same group of healthy individuals gave higher seroreactivity in fragments aa 16-30, aa 21-35, aa 546-560, and aa 728-742. When the groups of immunocompetent persistently infected and immunocompromised individuals were tested against the seroreactive regions, no significant differences could be seen in seropositivity compared to the healthy immunocompetent group. An exception was found in the carboxy terminal region of VP2 (aa 726-242), where sera from persistently infected individuals exhibited 3% seroreactivity compared to 23% in the healthy immunocompetent group ($p>0.05$). As in the case of NSP antigenic regions, temporal fluctuations of seropositivity could be observed in consecutively collected samples from persistently B19 infected individuals.

In conclusion, our results do not indicate a difference in linear humoral B19 epitope distribution between healthy immunocompetent individuals, immunocompetent individuals with persistent B19 infection and immunocompromised individuals. Furthermore, based on our results the inclusion of NSP antigens in a future B19 vaccine seems unfavourable as no peptide was universally recognised by the different study groups. Our study does not, however, exclude the presence of other antigenic regions in the B19 proteins, since we only studied linear epitopes and since the initial identification of the immunodominant regions were performed with sera from asymptomatic individuals.

– P A P E R V A N D V I

Both cellular and humoral immune responses participate in the defence against a virus. The literature in the subject of B19 cellular immune responses is quite sparse; possibly due to the difficulties in obtaining antigen for various T cell assays, since a model for continuous propagation of B19 in vitro is missing. Furthermore, the initial experiments performed using native B19 antigen failed to demonstrate a cellular response (Kurtzman *et al.*, 1989b). Examination of B19 cellular immune responses could be

important for the explanation of the altered or severe courses of B19 infection discussed above. The classification of persistently B19 infected individuals as immunocompetent is based on the presence of neutralising antibodies to B19 structural viral proteins, and the fact that no general immunodeficiency or history of susceptibility to other infections have been shown (Lundqvist *et al.*, 1999a). Selective inability to mount a cellular cytotoxic response to B19 could however be a possible genesis to disease manifestation in this group of patients, especially if the infection is tissue-localised and poorly cytopathic due to infection of less permissive cells (Zinkernagel *et al.*, 1986). Virus specific cellular immune response should also be addressed in context of B19 associated fetal loss, as a possible co-factor determining the outcome of fetal infection. Speculations about the aetiology to such a specific immunodeficiency include induction of tolerance due to exposure of the virus to an immature immune system, or frequent exposure of the antigen to the immune system.

As a first step to detect and quantify B19 specific cytotoxic T lymphocyte (CTL) responses, we were able to identify an immunodominant CTL epitope in the B19 NSP by screening overlapping peptides of the whole protein to mononuclear cells from a B19 IgG positive / IgM negative healthy donor. This epitope was shown to be HLA B35-restricted, situated at aa 391-399 (sequence QPTRVDQKM). The B19 specificity was ascertained by unlimited BLAST homology search. PBMC restimulated with the optimised peptide demonstrated high specific lysis in two HLA B35 donors (80-90%). CTL responses, as measured by IFN γ ELISpot also showed high reproducible levels of spot forming cells (300 spot forming cells / 10⁶ PBMC), even compared to influenza matrix and Epstein-Barr virus CTL epitopes. By tetramer staining using the same peptide, approximately 0.3% of circulating CD8⁺ lymphocytes demonstrated binding properties, which is in agreement with the ELISpot observations (assuming that 10% of total number of PBMC are CD8⁺ positive). Tetramer binding cells were predominantly CD28^{hi}, CD38^{lo}, CD45RO^{hi}, CD45RA^{lo}, CD56^{lo}, CD57^{lo}, and HLA-DR^{lo}, indicating a resting memory phenotype.

Assessment of the presence of B19 specific T cells were further evaluated in several HLA B35 matched healthy volunteers and immunocompromised (HIV-1 infected patients) in a second study (paper VI). In at least one longitudinal sample, ten out of 16 individuals exhibited frequencies of B19 specific CD8+ cells (range 0.13-0.34%) above the cut off 0.12%, by tetramer staining and 13/18 by B19 antigen (peptide or native) ELISpot, respectively. However, the frequencies of antigen specific fluctuated over time, also below level of detection on some occasions. Functional cytotoxic activity of the B19 specific CD8+ T cells also showed to correlate well to the frequencies of tetramer positive cells. The immunocompromised individuals generally exhibited similar frequencies of B19 specific T cells, but with a lower capacity of IFN γ secretion. This observation could possibly be due to unresponsiveness of the virus specific CTLs after loss of CD4+ cells (Matloubian *et al.*, 1994, Zajac *et al.*, 1998). In conclusion, we have by these initial studies, shown evidence of circulating functional B19 specific cytotoxic T cells in healthy and immunocompromised individuals with past B19 infection, and developed a method suitable for further investigation of B19 cellular immune responses in different clinical manifestations.

Conclusions

- Parvovirus B19 may be more commonly associated with intrauterine fetal death in the late stages of pregnancy than earlier appreciated.
- The majority of parvovirus B19 associated cases of intrauterine fetal death in late stages of pregnancy does not exhibit previously reported characteristics of hydrops fetalis and lack clinical and laboratory signs of acute maternal infection.
- The methods of direct visualisation of parvovirus B19 associated histopathological changes and viral antigens are subjected to low sensitivity in late pregnancy intrauterine fetal death as compared to polymerase chain reaction. Based on our results, we suggest that detection of parvovirus B19 DNA should be included in the routine investigation of intrauterine fetal death.
- The parvovirus B19 genotype does not explain the development of persistent parvovirus B19 infection in neither immunocompetent nor immunocompromised individuals.
- By linear epitope mapping, the anti parvovirus B19 non-structural protein specific antibody reactivity was found conserved among healthy subjects and patients with persistent parvovirus B19 infection.
- An HLA B35 restricted parvovirus B19 specific cytotoxic T cell epitope is contained within the non-structural protein. Approximately 65% of all matched immunocompetent individuals possess functional epitope specific cytotoxic T cells to this epitope but in immunocompromised individuals these cells exhibit lower cytolytic function ex vivo.

Future prospects

- We will continue to analyse factors that which may affect the outcome of B19 infection in pregnancy. Our clinical studies can substantiate evidence of fatal outcome in both the absence and presence of B19 IgG antibodies in different cases. What role does the anti-B19 cellular immune response play? Should local factors in the placenta be searched for? Based on our results it seems less likely for different viral genotypes to be of importance since the virus seems to be very conserved, at least in the patient groups we have examined.
- Our close collaboration with the Departments of Obstetrics and Gynecology at Huddinge University Hospital and Danderyd Hospital, has stimulated us to take part in the modification of the investigation protocol for IUFD, and regarding the investigation for other viral pathogens as well. It will be important to improve the diagnostic and therapeutic management of B19-associated cases of hydrops fetalis, in order to avoid fatal outcome. An initiative for a national protocol regarding B19-associated IUFD and hydrops fetalis, will be established in our ongoing collaboration with the obstetricians.
- As mentioned above, the viral genotype seems not to be important as a determinant for pathogenesis and persistence of infection in immunocompetent and immunosuppressed individuals. Persistent infection seems to be possible also in the presence of a B19 neutralising response. We will therefore concentrate on the less characterised cellular immune response to B19 a) by further mapping of immunodominant cytotoxic and helper T lymphocyte epitopes in the B19 NS and VP regions restricted to common HLA types, and b) by comparing these responses in immunocompetent and immunocompromised persistently B19 infected individuals.

- A vaccine to B19 is currently under evaluation in the US. Hopefully, it will prove effective in certain risk-groups, such as immunosuppressed non-immune children undergoing chemotherapy for various malignancies. We have previously reported that such children are at risk for life-threatening anaemia, and even pancytopenia, due to B19 infection. Our ongoing collaboration with the Department of Haematology and Oncology at Karolinska Hospital, where we evaluate a treatment protocol for B19 infection, may lead to our participation in a future vaccine trial. Furthermore, our characterisation of immunodominant B and T cell sites on B19 may influence the design for future vaccine candidates.

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