T-HELPER CELL IMMUNITY AGAINST HUMAN PARVOVIRUS B19

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Academic Dissertation

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ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by Roman numerals in the text.

I Franssila, R., Söderlund, M., Brown, CS., Spaan, WJ., Seppälä, I., Hedman, K. (1996). IgG subclass response to human parvovirus B19 infection. Clin Diagn Virol 6:41-9.

II Franssila, R., Hokynar, K., Hedman, K. (2001). T-helper cell-mediated in vitro responses of recently and remotely infected subjects to a candidate recombinant vaccine for human parvovirus B19. J Infect Dis 183:805-9.

III Franssila, R., and Hedman, K. (2004). T-helper cell mediated interferon-γ, interleukin-10, and proliferation responses to a candidate recombinant vaccine for human parvovirus B19. Vaccine 22:3809-15.

IV Franssila, R., Auramo, J., Modrow, S., Möbs, M., Oker-Blom, C., Käpylä, P., Söderlund-Venermo, M., and Hedman, K. (2005).T-helper cell-mediated interferon-gamma expression after human parvovirus B19 infection: persisting VP2–Specific and transient VP1u–specific activity. Clin Exp Immunol 142: 53-61.

ABBREVIATIONS

aa	amino acids
AAV	adeno-associated virus
B19	human parvovirus B19
BM	bone marrow
CVB4	coxsackievirus B4
CMV	cytomegalovirus
CPM	counts per minute
DCM	dilated cardiomyopathy
DNA	deoxyribonucleic acid
ds RNA	double-stranded RNA
EBV	Epstein Barr virus
EI	erythema infectiosum
EIA	enzyme immuno assay
ETS	epitope-type specificity
EMB	endomyocardial biopsy
EV	enterovirus
GCA	giant cell arteritis
HHV	human herpesvirus
ICTV	International Committee on Taxonomy of Viruses
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MS	multiple sclerosis
NS	nonstructural protein
nt	nucleotides
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PRCA	pure red cell aplasia
RA	rheumatoid arthritis
RNA	ribonucleic acid
SDS	sodium dodecyle sulphate
SLE	systemic lupus erythematosus
SNPs	single-nucleotide polymorphisms
ssDNA	single-stranded DNA
TAB	temporal artery biopsy
TCR	T cell receptor
TGF	transforming growth factor
TNF	tumor necrosis factor
VP	viral capsid protein

ABSTRACT

The purpose of this study was to characterize the nature of T-helper cell immunity against human parvovirus B19. At the start of this project, little was known about B19 specific cellular responses, and they were thought to be difficult, if not impossible, to study directly. B19 is a small nonenveloped DNA virus with an icosahedral capsid of two protein species, the major viral protein VP2 (58kDa) and the minor viral protein VP1 (83kDa). VP2 is contained within VP1, which has an additional unique portion (VP1u) of 227 amino acids. B19 is a significant human pathogen with clinical manifestations such as fifth disease (erythema infectiosum), joint symptoms, transient aplastic crisis (TAC), fetal hydrops and death during pregnancy and chronic infection. Due to the significant morbidity, recombinant vaccines for the B19 virus are being developed.

In the first phase, B19-specific IgG subclass responses were assessed in recent and remote infection. A restrictively VP1-specific IgG3 to IgG4 subclass shift was found in late convalescent phase. Since the appearance of IgG4 subclass reflects prolonged or repeated antigen exposure, the IgG4 subclass results suggests that B19 proteins should persist in the human body long after infection, or alternatively, reinfection or reactivation of endogenous B19 DNA may be common. Furthermore, as T-cell help is needed for IgG subclass switches, it became obvious that B19-specific cellular responses could be measured.

In later studies, B19-specific Th cell immunity was studied *in vitro* by using recombinantly expressed VP1/2 capsids, VP2-only capsids and VP1u antigen. VP1/2 capsids contained ~33%VP1 and 66%VP2, the ratios recommended for vaccine use. Strong B19-specific Th cell proliferation, IFN-γ and IL10 responses were found in recently infected adults. However, these strong Th cell responses were not confined to recent infection, as we found top responders among remotely B19-infected, healthy subjects. These top responders had B19-specific T cell activity comparable to the B19-specific responses among recently infected patients. IFN-γ turned out to be the dominant B19-specific cytokine in both recent and remote infection; yet B19-specific IL-10 responses were readily detectable among asymptomatic, recently or remotely infected subjects, consistent with a role in restoration of immune system homeostasis upon clearance of infection. However, patients with relapsing or persisting symptoms showed strikingly low IL-10 responses. Pregnancy was found to suppress strongly B19-specific proliferation and IFN-γ responses among both recently and remotely infected subjects; yet interestingly, also B19-specific IL-10 responses were suppressed, in contrast to earlier studies in which mitogen activated PBMC have shown higher IL-10 responses among pregnant than non-pregnant women. This suggests that pregnancy suppresses more strongly recall antigen-specific Th cell responses than mitogen-specific Th cell responses.

No Th cell activity could be located within the unique portion of VP1 (VP1u) in remotely infected subjects, and therefore, it appears that VP2 that contains the epitope(s) capable of inducing vigorous Th cell proliferation, IFN-γ and IL10 responses among remotely B19-infected subjects. Thus, if vaccines based on VP1u alone are used to boost pre-existing B-cell immunity, they may need a fusion partner containing known Th cell epitopes, as Th cells are needed for activation of memory B-cells into IgG secreting plasma cells, particularly if soluble proteins are used as antigens.

Unexpectedly, IFN-γ responses against VP1u turned out to be very strong in recent infection. This phenomenon might be useful in diagnostics of acute B19 infection. At present, it is not known why VP1u –specific Th cell responses are absent in remote immunity, and therefore, future experiments are needed to elucidate the reasons for this profoundly different maintenance of VP1u and VP2-specific Th cell immunity.

REVIEW OF THE LITERATURE

1. CLASSIFICATION AND STRUCTURE

1.1 Taxonomy

The classification of the family *Parvoviridae* relies on morphology and functional characteristics. Parvoviruses are common animal and insect pathogens. Until the recent discovery of the circoviruses and the related TT viruses, parvoviruses were among the the smallest DNA-containing viruses able to infect mammalian cells; hence, the name"parvum" (Latin), meaning small (Berns, 1996). Based on the ability to infect vertebrate or invertebrate cells the *Parvoviridae* are divided into *Parvovirinae* and *Densovirinae*, respectively (Berns, 1996; International Committee on Taxonomy of Viruses, 2000). *Parvovirinae* are subdivided into three genera according to their transcription maps, the nature of the terminal repeats, and the ability to efficiently replicate either autonomously (genus *Parvovirus*), with helper virus (genus *Dependovirus*), or preferentially in erythroid cells (genus *Erythrovirus*) (table 1). Only members of the *Dependovirus* and *Erythrovirus* genera are known to infect humans. So far no dependovirus has been definitively associated with human disease (Berns and Bohenzky, 1987). The members of the genus *Dependovirus*, which includes adeno-associated viruses (AAV) 1 to 9, require coinfection of target cells with adenovirus or herpesvirus for efficient replication. Human parvovirus B19 is autonomous in the sense that it does not require the presence of a helper virus and was, until recently classified in the genus *Parvovirus*. Since replication only occurs in erythrocyte precursors, B19 is now classified as a member of the *Erythrovirus* genus genus. B19 was discovered 1975 by Yvonne Cossart (Cossart and al, 1975), and by the year 2000, was the only accepted member of human erythroviruses (International Committee on Taxonomy of Viruses, 2000). Viruses closely related to B19 have been isolated during the recent years. These new isolates and B19 appear to cause similar diseases in humans (see section 1.5 strain variation). Some of these new isolates are now accepted as members of the *Erythrovirus* genus (table 1). Recently it has been shown that Simian parvovirus (SPV), which is closely related to B19, is able able to infect human bone marrow mononuclear cells (Brown and al, 2004). Whether SPV can cause disease among humans is still unknown (Brown and al, 2004).

Table1. Excerpt of the current classification of the *Parvoviridae* subfamily *Parvovirinae* (Modified from the International Committee on Taxonomy of Viruses database, 2004).

Genus	Virus	Natural host(s)
Parvovirus	*Aleutiann mink disease virus*	Mink, ferret, skunk, raccoon
	Canine parvovirus	Dog
	Mice minute virus	Mouse, rat
	Porcine parvovirus	Pig
Dependovirus	*Adeno-associated virus 1 to 9*	Human
	Avian adeno-associated virus	Birds
	Canine adeno-associated virus	Dog
	Bovine adeno-associated virus	Cow
Erythrovirus	*B19*	Human
	Erythrovirus A6	Human
	Human eythrovirus V9	Human
	Human eythrovirus VX	Human
	Chipmunk parvovirus	Chipmunk
	Simian parvovirus	Cynomolgus monkeys, Human ?
	Pig-tailed macaque parvovirus	Pig-tailed macaques
	Rhesus parvovirus	Rhesus monkeys

1.2 Morphology

The B19 virion has a simple structure composed of only two proteins and a linear, single-stranded DNA. The nonenveloped viral particles are 22 to 24 nm in diameter and show icosahedral symmetry (Berns, 1996). Mature infectious viral particles have a molecular weight of 5.6 x 10^6 and a buoyant density in a cesium chloride gradient of 1.41 g/ml (Brown and al, 1994; Berns, 1996). The B19 virion is an icosahedron consisting of 60 copies of the capsid proteins (Brown and al, 1994), and both positive and negative strands of DNA are packaged (Brown and al, 1994; Berns, 1996). The limited DNA content and the absence of a lipid envelope make B19 extremely resistant to physical inactivation. The virus is stable at 56°C for 60 min and lipid solvents have no effect (Schwarz and al, 1992). Inactivation of virus may be achieved by achieved by formalin, β-propiolactone, and gamma irradiation (Cohen and Brown, 1992).

1.3 Capsid structure

Agbandje and al (1994) determined the structure of empty B19 particles (baculovirus-expressed VP2 capsids) at 8.0 Å resolution. They showed that the central β-barrel structural motif of B19 was essentially in the same position and in a similar orientation as that in FPV with respect to the icosahedral asymmetrical unit. Another electron density region similar to that of FPV was the internal base portion of the cylindrical structures of the 5-fold axes. While the central parvoviral structural motif is maintained in B19, surface features of B19 differ greatly to those of FPV, as B19 lacks the antigenically important, prominent spikes in the threefold icosahedral axis observed in FPV and CPV (Agbandje and al, 1994). Chipman

and al (1996) determined the three-dimensional structure (at 26 Å resolution) of B19 VP2 capsids either alone, or complexed with its cellular receptor, globoside (Brown and al, 1993). A globoside receptor was located to surface depressions on the 3-fold axes of B19 VP2 capsids (Chipman and al, 1996). Kaufmann and al (2004) determined the structure of VP2 capsids at ~ 3.5Å resolution. The polypeptide fold of VP2 is a "jelly roll" with a β-barrel motif similar to that found in many icosahedral viruses. The large loops connecting the strands of the β-barrel form surface features that differentiate B19 from other parvovirus. The recombinant B19 particles were found to be structurally most similar to AAV-2. Finally, the binding site for the common B19 and AAV-2 coreceptor, α5β1 integrin (Weigel-Kelley and al, 2003), was suggested to be situated in the surface loops which are structurally conserved in B19 and AAV-2. These regions correspond to B19 (VP2) amino acids 79-88 and 178-187 (Kaufmann and al, 2004).

1.4 Genomic structure

The single-stranded genome contains 5596 nucleotides (nt), composed of an internal coding sequence of 4830 nt flanked by the terminal repeat sequences of 383 nt each (Deiss and al, 1990). The terminal sequences are palindromic and are capable of assuming hairpin duplex configurations, serving as primers for the synthesis of complementary strands (Astell, 1990). The B19 genome has two large open reading frames (ORFs), with the single nonstructural (NS1) protein encoded by genes on the left side of the genome and the two capsid proteins (VP1 and VP2) on the right side (Ozawa and al, 1987). The VP2 sequence is encoded by sequences from nt 3125 to 4786, and VP1 is encoded by the sequence from nt 2444 to 4786 (Heegaard and Brown, 2002). Transcription produces at least nine overlapping mRNA transcripts, all initiating from the single P6 promoter at the extreme left side of the genome (Ozawa and al, 1987; Deiss and al, 1990). The most important viral proteins include the major nonstructural protein NS1 and the two structural proteins VP1 and VP2 (Ozawa and al, 1987). In addition to the transcripts that encode the NS1,VP1 and VP2 proteins, B19 is unique among *Parvovirinae* in that two classes of small relatively relatively abundant mRNAs are also produced (Ozawa and al, 1987). These two small abundant RNAs belong to two size classes: 700 to 800-nt RNA class terminating in the middle of the B19 genome and 500 to 600-nt RNA class terminating at the far right of the genome. The 700 to 800-nt RNAs express a 7.5-Kda protein whereas the 500 to 600-nt RNA express a family of three 11-Kda proteins (Astell and al, 1997). The function of the 7.5-Kda is not known (Astell and al, 1997), whereas the 11-Kda proteins may pertubate normal cellular signalling pathways by interacting with growth factor binding protein 2 (Fan and al, 2001).

1.5 Strain variation

Genetic analysis of human erythrovirus has so far focused on parvovirus B19 strains. The genetic diversity among B19 virus isolates has been reported to be very low, with less than 1% nucleotide divergence in the full length or nearly full-length sequences of different B19 virus isolates, as recently reviewed by Gallinella (Gallinella and al, 2003). These isolates include the prototype isolates Wi (Blundell and al, 1987), Au (Shade and al, 1986), Stu (Hicks and al, 1996), the reference isolate HV (Gallinella and Venturoli, 1999), the isolates I/1, 2/II, SP2 (Hemauer and al, 1996), the isolate SLE (Hemauer and al, 1998), the isolates N8, Mi, Rm (Ishii and al, 1999), the isolates Kati 1-4 (Hokynar and al, 2000) and the isolate E 1.1 (Tolfvenstam and al, 2001c).

1.6 New variant genotypes of parvovirus B19

Nguyen and al (1999) identified a variant isolate in the serum of a child with aplastic anaemia. With this isolate, defined as V9, the base sequence in the VP1 unigue (VP1u) region was more than 11% divergent from the sequence of other B19 isolates. The detection of this variant isolate prompted the debate whether other B19-related variant isolates could be present in the human population. Subsequently, Nguyen and al (2002) identified and cloned another parvovirus variant, termed A6, from an anaemic HIV-positive patient. This isolate, in turn, exhibited 88% similarity to B19 and 92% to V9 (Nguyen and al, 2002).

Hokynar and al (2002) examined skin biopsies from constitutionally healthy adults or from patients with B19-unrelated skin disease. They detected a new B19 virus genotype, K71, which is persistently carried in human skin, and differs extensively from the B19 reference sequence Au (10,8%) and with the V9 sequence (8,6% divergence) (Hokynar and al, 2002).

In order to identify variant erythroviruses, to evaluate the possible circulation, and to specify the taxonomic groupings of these viruses, Servant and al (2002) analyzed 1084 plasma samples. Altogether 270 samples came from the USA and the rest were from France. A total of 394 samples were found positive in the consensus PCR assay, designed for detection and discrimination of B19 and V9 DNAs. Of these, 385 had a B19-type restriction pattern, while 11 had a V9-type restriction pattern. All the V9-positive samples came from France. The authors concluded that the V9-related viruses circulate at significant frequency (11.4%) in France, and proposed a link between geography and the prevalence of genotype 3. Analysis of the genetic diversity between 12 B19 isolates, two K71 isolates (Lali and HaAM) and two variant isolates from France (V9 and D91.1) was also carried out. A novel division of human erythroviruses into genotype 1 (representing B19 isolates), genotype 2 (prototype strain Lali) and genotype 3 (prototype strain V9) was proposed (Servant and al, 2002).

Hokynar and al (2004) studied the prevalence the three human erythrovirus genotypes (genotypes 1, 2 and 3) in pooled plasma samples obtained from 140,160 Finnish units of donor blood. None of the 480 U pools contained detectable levels of genotypes 2 or 3, whereas up to 17.5% of the plasma pools contained genotype 1 DNA (Hokynar and al, 2004).

In a recent study, Candotti and al (2004) screened the presence of human erythrovirus (genotypes 1, 2 and 3) in 2440 blood donations from United Kingdom and sub-Saharan Africa (Ghana, Malawi and South Africa). The genotype 3 was prevalent in Ghana, whereas genotype 1 was prevalent in United Kingdom, Malawi and South Africa (Candotti and al, 2004).

2. CAPSID AND NONSTRUCTURAL PROTEINS

2.1 Capsids proteins VP1 and VP2

Human parvovirus B19 is a small nonenveloped DNA virus with an icosahedral capsid of two protein species, the major viral protein VP2 (58kDa) and the minor viral protein VP1 (83kDa). VP2 is contained within VP1, which has an additional unique portion (VP1u) of 227 amino acids (Ozawa and Young, 1987; Ozawa and al, 1987). Most of the capsid protein is VP2, with only ~ 5% VP1 (Kajigaya and al, 1989). This low expression of VP1 is caused by minor synthesis VP1 transcripts (Ozawa and al, 1987), and by minor translation, as VP1 mRNA contains an upstream AUG rich region which downregulates its translation (Ozawa and al, 1988).

The major structural protein VP2 contains 554 amino acids, whereas the minor structural protein VP1 contains 781 amino acids (Astell and al, 1997). In mammalian and insect cells, expression of VP2 can self assemble in the absence of viral DNA to produce virus-like particles (VLP) that are physically, antigenically and immunologically similar to native virions (Kajigaya and al, 1989; Kajigaya and al, 1991; Brown and al, 1991). Deletion of more than 30 N-terminal amino terminal acids of VP2 has been shown to disable capsid formation (Kawase and al, 1995). Although VP1 alone cannot form capsids (Kajigaya and al, 1991), the presense of VP1u moieties in B19 capsids is extremely important for the B19 virus immunogenicity and infectivity. First, the presence of VP1u moieties in a B19 capsid reveal neutralizing determinants resident on VP2, whereas they are not visible to the immune system in a VP2-only capsid (Bansal and al, 1993; Rosenfeld and al, 1994). Second, a phospholipase A2 motif has been identified in the VP1u region of several parvoviruses, including B19 (Zadori and al, 2001; Dorsch and al, 2002). In a porcine parvovirus (PPV) model VP1u-mediated phospolipase A2 activity was essential for the transfer of viral genome from perinuclear vesicles into the nucleus (Zadori and al, 2001). Dorsch and al (2002) showed that the presence of VP1 in recombinantly expressed B19 capsids is necessary for phospholoipase A2 activity. They suggested that the enzymatic activity of VP1u might be necessary for viral entry, nuclear transport and virus release (Dorsch and al, 2002).

2.2 Structural proteins of human erythrovirus genotypes 2 and 3

Most extensive intergenotypic variations (compared with 12 B19 isolates) at the amino acid level are found in the VP1u region: 3.8-4.7% with genotype 2, and 6.4-8.7% with genotype 3. Intergenotypic variations in the VP2 region are considerably smaller, 1.0-2.1% with genotype 2 and 1.0-2.3% with genotype 3 (Servant and al, 2002). The extensive divergence of the VP1u region may be clinically important, as important neutralizing epitopes are located in this area (see chapter 9). The high degree of homology of the VP2 region, on the other hand, is though to make B19 VP2-based antibody assays also suitable for serodiagnosis of genotypes 2 and 3 (Servant and al, 2002).

2.3 Nonstructural protein NS1

In the left side of the B19 genome is the ORF for a 77 Kda nonstructural protein NS1 (Ozawa and al, 1987). NS1 has been found to be essential for replication of viral DNA, and for the regulation of its own promoter (Brown and al, 1994). NS1 induces apoptosis in erythroid

lineage cells (Moffat and al, 1988; Yaegashi and al, 1999). It also possesses DNA-binding properties (Raab and al, 2002) and biochemical activities, such as ATPase, helicase, site-specific endonuclease activities, and nuclear localization signals, as reviewed by Corcoran and al 2004.

2.4 NS1 proteins of human erythrovirus genotypes 2 and 3

Intergenotypic variations (compared with 12 B19 isolates) at the amino acid level of the NS1 protein are 5.9-6.4% with genotype 2, and 5.1-6.2% with genotype 3 (Servant and al, 2002). Importantly, with the dermal erythrovirus isolate K71 (genotype 2), the most extensive DNA divergence (26.5%) was found in the p6 promoter region (Hokynar and al, 2002). Whether the divergent NS1 proteins and p6 promoters have a role in tissue-type specifity, host cell type, and disease associations of the new erythrovirus is not known.

3. B19 TARGET CELLS

Autonomous parvoviruses have a near-absolute requirement for rapidly dividing cell populations in the S phase of the cell cycle. Identification of targets of virus infection can often explain the precise pathogenesis (Bloom and Young, 2001). B19, now classified as an erythrovirus, shows a remarkable tropism for human erythroid progentitor cells. B19 has been shown to replicate in late erythroid progenitor cells and in burst forming erythroid progenitors (Mortimer and al, 1983). Erythroid progenitor cells from human bone marrow, fetal liver, erythroleukaemia, chronic myelogenous leukaemia and normal peripheral blood all support replication of B19 when cultured in the presence of erythropoietin (Astell and al, 1997). Besides the need of rapidly dividing target cells, factors causing the narrow tropism of productive B19 infection are:

(1) The restricted presence of the B19 cellular receptors, the blood group P antigen and the coreceptors α5β1 integrin and Ku80 autoantigen. Blood group P antigen (synonyms: globoside, globotetraosylceramide, Gb4), a neutral glycosphingolipid, is the principal receptor for parvovirus B19 (Brown and al, 1993; Brown and al, 1994b) mediating cellular binding (Weigel-Kelley and al, 2001), whereas viral entry is mediated by the coreceptors α5β1 integrin (Weigel-Kelley and al, 2003; Munakata and al, 2005). Recently, the Ku80 autoantigen has been shown to be an important coreceptor for cellular binding. This autoantigen is expressed in erythroblasts, macrophages, T cells and B cells in bone marrow, but not in circulating mononuclear cells, possibly explaining the clinical manifestations associated with non-erythroid cells (Munakata and al, 2005).

(2) Restriction of putative intracellular factors largely to human erythroid cells. These factors are reguired for optimal transcriptional activation of the B19 p6 promoter and viral replication (Ozawa and al, 1987; Takahashi and al, 1990; Gallinella and al, 2000).

(3) Reduced B19 capsid protein expression in nonpermissive cells due to a block in full-length transcription of the viral genome, atypical mRNA splicing, and impaired ribosome loading of structural gene transcripts (Liu and al, 1992; Pallier and al, 1997; Brunstein and al, 2000).

(4) Hypoxic conditions. Hypoxia has been shown to enhance B19 gene expression. The precise mechanism in the oxygen-sensitive upregulation of B19 gene expression is not known, but upregulated expression of hypoxia-inducible factor-1 (HIF-1) may have a role. This transcription factor is involved in cellular response to hypoxia, and it has been shown to bind to B19 promoter region (Pillet and al, 2005). Finally, hypoxia may also increase B19 binding to target cells by upregulating cell surface expression of Ku80 (Lynch and al, 2001), which is a B19 coreceptor (Munakata and al, 2005).

B19 has been found to infect cell types other than erythroid progenitors, at least occasionally. Viral mRNA of structural proteins has been detected in the livers from patients with B19-associated hepatitis (Karetnyi and al, 1999) and B19 capsid proteins have been detected in synovial lymphocytes from patients with rheumatoid arthritis (Takahashi and al, 1998; Mehraein and al, 2002). Replication within vascular endothelial cells has been suggested by detecting B19-RNA and DNA *in situ* (Magro and al, 2002; Cioc and al, 2002; Bültmann and al, 2003). However, in a recent study, no significant increase of B19-DNA and mRNA levels were found in endothelial cells after *in vitro* infection, suggesting that productive infection may not occur in these cells, or alternatively, yet unknown growth factors and cytokines may be necessary to make endothelial cells permissive for B19 (Zakrzewska and al, 2005).

4. PERSISTENCE OF B19 DNA IN HEALTHY HUMANS

By detecting DNA, B19 has been proposed to be associated with various diseases (see chapter 6). However, accumulating data shows that B19 DNA can persist in various tissues or cell types among apparently healthy individuals:

4.1 B19 DNA in bone marrow (BM)
Heegaar and al. detected B19 DNA in 4/190 (2.1%) BM samples from healthy individuals, whereas higher prevalences were reported by Cassinotti and al. and by Eis-Hübinger and al: 4/45 (9%) and 3/17 (17.6%), respectively (Cassinotti and al, 1997; Eis-Hübinger and al, 2001; Heegaard and al, 2002).

4.2 B19 DNA in synovial tissue
B19 DNA also tends to persist in synovial tissue; In a pioneering study Söderlund and colleagues found B19 DNA in 13/27 (48%) synovial tissue samples from healthy seropositive subjects (Söderlund and al. 1997), whereas a smaller prevalence was later reported by Cassinotti and al, when they found B19 DNA in only 1/9 (11%) of such synovial tissue samples (Cassinotti and al. 1998). Latest reports suggest that the prevalence of B19 DNA in synovial tissues of healthy seropositive subjects is likely closer to 50% than to 10%, as prevalences of 67% (12/18) and 50% (12/24) have been reported (Hokynar and al, 2000; Peterlana and al, 2003). Importantly, B19 DNA appears to persist as its full size in synovial tissue (Hokynar and al, 2000).

4.3 B19 DNA in liver tissue
Persistence of B19 DNA in liver appears to be also common, as it has been detected in 4/17 (24%) of livers from randomly selected, B19 seropositive autopsy patients (Eis-Hübinger and al, 2001).

4.4 B19 DNA in skin
B19 DNA was first detected in the skin of patients with erythema infectiosum (Schwarz and al, 1994). Vuorinen and al. studied patients with chronic urticaria, with other skin manifestations and healthy controls, and found B19 DNA in 18/36 (50%), 11/32 (34%) and 14/22 (64%) of skin samples, respectively. The authors concluded that whereas the association with chronic urticaria and B19 remains uncertain, skin may constitute a reservoir for B19 (Vuorinen and al, 2002). Other studies have later confirmed the findings of Vuorinen and colleagues: Ohtsuka and Yamazaki found B19 DNA in 51/97 (53%) of skin tissue samples from normal donors. However, the prevalence of B19 DNA was increased to 36/48 (75%) in patients with systemic sclerosis, and the authors suggested that B19 might be associated with the formation of skin tissue abnormalities in the disease (Ohtsuka and Yamazaki, 2004). Hokynar and al. found 14/19 (74%) of skin samples from seropositive subjects to be VP1-DNA positive, but interestingly, only 5/14 VP1-positive samples gave expected results in conventional B19 NS1- and VP2-PCRs. Sequence analysis of samples which were only VP1 positive revealed a new parvovirus genotype, termed K71, to be persistently carried in human skin (Hokynar and al, 2002). Whether this new genotype is infectious or replication deficient remains to be seen.

4.5 Mechanisms of persistence
At present it is not known how and why B19 genome persists in human tissues. Furthermore, it is not known whether the persistent genome is reactivable or does the B19 DNA persist as a harmless “innocent bystander”. Different persistence strategies are known to be used by other parvoviruses, and possibly, some of these strategies might also be operative with B19.
The closely related adeno-associated viruses (AAV) can integrate site-specifically in human chromosome-19, and remain latent until activated by a helper virus, such as adenovirus (McCarty and al, 2004). Recent data obtained with patients with myocardial diseases suggest that other viruses might also have influence on the pathogenetic potential of B19 (see chapter 6.11).
Some autonomous animal parvoviruses, such as rodent parvoviruses and Aleutian disease virus (ADV) of mink, can establish persisting infection in their hosts (Siegl, 1984; Tattersall and Cotmore, 1986; Christensen and al, 1993). The ability of ADV to cause persistent infection has been suggested to be linked with the weak activities of ADV promoters (Christensen and al, 1993). Interestingly, in ADV infected cells, transcripts encoding non-structural proteins are predominant (Stoorgaard and al, 1997), as is the case with B19 in non-permissive cells (Liu and al, 1992).
Finally, it has been proposed that B19 DNA could retain in the synovium as whole, unprocessed virions on the surface of follicular dendritic cells (Hokynar and al, 2000). Further studies are needed to clarify in detail the mechanisms of B19 persistence, whether it is active (i.e. expresses mRNA), and whether it integrates into the human chromosomes like AAVs, or exist in an episomal form like herpes simplex viruses (Söderlund-Venermo and al, 2002). If the persistent B19 DNA is not active, the most important issue will be whether the

latent B19 infection can be reactivated under any conditions, such as stress, immunosuppression or co-infections by other viruses.

5. EPIDEMIOLOGY

5.1 Prevalence and incidence

Parvovirus B19 is a common infection in humans. By the age of 15 approximately 50% of children have detectable IgG to B19. Infection occurs throughout adult life, so that 80% of the elderly are seropositive (Cohen and Buckley, 1988). Women of child-bearing age show an annual seroconversion rate of 1.5% (Koch and Adler, 1989). Although antibody is prevalent in the general population, viremia is rare. The frequency of B19 viremia in voluntary blood donors has been estimated from 0.003% (Tsujimura and al, 1995) to ~1.0% (Candotti and al, 2004; Lefrère and al, 2005).

5.2 Transmission

Transmission of infection occurs via the respiratory route, through blood products administered parenterally, and vertically from mother to fetus (Heegaard and Brown, 2002). B19 DNA has been found in the respiratory secretions of patients at the time of viremia (Anderson and al, 1985; Chorba and al, 1986), suggesting that B19 infection is generally transmitted by the respiratory route. The transplacental transmission rates during maternal infection have been estimated to be 33-51% (PHLS, 1990; Yaegashi, 2000). B19 infection can be transmitted by blood and blood products. Even after the introduction of virus-inactivated clotting factor concentrates, a ~ 90% prevalence of B19 IgG has been detected among hemophiliacs, which correlated to the amount of clotting factors received (Rollag and al, 1991; Azzi and al, 1999). B19 may also be transmitted by bone marrow (Heegaard and Laub, 2000) and by solid organ transplants (Marchand and al, 1999).

6. DISEASE MANIFESTATIONS

6.1 Pathogenesis of B19-associated disease

The pathogenesis of B19 virus infection is complex, particularly when the less common clinical manifestations/associations are included, and a combination of several pathogenetic mechanisms, including chronic infection in immunocompetent subjects, may come into play (table 2).

6.2 Erythema infectiosum (EI)

About 25-68% cases of parvovirus B19 infection are asymptomatic (Woolf and al, 1989; Noyola and al, 2004). The most common clinical manifestation is erythema infectiosum, or fifth disease, a childhood exanthema characterized by a “slapped cheek” rash. Intranasal inoculation of normal volunteers has produced fifth disease, and the experimental setting has allowed detailed correlation of clinical manifestations with virological and immunological events (Anderson and al, 1985): Fever and nonspecific influenza-like symptoms occurred one

week after inoculation, during the phase of parvoviremia which was observed six to 14 d pi by dot-blot hybridization. Viral excretion in the upper respiratory tract was detected seven to 11 d pi.Two weeks after infection cutaneus eruption and rheumatoid symptoms occured, corresponding to the appearance of antiviral antibodies and disappearance of viremia by dot blot. The second phase lasted up to 24 days after inoculation, after which no recurrence of symptoms occurred (Anderson and al, 1985). However, the less sensitive PCR technique (see chapter 7.3) used by Anderson and al. propably underestimated the true duration of B19 viremia, since in a recent study using modern PCR, B19 DNA was detectable >77 weeks among immunocompetent patients with primary B19 infection (Lindblom and al, 2005).
The pathogenesis of EI has classically been explained to be mediated by the formation and deposition of immune complexes in the skin and elsewhere (Frickhofen and al, 1990; Kerr, 2000; Young and Brown, 2004; Corcoran and Doyle, 2004). This hypothesis is supported by the fact that symptoms of EI manifest simultaneously with the appearance of specific antibody responses (Anderson and al, 1985), or in some cases, after intravenous infusion of immunoglobulins (Kurtzman and al, 1988; Frickhofen and al, 1990) in patients with chronic B19 infection with absent antibody responses (see chronic infection). However, it appears obvious that other factors than the formation of immune complexes must have a significant contribution to the pathogenesis of EI. First, most cases of B19-infection are asymptomatic and B19 associated joint symptoms are age- and sex related (see below). Second, rash is not always accompanied with immunoglobulin infusions (Kurzman and al, 1989a; Frickhofen and al, 1990; Griffin and al, 1991). Third, reticular rash has been observed in chronically B19 infected patients with absent virus-specific antibodies, suggesting that rash might be a consequence of a direct virus effect on the skin (Schwarz and al, 1994; Schleuning and al, 1999). Fourth, inherent variability in cytokine responses may have a bearing on the symptomatology of B19 infection. The presence of the +869T allele of transforming growth factor β1 (TGFβ1) has been shown to predispose for the skin rash during the acute phase of B19 infection (Kerr and al, 2003). However, as this allele has been associated both with high levels (Awad and al, 1988; Perrey and al, 1988) and low levels (Yamada and al, 2001; Suthanthiran and al, 2000) of transcriptional activity, it is currently unknown how this allele might affect to clinical picture. Cell mediated immunity appears to have importance in the clinical picture of B19 infection, since *lower* serum levels of TNF-α, IL-6, and GM-CSF cytokines have been detected in patients with B19 infection and arthritis than in patients without it (Kerr and al, 2004), and human leucocyte antigens HLA-DRB1*01, *04, *07 and HLA-B49 alleles have been shown to associate with symptomatic B19 infection (Kerr and al, 2002). Finally, genes not directly linked with immune responses may predispose to symptomatic B19 infection, since single nucleotide polymorphisms (SNPs) in genes linked to apoptosis, cell cycle and growth, and function of cytoskeleton or chromatin, were found to be associated with symptomatic B19 infection (Kerr and al, 2005). Furthermore, B19 infection may have a long-term effect on the function of several genes, since higher levels of mRNAs of genes performing functions in the cytoskeleton, in integrin signalling, in HLA class III and in tumor suppression were found in remotely B19 infected subjects than in healthy controls. The mechanism of this altered genetic activity is not known, but possible explanations might be a prolonged immune response or persistent B19 infection (Kerr and al, 2005).

Table 2. Pathogenetic mechanisms known or proposed to account for various clinical syndromes associated with parvovirus B19 infection. Modified from Kerr, 2000.

	Local viral replication†	NS1 cytotoxicity*	Immune complex deposition#	Erythroblast apoptosis§	Autoantibody production¶	Cytokine upregulation++	Persistence∞
EI	+	+	+	+		+	
Arthropathy		?	+		?	?	?
Hydrops fetalis/fetal death	+			+			
Chronic PRCA	+			+			+
TAC	+			+			
Chronic infection with cytopenias	+			+	+	+	+
Hepatitis	+						?
Encephalopathy/meningitis	+					+	+
CFS						+	+
RA	+				+	+	+
SLE	+				+	?	+
Vasculitis	+		?		?	?	+
Myocarditis	+				?	+	+

TAC: transient aplastic crisis, EI: erythema infectiosum, PRCA: pured red cell aplasia, CFS: chronic fatigue syndrome, RA: rheumatoid arthritis, SLE: systemic lupus erythematosus.

†Local B19 replication occurs primarily in the erythroblast, but also occurs in macrophages, myeloid cells, lymphocytes, hepatocytes, fetal myocardial cells, and dendritic cells. Replication of B19 in endothelial cells may be important in myocarditis in adults.

*B19 NS1 cytotoxicity is thought to account for haematological abnormalities in EI and cytopenias and possibly for arthralgia/arthritis.

Immune complex deposition is thought to account for the rash of EI, arthralgia, peripheral neuropathy, and may contribute to other B19 associated skin rashes and vasculitides.

§ Erythroblast apoptosis, mediated by the NS1 protein, occurs in the TAC, EI, and hydrops fetalis and probably also in chronic anaemias/cytopenias.

¶ Autoantibodies may be significant in the pathogenesis of arthritis/RA, cytopenias and SLE. Antiphospolipid antibodies occuring after B19 may be important in the pathogenesis of symptomptoms which mimic SLE. For further information: **see table 3**.

++ Upregulation of human IL6 and/or TNF-α, mediated by NS1 protein, may be important in aplastic anaemia/cytopenias and B19 associated RA; possibly also in B19 associated skin rashes, and B19 associated CFS. The level of expression of TGFβ may have importance in the development of skin rash. Cytokines may also have importance in B19 associated myocariditis and meningitis.

∞ Persistence of B19 is important in PRCA, may be important in B19 associated skin rashes, arthritis, CFS, RA, SLE, vasculitides, cardiomyopathy and encephalitis/meningitis.

6.3 Arthropathy

On average 50% of adults (Woolf and al, 1989) and 10% of children (Heegaard and Brown, 2002) with EI have joint manifestations. B19 arthritis is usually symmetrical, affecting mainly the small joints of the hands, wrists and knees (Reid and al, 1985). It is more common in females than in males, since ~ 60% of females and 30% of males with symptomatic disease have joint manifestations (Joseph, 1986; Woolf and al, 1989). Symptoms usually subside within 3 weeks without any damage to the joints, although in 20% or more of affected women, arthropathy may persist for months (Woolf and al, 1989). In those with prolonged symptoms there is no corresponding increase in the amount or duration of anti-B19 IgM. Arthralgia may also occur without the rash (Kerr, 2000).

6.4 Rheumatoid arthritis (RA) after B19 infection

Evidence for a role in pathogenesis

RA is a chronic disorder of the joints in which the synovium becomes hyperplastic, secondary to proliferation of the lining layer and infiltration by inflammatory cells (van Boxel and Paget, 1975). Its pathogenesis is unknown but linkage to the class II region of the MHC, the presence of circulating autoantibodies, and response to some T-cell targeted therapies, suggest an autoimmune contribution (Fox, 1997). Some evidence suggests that B19 could have a role in the pathogenesis of RA. B19 arthritis often meets the clinical diagnostic criteria for rheumatoid arthritis (RA) (White and al, 1985; Naides and al, 1990), can be erosive (Cohen and al, 1986; Tyndall and al, 1994), is sometimes accompanied, at least transiently, by the development of rheumatoid factor (table 3) and B19 DNA may be detected in synovial fluid (Dijkmans and al, 1988), cells (Kandolf and al, 1989) and tissue (Saal and al, 1992) of affected joints. B19 structural proteins have been detected in synovial lymphocytes in patients with RA (Takahashi and al, 1998; Mehraein and al, 2002). The RA associated HLA-DR4 antigen was present in 12 of 18 (67%) patients with B19 associated arthropathy in one study (Klouda and al, 1986), but this association was not borne out in other studies (Dykmans and al, 1986; Woolf and al, 1986; Gendi and al, 1996, Kerr and al, 2002). Incubation of normal human synovial fibroblast cells with B19-containing sera has been shown to induce invasive properties (for cartilage memrane) in these cells (Ray and al, 2001).

Mice that were naturally resistant to collagen II induced arthritis (resembling human RA), developed arthropathy after collagen II immunization following introduction of B19 NS1 gene, which was isolated from synovial tissue from a human patient with RA (Takasawa and al, 2004). B19 infection of macrophage cell line U937 or bone marrow cells from normal donors has led to increased secretion of interleukin-6 (IL-6) and tumor necrosis factor α (TNF−α) cytokines by these cells (Takahashi and al. 1998). This increased cytokine secretion is mediated by B19 non-structural protein (NS1), which is able to activate IL-6 (Moffat and al, 1996) and TNF-α (Fu and al, 2002) promoters. Overproduction of IL-6 is thought to be important in the pathogenesis of RA, since it is responsible for the activation of autoreactive T cells and the appearance of autoantibodies (including rheumatoid factor). Furthermore, it induces acute-phase proteins, such as C-reactive protein (CRP) synthesis in the liver and is involved in bone destruction by activation of osteoclast precursors (Nishimoto and Kishimoto, 2004). TNF−α, in turn, has a primary importance in rheumatoid arthritis, since it regulates IL-6 secretion (Feldman and Maini, 2001), and stimulates fibroblasts, chondrocytes, and osteoclasts to produce matrix metalloproteinases which destroy joint tissue (Shingu and

al, 1993). Importantly, administration of inhibitors of TNF−α and its action protect the majority of patients from joint damage (Feldman and Maini, 2001). Finally, Lunardi and al have shown that in patients with RA and chronic B19 arthritis, anti- VP2 IgG specific for a linear epitope spanning VP2 aa 292-301, is cross reactive with autoantigens such as collagen II (table 3). As type II collagen is a target antigen of autoantibodies (Rowley and al, 1986; Lettesjö and al, 1988) and clonally expanded T cells in the RA synovium (Alam and al, 1996; Snowden, 1997), this finding has been suggested to have a considerable significance in the proposed link between B19 infection and RA (Kerr, 2000).

Evidence against a role in pathogenesis of RA

There is also significant evidence that B19-infections are ***not*** associated with RA: 1) In patients with RA the seroprevalence of B19 IgG is within the expected range for the age group tested and considerably less than 100%, indicating that many RA patients are unlikely to have been previously infected by B19 (Török, 1997), assuming that B19-infection invariably leads to seroconversion and B19-IgG persists for life (see chapter "antibody response"). 2) inflammatory arthritis was not seen in long term follow up (median 5 years) among 54 patients, all of whom had arthalgia in the acute phase (Speyer and al, 1998). 3) B19 DNA has been shown to persist in synovial membranes of healthy adult donors at similar or higher frequencies than in synovial membranes of patients with rheumatoid arthritis (see chapter 4, Söderlund and al, 1997; Peterlana and al, 2003). 4) Whereas young patients with rheumatic disease may be chronically viremic at significantly higher ($p< 0.0001$) frequency than their age-matched healthy controls (Lehmann and al, 2002), the prevalence of B19 viremia was similar in adult patients with RA (33.3%) than in their age-matched controls (20.8%) (Peterlana and al, 2003). 5) As B19 is a ubiquitous pathogen and RA is fairly common (prevalence 1%), simple coincidental occurrences of both are probable common (Török, 1997).

6.5 Systemic lupus erythematosus (SLE)

SLE is a multisystem inflammatory disease of unknown etiology, which is associated with production of autoantibodies reactive with nuclear, sytoplasmic and cell membrane antigens (Steinberg, 1992). Many prominent features of both B19 infection and SLE overlap (fever, rash, arthralgia, cytopenias, anemia, hepatitis and antinuclear antibody) (Soloninka and al, 1989; Kalish and al, 1992; Nesher and al, 1995). Autoantibodies and their targets encountered with B19 infection are shown in table table 3. B19 specific IgG recognizing a linear epitope spanning VP2 aa 292-301 has been shown to be cross-reactive with autoantigens such as keratin, collagen II, thyreoglobulin, DNA and cardiolipin (table 3).

Antiphospholipid antibodies produced during acute B19 infection seem to have the same specificity as those produced in SLE (Loizou and al, 1997). Their synthesis may be mediated by the phospolipase A_2-like activity by the VP1u region of the structural protein VP1, as it may generate unnatural gleavage products from cellular compounds, and therefore, induce antiphospholipid antibodies in subjects with predisposing genetic background (von Landenberg al al, 2003).

Besides mimicking SLE, acute B19 infection has been shown to be associated with induction or exacerbation of SLE (Chassagne and al, 1993; Vigeant and al, 1994; Fawaz-Estrup, 1995; Tanaka and al, 1998; Hemauer and al, 1999; Chou and al, 2000; Von Landenberg and al, 2003; Magro and al, 2002), and parvovirus infection of endothelial cells may have a role in a SLE-like disease manifested after B19 infection (Magro and al, 2002). However, as B19 seroprevalence was not elevated in 99 patients with SLE compared with 99 age and sex-matched controls (Bengtsson and al, 2000), it seems likely that B19 may mimic or precipitate SLE only in a minority of cases.

Table 3. Autoantibodies found in B19 infection

Autoantibody	References
Rheumatoid factor	Luzzi and al, 1985; Naides and al, 1988; Sasaki and al, 1989; Sasaki and al, 1995; Kerr and Boyd, 1995; Hansen and al, 1998
Anti-cardiolipin	Fawaz-Estrup, 1996; Hansen and al, 1998; Lunardi and al, 1998; Chou and al, 2000
Antinuclear (ANA)	Sasaki and al, 1989; Kerr and Boyd, 1995; Nesher and al, 1995; Fawaz-Estrup, 1996; Hansen and al, 1998; Tanaka and al, 1998; Lunardi and al, 1998; Crowson and al, 2000
Anti-DNA	Soloninka and al, 1989; Vigeant and al, 1994; Nesher and al, 1995; Fawaz-Estrup, 1996; Hansen and al, 1998
Anti neutrophil cytoplasmic antibody (ANCA)	Crowson and al, 2000; Chou and al, 2000; Hermann and al, 2005
Antiphospholipid	Loizou and al, 1997; von Landenberg and al, 2003
Antireticulin	Kerr and Boyd, 1995
antimitochondrial	Kerr and Boyd, 1995
Anti-smooth muscle	Kerr and Boyd, 1995
Anti-keratin	Lunardi and al, 1998.
Anti-collagen II	Lunardi and al, 1998.
Anti-thrombocyte	Toyoshige and Takahashi, 1988; Murray and al, 1994; Scheurlen and al, 2001
Anti-leukocyte	Soloninka and al, 1989; Hanada and al, 1989; Mc Clain and al, 1993; Murray and Morad, 1994

6.6 Complications in pregnancy

The B19-specific seroconversion incidence in susceptible women may rise from 1.0 to 1.5% during endemic to 13to 13.5% during epidemic periods (Koch and Adler, 1989; Valeur-Jensen and al, 1999; Jensen and al, 2000). The transplacental transmission rates during maternal infection have been estimated to 33-51% (PHLS, 1990; Yaegashi, 2000). Fetal B19 infection may be asymptomatic but it may also result in fetal hydrops and death (Brown and al, 1984; Anand and al, 1987; Kinney and al, 1988; Tolfvenstam and al, 2001). Fetal B19 infection may also cause fetal or congenital anaemia or stillbirth (Heegaard and Brown, 2002). Only rare cases of fetal anomalies or malformations have been associated with B19 infection. These include liver siderosis, ocular malformations, and central nervous system abnormalities (Van Elsacker-Niele and al, 1989; White and al, 1995; Katz and al, 1996). The chance of an adverse fetal outcome after infection seems to be greatest between 11 and 20 weeks of gestation (Yaegashi and al, 1998; Enders and al, 2004), which correlates with the hepatic period of hematopoietic activity (Yeagashi and al, 1998). At this time point, the life-span of red blood cells is only 50-75 days, compared with the 120 days in adults (Chisaka and al, 2004), and red blood cell mass increases three- to fourfold (Rodis and al, 1988). As the fetal immune system is immature, infection may not be overcome (Eis-Hübinger and al, 1998), and the B19-infected fetuses may subsequently develop severe anaemia and high-output cardiac failure, leading to potentially lethal hydrops fetalis (Wright and al, 1996; Forestier and al, 1999; Heegaard and Brown, 2002). Impaired circulation due to fetal myocarditis may contribute to the accumulation of fluids (Morey and al, 1992). Hydrops may resolve spontaneously with a delivery of a healthy newborn (Pryde and al, 1992; Enders and al, 2004). However, in severe hydrops fetal survival is impaired, but risk of fetal death can be reduced by intrauterine blood transfusions (Fairley and al, 1995; Enders and al, 2004). Hydrops fetalis has also been successfully treated with intravenous gammaglobulin (Selbing and al, 1995).

Fetal deaths that present in the first 16 weeks of gestation may be unaccompanied by signs of hydrops fetalis, possibly because small fetuses may succumb to severe anaemia relatively quickly (Enders and al, 2004) and they may have more intense immune responses with proinflamatory cytokines, such as TNF-α and IFN-γ (Enders and al, 2004). These cytokines are known to be harmful to the conceptus (Raghupathy, 2001). B19-associated fetal deaths in late gestation without hydropic presentation have also been described (Norbeck and al, 2002). However, fetal deaths of this type were not found in a large prospective study of 1018 pregnant women with serologically confirmed B19 infection (Enders and al, 2004). In this study, B19 infection occurred by gestation week 20 in 579 women and later in 439 women. All 64 fetal deaths among 1018 pregnant women occurred when infection occurred before completed gestation week 20 (fetal death rate 11%), whereas none of the 439 women infected after gestation week 20 suffered a fetal loss (Enders and al, 2004).

6.7 Transient aplastic crisis (TAC)

Aplastic crisis is a severe anemia with abrupt onset and absence of reticulocytes. This condition was first described in patients with hereditary spherocytosis (Owren 1948). TAC occurs as a unique episode in the patient's life, and frequently there is a preceding prodromal illness (Brown, 1997). TAC was the first clinical illness associated with B19 infection (Pattison and al, 1981). As B19-infection temporarily suppresses erythropoesis (Anderson and al, 1985), life-threating aplastic crisis may occur in patients with shortened red cell survival (Saarinen and al, 1986; Goldstein and al, 1987). However, this condition

is transient and has an excellent prognosis if diagnosed promptly and managed appropriately with blood transfusions (Saarinen and al, 1986; Goldstein and al, 1987).

6.8 Chronic pure red cell aplasia (PRCA)

Patients with wide variety of conditions of immunosuppression, such as HIV infection (Frickhofen and al, 1990; Griffin and al, 1991), congenital immunodeficiency (Kurtzman and al, 1989 a and b ; Gahr and al, 1990; Seyama, 1998) or immunodeficiency secondary to immunosuppressive or cytotoxic drugs (Kurzman and al, 1988; Koch and al, 1990; Marchand and al, 1999; Geetha and al, 2000; Cavallo and al, 2003) may not be able to clear B19 from bone marrow. Persistent B19 infection with congenital anaemia may also develop after transplacental infection (Brown and al, 1994c). The typical manifestation of chronic B19 infection among immunocompromised patients is chronic pure red cell aplastic anaemia or PRCA (Kurzman and al, 1989b; Frickhofen and al, 1990; Gahr and al, 1990; Brown, 1997). Chronic B19 infection is only one cause of PRCA, as there is a congenital form linked to genetics defects affecting erythropoeisis, and acquired forms linked to (1) immunologic interactions, (2) myelodysplasia and (3) viral infections such as B19 (Chisaka and al, 2003). Importantly, anemias caused by chronic B19 infection may be cured or ameliorated by infusions of virus-neutralizing immunoglobulins (Kurzman and al, 1989 a and b; Frickhofen and al, 1990; Koch and al, 1990; Seyama and al, 1998; Marchand and al, 1999), however, relapses of anemia may occur (Frickhofen and al, 1990; Moudgil and al, 1997; Koduri and al, 1999), and infection may persist despite treatment in heavily immunosuppressed transplant patients (Moudgil and al, 1997; Schleuning and al, 1999; Lui and al, 2001). For the rationale of immunoglobulin treatment, see chapter 9. Resolution of anemia has also been achieved by temporary cessation of maintenance chemotherapy, or by alleviation of immunosuppression (Smith and al, 1988; Geetha and al, 2000). Finally, PRCA cases were not found in chronically B19 infected HIV-patients, whose immunosuppression were not yet severe (La Monte and al, 2004), and on the other hand, few cases of PRCA after B19 infection have been reported in immunocompetent patients (Frickhofen and al, 1994; Lugassy, 2002).

6.9 Thrombocytopenia

B19 infection may result in subclinical or overt thrombocytopenia in (Anderson and al, 1985; Potter and al, 1987; Lefrere and Got, 1987; Yoto and al, 1993; Murray and al, 1994). Clinically significant thrombocytopenia may be of central origin be due to BM suppression (Nagai and al, 1992; Sristava and al, 1997; Bhattacharyya, 2004) or of peripheral origin (due to excessive consumption of platelets).
Central thrombocytopenia may be mediated by the cytotoxic effects of NS1 protein (Ozawa and al, 1988; Srivastava and al, 1990), whereas peripheral thrombocytopenia may be immunologically mediated (table 3), manifesting as an idiopathic thrombocytopenic purpura (ITP) (Lefrere and Got, 1987; Inoue and al, 1991; Murray and al, 1994; Heegaard and al, 1999; Scheurlen and al, 2001; Aktepe and al, 2004), or of thrombotic thrombocytopenic purpura (TTP) type, associated with microangiopathic hemolysis and thrombotic occlusions (Kok and al, 2001).

ITP after B19 infection is probably much more common in pediatric patients (Murray and al, 1994; Heegaard and al, 1999; Aktepe and al, 2004) than in adults (Elsacker-Niele and al, 1996). ITP or amegakaryocytic thrombocytopenia has rapidly responded to intravenous immunoglobulin (Heegaard and al, 1999; Bhattacharyya, 2004), whereas thrombocytopenia has become chronic in children treated with high-dose steroids (Heegaard and al, 1999).

6.10 Neutropenia

A transient, mild neutropenia is commonly observed during acute B19 infection (Anderson and al, 1985), possibly caused by infection and depletion of bone marrow granulocytes (Barlow and McKendrick, 1999). Neutropenia may be more severe (Barlow and McKendrick, 2000), even reaching aganulocytosis (Pont and al, 1992; Istomin and al, 2004). Chronic neutropenia has been detected in patients with chronic B19 infection (Koch and al, 1990; Pont and al, 1991; Mc Clain and al, 1993; Scheurlen and al, 2001; Istomin and al, 2004), and immediate recovery of granulopoiesis has been shown after high dose intravenous immunoglobulin treatment (Pont and al, 1991). Anti-neutrophil antibodies appear to be common in patients with B19 infection and neutropenia (table 3).

6.11 Myocarditis and myocardial diseases

Myocarditis represents a nonischemic inflammatory disease with a highly variable clinical outcome. In most cases this disease is self limiting; however, it may lead to acute heart failure, resulting in early death or heart transplantation (Virmani and al, 1995). So far, enteroviruses and adenoviruses have been recognized as the major causes of viral myocarditis (Liu and Mason, 2001; Figulla, 2003; Bowles, 2003; Pauschinger and al, 2004).
B19 infection has mainly been associated with fetal myocarditis, as histologic examinations and the presence of B19 DNA in the nuclei of fetal myocytes have demonstrated the tropism of B19 for these fetal cells (Porter and al, 1988; Naides and Weiner, 1989; Morey and al, 1992; Lambot and al, 1999; O'Malley and al, 2003). Importantly, clinically significant (occasionally fatal) myocarditis cases have also been diagnosed among pediatric (Nigro and al, 2000; Murry and al, 2001; Papadogiannikis and al, 2002; Dettmeyer and al, 2003) and adult patients with recent B19 infection (Tsuda and al, 1993; Malm and al, 1993; Orth and al, 1997).

Causes of clinically severe or fatal myocarditis may be different than the causes of clinically milder myocarditis (adeno- and enteroviruses), and B19 may be important in severe myocarditis, either acting alone or as a partnervirus in multiple infections (Rohayem and al, 2001; Bültmann and al, 2003; Kühl and al, 2003; Lamparter and al, 2003; Kytö and al, 2005). The pathogenesis of B19 myocarditis in children and adults is puzzling, since B19 is thought to replicate only in rapidly dividing cells, such as cells of hematopoietic origin (see chapter target cells). However, myocardial cells express P-antigen, the B19 receptor (Rouger and al, 1987; Cooling and al, 1995).

Immunological cross-reaction to epitopes shared between the B19 virus and the myocardium has been suggested (Dettmeyer and al, 2003). Cytokines, particularly TNF-α, which is a key pathogenetic mediator of myocarditis and heart failure (Ferrari, 1999; Jibiki and al, 2000; Paulus, 2000; Liu and Mason, 2001; Calabrese and al, 2004), may also be involved in the pathogenesis (Török, 1997), as high titres of it has been detected in adult and pediatric patients with B19 myocarditis (Tsuda and al, 1994; Nigro and al, 2000).

As B19 may replicate in vascular endothelial cells (Magro and al, 2002), they have been proposed to be the principal intracardial targets in B19 myocarditis (Bültmann and al, 2003). Endothelial cell infection with subsequent intravascular accumulation, adhesion and penetration of inflammatory cells in small intramyocardial vessels has been demonstrated in heart tissue from a patient with fatal B19 myocarditis (Bültmann and al,

2003; Klingel and al, 2004). Also others have suggested that endothelial cells (or mononuclear inflammatory cells) may be the intracardial targets for B19 (Mantke and al, 2004). Nevertheless, other studies suggest direct myocardial cell involvement of B19 infection in the pathogenensis of B19-associated heart diseases, as by ISH, B19 DNA has been located on myocardial cells in adult or pediatric patients with myocarditis or congenital heart diseases (CHD) (Wang and al, 2004; Kytö and al, 2005).

Viral myocarditis may lead to dilated cardiomyopathy (DCM), a condition with systolic cardiac dysfunction (Richardson, 1996). Myocardial persistence of various viruses appear to be common, and suggests a role in the pathogenesis of DCM (Liu and Mason, 2001; Calbrese and Thiene, 2003; Kühl and al, 2005).

Mantke and al (2004) studied 110 explanted hearts either from heart transplant (HTx) patients (n=56) or from multi-organ donors (MOD) (n=54). They showed that the presence of B19 within myocardium is relatively common in cardiological patients and in "healthy" controls, as they detected B19 DNA in 9 (4 HTx, 5 MOD) explanted hearts, and by ISH, found B19 DNA in interstitial cells but not in myocytes. All B19 positive HTx-hearts showed co-infections with one or two cardiotropic viruses (EV, ADV or CMV). Two of five B19 positive MOD-hearts also had co infections (EV or CMV), and one (EV co-infection) showed myocarditis in histological examination (Mantke and al, 2004). The authors concluded that co-infections of B19 and other cardiotropic viruses contribute to severe clinical outcome (Mantke and al, 2004).
In a recent study of 245 patients with "idiopathic" DCM, viral genomes were amplified in 165 samples (67%), and multiple viral genomes were found in 45 (27.3%) of 165 virus-positive endomyocardial biopsies. B19 was involved in 41 of 45 of such multiple infections (Kühl and al, 2005). Among all 245 samples, B19 genomes were most often amplified, in 126 (51.4%) samples, whereas genomes of other viruses were less prevalent: enterovirus in 23 (9.4%), adenovirus in (1.6%), HHV-6 in 53 (21.6%), EBV in 5 (2.0%), and CMV in 2 (0.8%) (Kühl and al, 2005).

Kytö and al. studied myocardial samples from 40 Finnish pediatric and adult patients with fatal myocarditis, and found viral nuclear acids in 43% of samples. In their study, CMV DNA was detected most frequently, in 38% of samples, and the presence of B19 DNA was next abundant, in 10% of samples. HHV-6 or enterovirus nuclear acids were detected in 2.5% of samples. The majority (75%) of B19-positive samples contained also CMV DNA, which was proposed to have contributed to the poor outcome of myocarditis (Kytö and al, 2005).

Tschöpe and al studied EMBs from 37 patients with isolated diastolic dysfunction for the presence of storage or infiltrative diseases or myocarditis, including molecular screening for cardiotropic virus genomes. Viral genomes were found in 35/37 (95%) of myocardial samples, and B19 genomes were found most frequently, in 84% of myocardial samples. Twenty four samples (65%) had B19 monoinfection and 6 samples (16%) had coinfection with HHV-6. HHV-6 and enterovirus monoinfections were found in 2 of 37. In a subgroup of patients with endothelial dysfunction, B19 DNA was detected in 10 of 10 (100%) patients. The authors suggested that B19 might have a role diastolic dysfunction, possibly mediated by B19 VP1 protein which has phospholipase activity and therefore, could induce locally inflammatory eicosanoids, prostaglandins, and leukotrienes leading host cell dysfunction (Tschope and al, 2005).

In conclusion, recents studies suggest that B19 is accociated with the development of myocarditis and subsequent cardiomyopathies more frequently than previously expected. B19 should therefore be recognized as a potential cardiotropic pathogen in patients of all ages.

6.12 Meningitis and encephalitis

An increasing number of cases of meningitis and encephalitis associated with B19 infection have been described. During an epidemic season in the United Kingdom, 4.5% of all undiagnosed meningoencephalitis cases have been estimated to be caused by B19 (Barah and al, 2001).

The pathogenesis of B19 meningoencephalitis is not fully understood. There is evidence that both the presence of virus in CNS and host´s immune responses are involved in the pathogenesis. First, B19 appears to be able to infect cells of endothelial and macrophage/microglial origin (Isumi and al, 1999), and B19 DNA has been detected in cerebrospinal fluid (CSF) (Cassinotti and al, 1993; Okamura and al, 1993; Druschky and al, 2000; Barah and al, 2001; Yoto and al, 2001; Barah and al, 2003) and brain tissue (Druschky and al, 2000) of immunocompetent patients.

Second, inappropriate activation of immune system appears important, as high levels of TNF-α, IFN-γ, IL-6, MCP-1 and GM-CSF cytokines and increased prevalence of HLA-DRB1 *04 allele have been found in patients with B19-associated meningoencephalitis (Kerr and al, 2002). Finally, B19-encephalitis has manifested after introduction of highly active antiretroviral therapy, (upon recovery of cellular immunity) as an "immune restoration disease" in a HIV-infected patient with chronic B19 infection. This patient had also detectable B19 DNA in CSF and brain tissue samples (Nolan and al, 2003).

6.13 Hepatitis

B19 has been associated with mild hepatitis where there are transiently elevated liver enzymes in pediatric and adults patients (Yoto and al, 1996; Nikkari and al, 1996; Barah and al, 2001), as well as with fulminant hepatitis (Karetnyi and al, 1999). Direct involvement of B19 in liver injury has been suggested, as B19-DNA, RNA and virions have been detected in liver tissue samples from patients with fulminant (non-A, non-B, non-C, non-E) hepatitis requiring liver transplantation (Karetnyi and al, 1999). However, persistence of B19-DNA in livers of B19 IgG-seropositive subjects appears to be common. B19-DNA was found in 24% of randomly selected autopsy liver samples as well as in 43% of livers explanted for various reasons (Eis-Hübinger and al, 2001). In a later study, liver tissue samples from patients with fulminant hepatitis (FH), nonviral liver diseases, and hepatitis B (HBV) virus or hepatitis C (HCV) virus infections were screened for B19-DNA and RNA: although B19 DNA was present in 35% of liver-tissue samples from patients with FH, it was also found in 33% of liver-tissue samples from patients with known hepatitis HBV and/or HCV infection, whereas none of the B19-DNA positive liver samples contained B19-RNA (Wong and al, 2003). Interestingly, there was a significant increase of variant erythrovirus genotypes in liver-tissue samples of patients with HBV or HCV hepatitis, the reason of this was not explained (Wong and al, 2003). One should notice that in Wong's study, it was not reported whether the patients with FH suffered from non-A, non-B, non-C, non-E hepatitis as did the patients in Karetnyi's study (Karetnyi and al, 1999). In conclusion, further studies are needed in order to elucidate whether B19 is an "innocent bystander" in the liver or whether the presence of B19 in liver may have biological and clinical significance.

6.14 Vasculitis

Vasculitis is a clinicopathologic process characterized by inflammation of blood vessel walls (Wolff, 1992).There is also evidence of the involvement of chronic B19 infection with various forms of vasculitides, such as Wegener's granulomatosis (Nikkari and al, 1994; Finkel and al, 1994), giant cell arteritis (Gabriel and al, 1999), polyarteritis nodosa (Finkel and al, 1994; Viguier and al, 2001), connective tissue disease associated vasculitis (Crowson and al, 2000; Magro and al, 2002), and adult Henoch Schönlein purpura (Cioc and al, 2002). Direct infection and viral replication within vascular endothelial cells appear to be involved in B19-associated vasculitides (Magro and al, 2002; Cioc and al, 2002). In addition, TNF-α mRNA has been detected *in situ* in the area of parvoviral infected cells (Cioc and al, 2002; Magro and al, 2002), and anti-tumor necrosis factor alpha treatment has yielded dramatic improvement, whereas traditional immunosuppressive therapy has caused worsening of symptoms (Magro and al, 2002). Rapid improvement of vasculitis has also been achieved with intravenous immunoglobulin treatment (Finkel and al, 1994; Viguier and al, 2001).

However, several case-controlled studies also suggest that B19 is not the causative factor for the majority of vasculitis cases. First, no association with B19 infection in Henoch-Schonlein purpura (Heegaard and Taaning, 2002) or in ANCA-positive vasculitis (Eden and al, 2003) were found. Second, Helweg-Larsen and al. did not detect B19-DNA in any temporal artery biopsy (TAB) samples from ten patients with giant cell arteritis (GCA) and 15 patients with polymyalgia rheumatica (Helweg-Larsen and al, 2002). In another study, TABs from patients with GCA or polymyalgia rheumatica contained B19 DNA at similar frequencies (64.5-76.7%) than in age matched healthy controls (73.7%) (Salvarani and al, 2002), suggesting that B19 DNA can persist as an "innocent bystander" in vascular endothelium. Whether any of these TABs also contained B19-RNA was not reported (Salvarani and al, 2002). However, the load of B19 virus may have importance in the aetiopathology of GCA, as in a recent sudy, the prevalence of B19-DNA in TABs of GCA patients and in histologically normal controls was again similar (54 vs 38%), whereas the viral loads (in genomes/ g of DNA) were significantly higher in GCA patients than in controls (Álvarez-Lafuente and al, 2005).

6.15 Chronic fatigue syndrome (CFS)

CFS is an illness with debilitating fatique lasting in excess of 6 months that occurs in association with other non-specific signs and symptoms (Holmes and al, 1988; Fukuda and al, 1994). CFS cases after B19 infection have been described (Kerr and al, 2001 and 2002b). Persistent infection (Kerr and al, 2001) and strong immune responses may be important in the pathogenesis of B19-associated CFS, as both permanently elevated serum levels of IFN-γ and TNF-α (Kerr and al, 2001) and the presence of HLA-DRB1*01 and *04 alleles (Kerr and al, 2001) have been shown to be predisposing factors.

6.16 Other disease associations

There are numerous other diseases having at least a putative association with B19, including hematological, connective tissue, neurological, dermatological, ocular and renal diseases. These clinical manifestations are described in more detail elsewhere (Török, 1997; Kerr, 2000; Heegaard and Brown, 2002; Chisaka and al, 2003; Barah and al, 2003).

7. DIAGNOSIS OF B19 INFECTIONS

7.1 Antibody assays

Acute B19 infections are confirmed by B19-specific IgM reactivity, whereas past infections are detected by IgG reactivity (see chapter 8). Most sensitive B19 enzyme immunoassays (EIA) are based on recombinantly expressed VP2 capsids. B19 IgM and IgG antibodies can be detected by these EIAs (Doyle and al, 2000; Heegaard and al, 2002b). Furhermore, the time of B19 infection can be serologically determined by measuring of VP1-IgG avidity (Söderlund and al, 1995a) or by measuring by VP2-IgG epitope type specificity (see below).

7.2 Antigen detection

Much of the early work on B19 was accomplished using the classic methods of immunoelectronmicroscopy (IEM) and counter-immunoelectrophoresis (CIE) (Erdman, 1997; Zerbini and al, 2002). However, these methods have been replaced by more sensitive and convenient antigen detection techniques which use procedures adapted from capture EIA formats (Cohen and al, 1983; Anderson and al, 1986; Erdman, 1997). In addition, a simple receptor-mediated hemagglutination method (RHA) based on interaction of B19 and P antigen on human erythrocytes appears to be useful in order to screen blood donors (Sato and al, 1995). Detection of B19 antigen in serum can complement serologic diagnosis. However, antigen detection in serum may not be helpful in cases in which the titer of circulating virus is too low or in which its presence is masked by B19-specific antibodies (Erdman, 1997).

7.3 DNA detection

Dot-blot hybridization

Dot –blot hybridization using ^{32}P-labelled probes (Clewley, 1985) or nonradiolabelled probes (Prato and al, 1991) provides a sensitive and specific method for direct detection of B19 DNA in clinical specimens. These tests can detect between 10^3 to 10^5 genome copies/ml, with longer probes combined with high-emission indicator systems, such as chemiluminesence, offering the highest sensitivity for hybridization (Erdman, 1997).

In situ hybridization

In situ hybridization (ISH) with B19-specific probes for detection of B19 in tissue specimens can provide an important adjunct to immunohistochemical studies, as ISH identifies the tissues and cells infected by the virus (Erdman, 1997; Zerbini and al, 2002).

Polymerase chain reaction.

PCR is currently the most sensitive method for detecting B19 DNA. Between 10^2 to 10^4 B19 genome copies/sample can be detected by PCR by using standard gel electrophoresis and ethidium bromide staining (Erdman, 1997). When combined with Southern blot, dot-blot, or liquid-phase hybridization techniques, PCR sensitivity can be increased to 1-100 genome copies/sample (Cunningham and al, 1988; Clewley, 1989; Salimans and al, 1989; Sevall, 1990; Koch and Adler, 1990; Durigon and al, 1993; Erdman and al, 1994). Improved sensitivity can also be achieved with a second amplification using internal or nested primers (Durigon and al, 1993; Patou and al, 1993; Zerbini and al, 2002), but with a concomitant increased risk of DNA contamination and false-positive results. B19 DNA

may be detectable for extended period of time in serum, synovial membranes and bone marrow, even in healthy individuals, and therefore, the presence of low levels of B19 DNA alone cannot be used to diagnose acute B19 infection.

8. IMMUNE RESPONSE

Two categories of humoral, as well as of cellular immunity exist: the innate and the adaptive. Innate immunity (i) does not require gene rearrangements, which are essential for adaptive immunity, (ii) recognizes conserved pathogen-associated molecular patterns (PAMPs), (iii) has rapid activation of effectors, (iv) has perfect self-nonself discrimination (selection over evolutionary time), yet (v) has no immunological memory (Janeway and Medzhitov, 2002). Mannan-binding lectin (MBL), C-reactive protein (CRP), complement factor 3 (C3) and serum amyloid protein (SAP) are the secreted proteins of the innate immunity (Fearon and Locksley, 1996), whereas granulocytes, natural killer cells (NK cells), natural killer T cells (NKT cells), macrophages and dendritic cells provide the cellular components of the innate immune system (Guidotti and Chisari, 2001).
γδ T cells, a minor T cell population in peripheral blood, may also be classified as a part of innate immunity (Modlin and Sieling, 2005; Holtemeier and Kabelitz, 2005).

Adaptive immunity, in turn, (i) is dependent on gene rearrangements, (ii) recognizes details (or epitopes) of molecular structures, (iii) has delayed activation of effectors (as it needs to be activated by innate immunity), (iv) has imperfect self-nonself discrimination (cells may obtain an autoreactive receptor because of the random nature of their receptor gene rearrangement) and (v) generates memory. Humoral adaptive immunity is mediated by antibody proteins secreted by B cells. Cytotoxic T-cells and T-helper cells are the cellular components of the adaptive immune system (Guidotti and Chisari, 2001; Janeway and Medzhitov, 2002).

9. HUMORAL IMMUNE RESPONSE

Antibodies are thought to provide an important defence mechanism against B19 (see below), whereas the roles (if any) of the innate humoral defence proteins (MBL, CRP, SAP or C3) are currently unknown. These innate proteins mainly target against bacterial cell wall components such as phosphorylcholine (CRP and SAP) and terminal mannose residues (MBL) (Janeway and Medzhitov, 2002) or OH-groups on carbohydrates (C3) (Fearon and Locksley, 1996), which are not present in the B19 virus.

9.1 Antibody responses towards the B19 structural proteins VP1 and VP2

Virus capsid-specific immunoglobulin M (IgM) and IgG antibodies are produced following experimental (Anderson and al, 1985) and natural (Saarinen and al, 1986; Erdman and al, 1991) B19 infection. After experimental infection, B19-specific IgM response developed during the second week after inoculation, whereas virus-specific IgG began to develop at the end of the second week and early in the third week (Anderson and al, 1985). B19-specific IgM is directed against the unique portion of VP1 (Söderlund and al, 1992) and VP2 (O'Neill and al, 1995). After natural infection, B19 IgM titers begin to fall at the second month after onset of illness, but may be found for several months (Anderson and al, 1986, Erdman and al, 1991). Virus-specific IgA antibodies are detectable in about half of IgG-positive subjects (Erdman and al, 1991). Also B19 antibodies of the IgE class have been detected in acute and recent B19 infection (Bluth and al, 2003)
IgG against conformational epitopes of VP2 and linear epitopes of VP1u persist for years or decades after natural infection (Kurtzman and al, 1989; Söderlund and al, 1995; Zuffi

and al, 2001). The avidity of VP1u-specific IgG is low in the acute phase, and maturates thereafter (Söderlund and al, 1995a). The maturation of IgG avidity for conformational VP2 is not known.
The VP1u-specific IgG is somewhat less prevalent (84-86%) than the conformational VP2-specific IgG among the remotely B19-infected subjects (Söderlund and al, 1992; Zuffi and al, 2001). However, lower prevalence (39%) of IgG towards linear VP1 was recently reported (Corcoran and al, 2000), and subsequently, IgG response againts linear VP1 was claimed to decrease post-infection (Corcoran and Doyle, 2004).

IgG reactivity against linear VP2 epitopes is strong in the acute phase, but virtually disappears during the late convalescence in humans (Kurtzman and al, 1989; Söderlund and al, 1995b). Interestingly, mice immunized with B19 structural proteins are able to maintain IgG responses against linear VP2 (Corcoran and Doyle, 2004). With human IgG, the dominant acute-phase epitopes within VP2 have been located to VP2 aa 65-75 (Fridell and al, 1989; Kaikkonen and al, 1999), to VP2 aa 266-273 (Sato and al, 1991; Kaikkonen and al, 1999) and in particular, to an immunodominant heptapeptide KYVTGIN, corresponding to VP2 aa 344-350 (Kaikkonen and al, 1999). Based on this strong acute-phase reactivity against linear VP2, an epitope type spescificity (ETS) immunoassays was set up and was shown to be useful for verification of recent B19 infection (Kaikkonen and al, 1999; Kaikkonen and 2001).

The disappearance of IgG towards linear epitopes within VP2 is incompletely understood. The simplest explanation, i.e. cryptic residence, appears unlikely as the dominant acute-phase epitope KYVTGIN is located externally on the surface of the VP2 capsid (Chipman and al, 1996; Kaufmann and al, 2004).

Söderlund (1995b) hypothesized that the primary structure of VP2 might resemble “self components”, against which a transient B-cell response could be mounted because of a short-living breach of immunologic tolerance during the acute phase. This theory is indirectly supported by the findings of Lunardi and al (1998). They showed that patients with chronic parvovirus infection have a persistent IgG response to an acute phase epitope (VP2 aa 65-88), and that these antibodies have autoantigen binding properties. However, such cross-reactive antibody responses have not been shown towards other acute phase VP2-epitopes.

Kaikkonen (1999) suggested that a transient IgG response towards linear VP2 epitopes could simply be an example of a common immunological tendency favouring conformational epitopes over linear ones. This theory is based on the findings from other, unrelated viruses: For example, a transition from a linear-epitope to a conformational-epitope specifity of IgG has been shown with human immunodeficiency virus (Cole and al, 1998) and equine infectious anaemia virus (Hammond and al, 1997) and furthermore, a linear epitope in the envelope glycoprotein of Sin Nombre virus appears to be specific for acute-phase IgG (Hjelle and al, 1997). However, even this theory does not explain why IgG towards linear eptitopes within VP1u, nevertheless, persists for years or decades after infection.

Most recently, the survival of B cells recognizing conformational VP2 epitopes has been suggested to be associated with their (hypothetical) better ability to receive T-cell help, stronger B-cell receptor cross-linking or “maintenance” signals than linear VP2 specific

B-cells (Corcoran and al, 2004). Of note, also these new theories fail to explain why IgG against linear VP1u persists.
Antibodies towards linear B19 epitopes within VP1u and VP2 seem to be functionally important, as their ability to react on immunoblot appears to correlate with their capacity to neutralize virus infectivity, and patients with underlying immunodeficiency and chronic B19 infection may have B19-specific antibodies that fail to recognize linear epitopes (Kurtzman and al, 1989). By using human IgG after natural B19 infection, defined neutralizing linear epitopes have been localized within the amino terminal portion of VP1u (Gigler and al, 1999) and the carboxyl terminal half of VP2 (Sato and al, 1991). Rabbit immunization experiments (with fusion proteins containing B19-specific regions of 56 to 111 amino acids) have also revealed neutralizing linear epitopes within the amino terminal portion of VP1u and within the VP1-VP2 junction area (Saikawa and al, 1993). A linear neutralizing epitope in the amino terminal region of the VP2 protein has been identified by a monoclonal antibody made after immunizing mice with purified B19 virus (Yoshimoto and al, 1991). Conformational VP2 also contains neutralizing epitopes, as shown by studies using monoclonal murine antibodies (Yoshimoto and al, 1991) or monoclonal human B19-specific IgG (Gigler and al, 1999; Arakelov and al, 1993), although about nine times less human monoclonal IgG to linear VP1u than to conformational VP2 was sufficient for 50% virus neutralization in one study (Gigler and al, 1999).

9.2 Antibody responses towards the nonstructural protein NS1

The first reports suggested that the presence of B19 NS1 IgG is confined within patients with prolonged B19-associated arthritis (Von Poblotzki and al, 1995 a) or persistent B19 infection (Von Poblotzki and al, 1995 b). Several other groups subsequently tried to confirm these findings, and found less restricted occurrence of B19 NS1 IgG in patients with chronic B19 infection (Jones and al, 1999), or patients with B19-arthropathy (Venturoli and al, 1998; Searle and al, 1998). IgG responses against linear NS1 epitopes occur more frequently during convalescence or remote immunity than during acute infection (Venturoli and al, 1998; Searle and al, 1998; Jones and al, 1999). Three linear antigenic regions (amino acids 191-206, 271-286 and 371-386) that are equally reactive with sera of healthy, remotely infected subjects and patients with persistent B19 infection have been mapped within NS1 (Tolfvenstam and al, 2000). IgM responses appear not to be directed against linear NS1 (Von Poblotzki and al, 1995 b; Hemauer and al, 2000). The prevalence of IgG against linear NS1 is only ~ 20-36% among remotely B19 infected healthy adults (Venturoli and al, 1998; Searle and al, 1998; Jones and al, 1999; Hemauer and al, 2000; Ennis and al, 2001). However, patients with acute B19 infection during pregnancy appear to make an interesting exception, because as many as 45- 61% of them may have IgG against linear NS1 (Searle and al, 1998; Hemauer and al, 2000). This higher IgG prevalence has been suggested to be associated with the pregnancy-associated immune suppression, which may lead to ineffective virus elimination (Hemauer and al, 2000).

Even if NS1 specific IgG is not specific for chronic infection or prolonged arthropathy after B19 infection, it nevertheless might be more prevalent among in those patients (Hemauer and al, 2000; Kerr and Cunniffe, 2000), with the highest prevalence (80%) among persistently infected, immunocompetent patients (Hemauer and al, 2000).

Recently, NS1-specific antibodies have been studied by using NS1 EIA containing conformational epitopes. Interestingly, IgM responses may also be detectable by this type

of NS1-EIA, suggesting that conformational epitopes are essential for NS1-specific IgM detection in recent infection (Ennis and al, 2001). IgG responses occur earlier against conformational than against linear NS1 epitopes, and among the remotely B19 infected subjects, the prevalence of IgG against conformational NS1 epitopes appear to be higher (78% vs 31%) than against linear epitopes (Heegaard and al, 2002). These new NS1 EIAs are likely to be useful in the confirmation of recent B19 infection (Ennis and al, 2001; Heegaard and al, 2002) and in monitoring the effect of future capsid-based B19 vaccines (Heegaard and al, 2002), as the appearance of the NS1 specific antibodies would indicate vaccine failure.

9.3 IgG subclasses

The human immunoglobulin isotypes are (in the order of appearance of their heavy chain genes in the long (q) arm of chromosome 14; IgM, IgD, IgG3, IgG1, IgA1, IgG2, IgG4, IgE and IgA2 (Schur, 1987). The great diversity in IgG isotypes is obtained during B-cell maturation by recombination of germline Ig genes and by mRNA splicing (Janeway and Travers, 1997a). The expression of the constant region of the heavy chain determines the IgG isotype. In normal adult serum, 70% of IgG is IgG1, 20% IgG2, 6% IgG3 and 4% IgG4 (Schur, 1987). Antibodies to protein antigens are usually of subclasses IgG1 and IgG3, whereas antibodies to carbohydrates and polysaccharides are usually IgG2 (Schur, 1987). Class switch to IgG4 reflects prolonged or repeated antigen exposure (Aalberse and al, 1983; Linde and al, 1988; Bird and al, 1989). Antibody class switch is mediated by helper (Th) cells, which activate B cells by secreting soluble (class switching) cytokines and by triggering the CD40 receptor on B-cells (Banchereau and al, 1994; Bachmann and Zinkernagel, 1997).

9.4 Antibody class switch cytokines in humans

B cells are induced to produce IgG1 and IgG3 by interleukin (IL)-10 (Banchereau and al, 1994) and IL-21 (Pene and al, 2004). IgA1 is induced by IL-10, whereas IgA2 is induced by IL-10 and transforming growth factor beta (TGF-β) (Banchereau and al, 1994). Interleukin-4 (Gascan and al, 1991) and interleukin-13 (Punnonen and al, 1993) are switch factors for both IgE and IgG4. At present, class switch to IgG2 is known to be mediated by Th cell triggering of CD40 receptor and secretion of (yet uncharacterized) cytokine(s) other than IL-4, -10, -13 or interferon (IFN)-γ (Servet-Delprat and al, 1995). However, IFN-γ and IL-6 additively enhance IgG2 secretion by surface IgG2-positive B cells (Kawano and al, 1994).

9.5 IgG subclasses in viral infections

In most viral infections IgG1 and IgG3 are the predominant subclasses, antibodies of the IgG4 subclass are found less often, and IgG2 (the characteristic anti-polysaccharide subclass) are least abundant (Skvaril, 1986; Linde and al, 1988). For viruses without latency, specific IgG3 suggests current or recent infection (Linde and al, 1988; Lundkvist and al, 1993).

9.6 The role of antibodies in B19 defence

Antiviral IgG specific for B19 structural proteins is currently thought to represent the major defence against B19. After infection virus-specific IgG antibodies persist presumably for life and are suggested to protect against reinfection (Brown et al, 1994). However, an asymptomatic reinfection with short viraemia and an anamnestic antibody response has been described in a human volunteer with low levels of B19 IgG (Anderson

et al, 1986). Therefore, even if prior B19 infection does not prevent reinfection, it is likely that it prevents disease (Kajigaya and Momoeda, 1997). Chronic B19 infections may often be cured with immunoglobulins containing neutralizing B19-specific antibodies (Kurtzman et al, 1989; Koch et al, 1990). However, chronig parvovirus infections have been observed in some apparently immunocompetent subjects (Faden et al, 1992; Hemauer et al, 2000) and complete virus clearance is not always achieved by immunoglobulin infusions (Frickhofen et al, 1990; Tang et al, 1994), suggesting that humoral immunity alone may sometimes be insufficient for virus eradication.

10. CELLULAR IMMUNE RESPONSE

Cellular immunity provides antiviral immune responses by secreting soluble antiviral cytokines, by direct killing of virus infected cells and by providing help for virus-specific B-cell immunity.

10.1 The cells of the innate immune system in B19 infection

Granulocytes are short-lived phagocytic cells, which are very important effectors against various bacterial infections (Mollinedo et al, 1999). They may contribute directly to the antiviral response by secreting antiviral molecules such as nitric oxide and tumor necrosis factor-α (TNF-α)(Dahesia et al, 1998; Wang et al, 2000). Granulocytes can also play an indirect role in antiviral immunity, as they are able to present at least rhinoviral antigens to antiviral T-helper cells (Handzel et al, 1998). The possible role (if any) of granulocytes in B19 immunity is currently unknown. Neutrophilia is not commonly associated with B19 infection, whereas mild neutropenia was reported among all voluntary subjects after experimental infection (Anderson et al, 1985).

Natural killer (NK) cells are the population of lymphocytes that can be activated to mediate cytotoxicity and to produce high levels of chemokines and antiviral cytokines, such as TNF-α and IFN-γ, providing important early defence against viruses and tumors (Djeu and al, 1982; Biron and al, 1999; Papamichail and al, 2004). NK-mediated cytotoxicity and antiviral cytokine responses peaks within the first several hours to days after primary infection, whereas adaptive T and B cell responses take more than a week to develop (Biron et al, 1999). NK cell function is tightly regulated by the balance between positive and negative signals provided by a diverse array of cell surface receptors. Negative signals to NK cells are provided by inhibitory MHC class I-specific receptors (iNKRs), whereas positive or activating signals are mediated by activating receptors, such as natural cytotoxicity receptors (NCRs) or lectin-like NKG2D receptor (Moretta and al, 2004; Smyth and al, 2005). In most instances, when simultaneous engagement of both activating and inhibitory (iNKR) receptors occurs, the HLA class-I specific inhibitory receptors predominate over the activating receptors, thus preventing NK-cell activation (Moretta and al, 2004; Smyth and al, 2005). However, if the expression of HLA class I molecules on target cells is lost (Moretta and al, 2004; Smyth and al, 2005) or foreign peptides are presented (Liberatore and al, 1999; Michaëlsson and al, 2002), inhibitory receptors are not engaged and NK cell triggering may occur (Liberatore and al, 1999; Michaëlsson and al, 2002; Moretta and al, 2004; Smyth and al, 2005).

The contribution of NK cells to defence against human viral infections is supported by data from natural infections. In humans, low NK cell cytotoxic activity is associated with

increased sensitivity to severe disseminating herpesgroup virus infections, such as HSV (Ching et al, 1979; Biron et al, 1989), Epstein-Barr virus (Merino et al, 1986; Joncas et al, 1989), and human cytomegalovirus (Biron et al, 1989; Quinnan et al, 1982).

At present, there are only two published studies concerning the role of NK-cells in B19 immunity. Wagner considered that NK cells might have contributed to a vigorous IFN-γ mRNA response in PBMC samples of a 65 years old female with acute B19 infection (Wagner et al, 1995). Bluth studied the percentages of NK-cells and T-cells (in total PBMC) in an allergic (tree, grass pollen, ragweed) pediatric patient with acute B19 infection: virtually no change in NK-cell and T-cell distributions were found 0, 14 and 210 after onset of symptoms (Bluth et al, 2003).

Natural killer T (NKT) cells are a population of T cells sharing characteristics with classical NK cells (Biron and al, 1999). These cells express a limited T cell receptor (TCR) repertoire, being predominantly TCR α/β expressing Vα24 rearrangement in humans (Kronenberg, 2005). NKT cells recognize glycolipid antigens presented by nonclassical MHC class I-like molecule CD1d (Moody et al, 1999; Guidotti and Chisari, 2001; Kroneberg, 2005). NKT cells are likely to have importance in immunity against intracellular bacteria and parasites (Guidotti and Chisari, 2001), and they may also have importance in immunity against viral infections (Kakimi et al, 2000; Exley et al, 2001). The role of NKT cells in B19-specific immunity is currently unknown.

Macrophages are long-lived phagocytic cells that can circulate in the blood or reside in different organs and tissues. Macrophages phagocytose particulate and foreign material (microorganisms, red cells, immune complexes, endotoxin) and they also present antigens very efficiently to T lymphocytes (Unanue, 1984; Laskin and Pendino, 1995). Macrophages have an important role in the control of intracellular bacterial, protozoan and funcal infections (Adams and Hamilton, 1984). Macrophage activation also provides important antiviral defence by providing cytokines with direct antiviral activity, such as IFN-α and -β, TNF-α, and nitric oxide (Wildy et al, 1982; Keller et al, 1985; Laskin and Pendino, 1995) as well as other cytokines with indirect immunoregulatory functions (e.g interleukins -1, -6, -8, -10, -12, -18 and GM-CSF) (Guidotti and Chisari, 2001).

Macrophages are likely to have significant importance in the B19-specific immune responses, both as antigen presenting cells to T-cells and as sources of proinflammatory cytokines. Vigorous production of IL-1 and IL-6 mRNA has been detected in peripheral blood monocytes from a patient with acute B19 infection (Wagner et al, 1995). Circulating IL-1 and IL-6 (Kerr and al, 2001) and IL-6 and IL-8 (Nigro and al, 2000) have been detected among B19-infected patients. IL-8 is an important mediator of chemotaxis for lymphocytes and granulocytes (Van Damme and al, 1990; Gesses And al, 1996). IL-1 and IL-6 are important inducers of acute phase proteins in the liver (Snick, 1990). IL-1 and IL-6 synergistically stimulate B-cell (Emilie and al, 1988) and T-cell proliferation (Houssiau and al, 1988). IL-6 is not a IgG subclass switch factor, but it enhances production of all IgG subclasses (IgG1-4) in class-switched B-cells (Kawano and al, 1994). IL-6 is thought to be an important mediator in B19 infection, since direct B19-infection of macrophages has been reported, leading to enhanced IL-6 and TNF-α production (Takahashi and al, 1998). By using cell lines, B19 non-structural protein (NS1) has been shown to be able to enhance activation of IL-6 (Moffat and al, 1996) and TNF-α (Fu and al, 2002) promoters causing the increased secretion of cytokine proteins (Moffat and al, 1996; Fu and al, 2002).

Dendritic cells (DCs) are highly specialized in capturing and presenting antigens to naïve T-cells, producing active antigen specific cytotoxic or helper T-cells (Banchereau and Steinman, 1998). Because of these functions, DCs are thought to be key modulators in the development of the adaptive immune responses during viral infection. Dendritic cells can also activate resting NK-cells by triggering their NKp30 receptor (Ferlazzo and al, 2002). Viruses may stimulate dendritic cells to secrete various immunoregulatory cytokines, such as IFN-α and- β, IL-6 and TNF-α (Kadowaki and al, 2000; Guidotti and Chisari, 2001) and IL-1, -12, -18 (Guidotti and Chisari, 2001).
The role of DCs in B19-specific immunity is currently undefined, but one may assume that DCs are likely to have an important role in initiating B19-specific Th-cell responses. The possible role of DCs in priming B19-specific cytotoxic T-cells is more uncertain, as classically, *de novo* or endogenenous synthesis of proteins has been considered as a prerequisite for MHC-I-restricted cytotoxic T-cell responses (Braciale and al, 1987; Rammensee and al, 1993). DCs are not known to be infected by B19. However, there is increasing evidence that the aforementioned 'classical' endogenous pathway is not the only mechanism for MHC-I restricted antigen presentation. Importantly, by using alternative pathways, unified under the name of cross-priming or cross-presentation, DCs have shown to be able to capture *exogenous* viral antigens and present them in a MHC-I restricted manner (Banchereau and Steinman, 1998; Rock and al, 2002; Morón and al, 2004). This mechanism has been shown to be operative with human DCs either primed with apoptotic, influenza-virus infected monocytes (Albert and al, 1998) or papillomavirus type-16 virus-like particles (Rudolf and al, 2001) as well as with murine DCs primed with porcine parvovirus-like particles (Morón and al, 2002). Importantly, a NS1 specific cytotoxic T-cell response has been described (Tolfvenstam and al, 2001). Moreover, NS1-protein is a known inducer of apoptosis (Moffat and al, 1998), and these CTLs may have been induced by DCs cross-primed with B19-infected, apoptotic cells containing NS1. Whether empty B19 capsids could induce similar cross-presentation as papillomavirus- or porcine parvovirus capsids is currently unknown and clearly, needs to be studied.

γδ T cells constitute a major population of intestinal intraepithelial lymphocytes, but a minor population of human peripheral blood T cells. These cells have a TCR composed of γ and δ chains, unlike theTCR α and β chains of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. Two main subsets of γδ T cells have been described in humans, one expressing TCR variable regions Vγ9/Vδ2, which are the dominant γδ T cell population in the circulation, and the other expessing Vδ1 paired with various Vγ elements, being the dominant γδ T cell population in mucosal surfaces (Holtmeier and Kabelitz, 2005). These cells have properties of innate immunity, since they respond rapidly, have limited TCR gene usage and express TCRs that act as pattern recognition receptors for phosphorylated isoprenoid precursors and alkylamines (Vγ9/Vδ2 T cells) or glycolipids (Vγ1 γδ T cells). The antigen presenting element for Vγ9/Vδ2 T cells is unknown, whereas glycolipid antigens are presented to Vδ1 γδ T cells by CD1 molecules (Holtmeier and Kabelitz, 2005). γδ T cells have also properties of adaptive immunity, as they have a memory phenotype and their TCRs require gene rearrangement (Holtmeier and Kabelitz, 2005; Modlin and Sieling, 2005).
γδ T cells appear to have a role in defence against HIV, EBV, CMV and HSV (Cai and Tucker, 2001) whereas their role (if any) in B19-specific immunity is currently unknown.

10.2 Antiviral cytokines in B19 infection

To date, IFN-γ and TNF-α are the best investigated antiviral cytokines in patients with recent B19-infection. $CD4^+$ T-cells, $CD8^+$ T-cells and NK-cells provide the main sources of IFN-γ (Boehm and al, 1997), whereas TNF-α is also secreted by macrophages (Herbein and O'Brien, 2000), as well as by NK-cells (Biron and al, 1999) and T-cells (Herbein and O'Brien, 2000).

IFN-γ receptors are ubiquitously expressed on most cells (Valente and al, 1992). Binding of IFN-γ to its receptor activates several antiviral mechanisms in virus-infected cells. Best known direct antiviral actions of IFN-γ are provided by transcriptional induction of double-stranded RNA activated protein kinase (PKR), 2′-5′oligoadenylate synthetase (2-5A synthetase), and dsRNA specific adenosine deaminase (dsRAD) genes (Boehm and al, 1997).
Both RNA and DNA virus produce RNA intermediates which can activate PKR leading to phosphorylation of initiation factor 2 and subsequent inhibition of protein synthesis.
2-5A synthetase is also activated by dsRNA. Upon activation 2-5A synthetase activates latent riboendonuclease RnaseL, which degrades single-stranded viral and cellular RNAs, inhibiting viral growth. The dsRAD uses viral dsRNA as a substrate, causing production altered viral mRNAs and non-functional viral proteins (Boehm and al, 1997). IFN-γ has also an apparent role in apoptotic cell death (Murphy and al,1988; Binder and al, 1988), as IFN-γ up-regulates a number of apoptosis associated proteins in vitro, including Fas (Maciejewski and al, 1995) and TNF-related apoptosis inducing ligand (TRAIL) (Sedger and al, 1999). IFN-γ has also important indirect antiviral effects, as it stimulates antigen presentation to helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells (Spellberg and Edwards, 2001).

TNF-α binds to two distinct, ubiquitousy expressed TNF receptors denoted TNFR1 and TNFR2, and which belong to a broader family of related TNFR proteins. TNF-α interferes with viral replication in several ways. TNF-α can block viral replication by interfering with the viral life cycle, especially viral entry. In addition, TNF-α can activate, differentiate, or in particular, kill target cells by inducing apoptosis (Herbein and O'Brien, 2000). In addition, synergistic antiviral action of IFN-γ and TNF-α has been shown to affect the early steps in herpes simplex virus replication at the level of early gene transcription and translation (Feduchi and al, 1989), while in murine cytomegalovirus they inhibit late gene transcription and DNA replication (Lucin and al, 1994) and in adenovirus, capsid formation (Mayer and al, 1992).

Direct cytotoxic-target cell contact is not necessary for the antiviral potential of these cytokines, and therefore, antiviral cytokines secreted upon target cell contact may provide a strong antiviral effect on numerous adjacent virus-infected cells (Guidotti and Chisari, 2001). Although apoptosis provided by these cytokines can be an effective strategy for virus clearance, it does not come without potential cost to the host. In particular, if viruses have extensively infected large or vital organs, cytotoxicity as the only antiviral mechanism might destroy it and kill the host (Guidotti and Chisari, 2001). At present, the noncytopathic antiviral defence has been most extensively studied with hepatitis B models: TNF-α and IFN-γ dependent, non-apoptotic clearance of hepatitis B virus genomic DNA has been described in murine (Guidotti and al, 1996) and primate (Guidotti and al, 1999) HBV infections. In addition, TNF-α and/or IFN-γ–mediated, noncytopathic

antiviral activity has been shown in LCMV (Guidotti and al, 1999b), adenovirus (Benihoud, 1998) and coxsakievirus (Horwitz and al, 1999) models.

In human parvovirus B19 infection, IFN-γ mRNA has been detected in PBMC from recently infected adult (Wagner and al, 1995) and child (Bluth and al, 2003). Elevated levels of circulating IFN-γ and TNF-α have been detected among pediatric patients with B19-associated myocarditis (Nigro and al, 2000) and recently infected adults (Kerr and al, 2001). Importantly, among those patients who developed chronic fatigue the IFN-γ and TNF-α levels remained elevated during follow up (Kerr and al, 2001). In addition, circulating tumor necrosis TNF-α has been detected among adult patients with recent B19 infection and haemophagocytic syndrome (Tsuda and al, 1994; Watanabe and al, 1994) and pediatric patients with self limiting infection (Barash and al, 2003).

At present, no studies concerning the effects of IFN-γ or TNF-α on parvovirus B19 replication are published, whereas these cytokines have been suggested to have a role in pathogenesis of several B19-associated diseases (see chapter 6). Among animal parvoviruses, raised levels of serum TNF-α has been associated with canine parvovirus (CPV) enteritis (Otto and al, 1997) and both IFN-γ and TNF-α have a role in Kilham rat-virus induced autoimmune diabetes mellitus in rats (Chung and al, 1997).

There is increasing evidence that B19 DNA may persist in the human body in vital and large organs (see chapters 4 and 6). If this parvoviral DNA is reacted, the noncytolytic control provided by antiviral cytokines may provide a very important antiviral defence mechanism also against B19.

10.3 Cytotoxic T-cell immune responses in B19 infection

Cytotoxic T-cells (CTLs) may kill virus-infected cells by delivering apoptotic signals via the Fas/FasL or the granule exocytosis pathway. The Fas/FasL system is mainly responsible for activation-induced cell death, but it also may have a role in virus clearance. The granule exocytosis pathway uses perforin to target granzymes A and B to appropriate locations in the target cells, where they cleave critical substrates that initiate DNA fragmentation and apoptosis (Russell and Ley, 2002). Cultured human $CD4^+$ and $CD8^+$ T cell lines can use both the Fas/FasL and perforin/granzyme pathways (Ju and al, 1994; Williams and Engelhard, 1996; Yasukawa and al, 1999). $CD4^+$ CTLs preferentially lyse their targets via Fas/FasL pathway, and their major role is thought to be immunoregulation (Hahn and al, 1995). Nevertheless, $CD4^+$ CTLs appear to be important in immunity against viruses which can prevent normal MHC class-I presentation pathway (Appay, 2004). Granule exocytosis pathway mediated by $CD4^+$ CTLs has been shown to be operative against HSV (Yasukawa and al, 1999) and EBV (Khanolkar and al, 2001).

$CD8^+$ T cells are the predominant CTLs. They recognize intracellularly produced, pathogen-derived or mutant (self) peptides bound to MHC class I molecules on the surface of APC (Janeway and Trawers, 1997b; Rock and al, 2002). Perforin/granzyme pathway dominates this class I pathway (Hahn and al, 1995; Russell and Ley, 2002). $CD8^+$ T cells are essential for control in certain virus infections, for example HSV (Cunningham and Mikloska, 2001; Khanna and al, 2004), CMV (Pahl-Seibert and al, 2005; Sacre and al, 2005), EBV (Torre-Cisneros and al, 2004; Gudgeon and al, 2005), and influenza (Christensen and al, 2000).

In B19 infection, NS1-specific $CD8^+$ T-cells may have significant importance. Vigorous cytolytic responses were shown among HLA-B35 positive subjects against an optimized 9-mer NS1 epitope QPTRVDQKM, aa 391-399 of NS1(Tolfvenstam and al, 2001). In order to determine the number of these NS1-specific CTLs, IFN-γ Elispot and HLA-B35 tetramer staining (using the optimized epitope) experiments were carried out: IFN-γ Elispot experiments showed that the frequency of NS1-specific $CD8^+$ T-cells was as high as ~ 300 spot forming cells/10^6 PBMC, a frequency comparable to frequencies of HLA-A2 restricted influenza- and Epstein-Barr virus specific CTLs. The Elispot results were confirmed by HLA-B35 tetramer staining with the optimized epitope: 0.3% of all $CD8^+$ T-cells could be stained, confirming that the frequency of NS1-specific CTLs was unexpectedly high (Tolfvenstam and al, 2001). Later, Klenerman and al. studied $CD8^+$ T responses among 6 remotely infected subjects by using peptide pools spanning the whole NS1 (Klenerman and al, 2002). Reactivity was found in several peptide pools, suggesting that NS1 may contain several other CTL-epitopes than the epitope within aa 391-399.

Recently, Norbeck and al. studied $CD8^+$ T cell responses among 5 recently infected women by using peptide pools spanning the whole NS1, VP1u and VP2 (Norbeck and al. 2005). CTL responses were well maintained during follow up, and epitopes were fine mapped to several other NS1 regions than NS1 aa 391-399 in all five subjects, whereas CTL reactivity within VP2 was found only with two subjects, and none showed reactivity within VP1u. Thus, NS1 appears to contain several principal epitopes for B19-specific $CD8^+$ T cells.

10.4 T-helper cells

T-helper (Th) or CD4$^+$ T cells are essential in antiviral immunity, as they participate in antiviral responses both directly (by producing antiviral cytokines and possibly by cytotototoxic mechanisms) and indirectly by providing help for B-cells and CTLs (Guidotti and Chisari, 2001). Th cells are also necessary for generation of functional CD8 T cell memory (Sallusto and al, 2004). On the other hand, in genetically predisposed individuals with disease-associated MHC alleles, Th cells can be dangerous mediators of autoimmune diseases, such as RA, MS or IDDM (Romagnani, 1994; Druet and al, 1996; Windhagen and al, 1996; Bach and Chatenoud, 2001; Roep, 2003; Sospedra and Martin, 2005). Autoimmunity may be induced by molecular mimicry, in which T cells respond to infectious agent and then cross-react with self antigens. Cross reactivity can occur between Th cell epitopes that have a limited number of common amino-acids (Wucherpfenning and Stominger, 1995), no common amino acids (Hemmer and al, 2000), and furthermore, with incompletely sequence-matched peptides binding to different class HLA-DR alleles (Lang and al, 2002; Mycko and al, 2004). Incomplete regulation of autoreactive Th cells (see below for regulatory Th cells), which are present in most normal individuals, may also be important in the pathogenesis of autoimmune diseases (Bach and Chatenoud, 2001; Roep, 2003; Sospedra and Martin, 2005). Several viruses may be important inducers of organ-specific autoreactive Th cells. In IDDM, enhanced PBMC responsiveness to CBV4 has been observed in patients with newly diagnosed disease (Klemetti and al, 1999), although the diabetogenic autoantigens recognized by the CBV4-specific Th cells remain to be characterized (Marttila and al, 2001). On the other hand, a Th cell cross reaction between islet cell autoantigen GAD65 and a peptide derived from cytomegalovirus major DNA-binding protein has been reported (Hiemstra and al, 2001). In MS, viral peptides from several different viruses have been shown to activate Th cell clones spesific for myelin basic protein, one of the important target antigens in the immunopathogenesis of MS (Wucherpfenning and Strominger, 1995).

T-helper cells recognize peptides derived from extracellular proteins and bound to MHC class II molecules on the surface of APC (Janeway and Trawers, 1997b; Wolf Bryant and al, 2002). All T-helper lymphocytes start out as naïve IL-2 secreting Th0 cells, which, after being activated, are capable of differentiating into mature Th0 cells secreting both IFN-γ and IL-4 (Spellberg and Edwards, 2001), which can, in the presence of polarizing factors, can differentiate further into more Th1 or Th2 – oriented effector cells (Röcken and al, 1991; Sad and Mosman, 1994; Spellberg and Edwards, 2001; Löhning and al, 2002) and fig 1. Five factors regulate the polarization of newly activated naïve T cells into mature Th1 or Th2 cells: 1) the local cytokine milieu, 2) the presence of immunologically active hormones, 3) the dose and route of antigen administration, 4) the type of antigen presenting cell stimulating the T-cell and 5) the “strength of signal” which is a summation of the affinity of the T-cell receptor for the MHC-antigen complex, combined with the timing and density of receptor ligation (Seder and Paul, 1994; Constant and Bottomly, 1997; Spellberg and Edward, 2001; Löhning and al, 2002). The key polarizing factors are cytokines IL-12 (Th1 polarization) and IL-4 (Th2 polarization), acting via intracellular transcription factors which participate to regulation and epigenic or heritable modification of cytokine genes and gene loci (Murphy and Reiner, 2002; Löhning and al, 2002). Signal transducer and activator of transcription 4 (STAT4) and transcription factor T-bet induce Th1 polarization, whereas STAT6 and transcription factor GATA-3 induce Th2 polarization (Spellberg and Edwards, 2001; Murphy and Reiner, 2002; Löhning and al, 2002). If polarizing signals are strong enough, effector memory Th cells reproducing the expression of effector cytokines are established, whereas weaker stimulation leads to the

generation of central memory Th cells, which have no memory for effector cytokines, but they need weaker TCR stimulation for proliferation than naïve Th cells do (Löhning and al, 2002; Sallusto and al, 2004).

Extremely polarized T-helper cells can be classified by their cytokine profiles as Th1 cells or as Th2 cells. This classification was originally compiled after a series of experiments with murine Th-cell clones stimulated repeatedly with antigens. Type1 Th cells (Th1 cells) produced stereotypically IL-2, IFN-γ, and TNF-β, whereas type2 Th cells (Th2 cells) produced stereotypically IL-4, IL-5, IL-6, IL-10 and IL-13 (Mosmann and al, 1986; Cher and Mosmann, 1987; Cherwinski and al, 1987; Fiorentino and al, 1989; Mosman and Coffman, 1989).

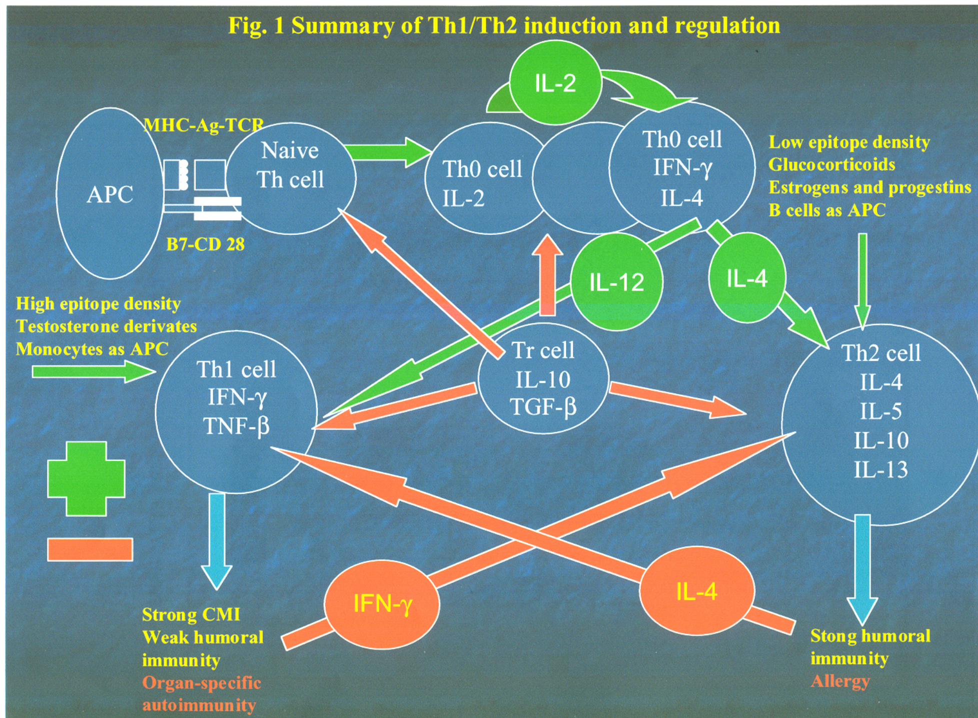
Later, $CD4^+$ T-cells (Umetsu and al, 1988; Salgame and al, 1991) and $CD8^+$ T-cells (Salgame and al, 1991) with cytokine profiles resembling those of murine Th1 or Th2 cells were also found among humans. However, in humans this division is not as stringent as in inbred mice, since some human Th1 cells may be able to secrete IL-10 (Yssel and al, 1992; Del Prete and al, 1993) and IL-13 (de Waal Malefyt and al, 1995).

Th1 cells are the key regulators of cellular immunity. The cytokine primarily responsible for their proinflammatory action is IFN-γ. IFN-γ induces various cells to sectere TNF-α and chemokines (Spellberg and Edwards, 2001). IFN-γ stimulates intracellular killing of microbes and antigen presentation to cytotoxic ($CD8^+$) and helper ($CD4^+$) T cells by upregulating MHC class I and II molecules (Boehm and al, 1997, Spellberg and Edwards, 2001). IFN-γ has direct antiviral activity (see section 9.2). B-cell help of Th1 cells is limited by their tendency to kill B-cells (Del Prete and al, 1991). Th1 cells regulate the development of Th2 cells, as IFN-γ secretion suppresses IL-4 secretion and therefore inhibits the differentiation of naïve Th0 cells into Th2 cells (Spellberg and Edwards, 2001; Murphy and Reiner, 2002; Löhning and al, 2002). Finally, Th1 cells are the key mediators of organ-specific autoimmunity (Druet and al, 1996; Bach and Chatenoud, 2001; Roep, 2003; Sospedra and Martin, 2005) and fig 1.

Th2 cells, on the other hand, support humoral and regulate Th1-oriented immunity. They are essential for generation of B-cell memory, antibody class switch and affinity maturation (De Kruyff and al, 1993). Little or no T-cell help seems to be needed for maintenance of B-cell memory whereas the activation of virus-specific memory B-cells into IgG secreting cells, again, is Th cell dependent (Vieira and Rajewsky, 1990; Bachmann and Zinkernagel, 1997; Ochsenbein and al, 2000). Th2 cells help B cells by triggering of CD40 receptor by membrane CD40 ligand expressed on activated Th cells and by secreting soluble cytokines (Banchereau et al, 1994). IL-4, IL-10, and IL-13 Th2 like cells activate B-cell proliferation, antibody production and class switching (Paul, 1991; Moore and al, 1993; Mc Kenzie and al, 1993; Punnonen and al, 1993). IL-10 is particularly important in human parvovirus B19-specific humoral immunity, as IL-10 is a class-switch factor for IgG1 (Brière and al, 1994), the predominant IgG subclass for B19 (Corcoran and al, 2000). IFN-γ, in turn, downregulates IgG1 secretion (Kawano and al, 1994).
Furthermore, IL10 increases B-cell growth and IgG secretion (Rousset and al, 1992), and is essential for maintenance of human germinal center B-cells in vitro (Pound and Gordon, 1997). Importantly, cultured CD40-activated B cells are differentiated into plasma cells by IL10 but not by IL4 (Rousset and al, 1995).

IL4 and IL-10 inhibit the secretion of IL-12 and IFN-γ, thus blocking the polarization of Th0 cells to Th1cells (D'Andrea and al, 1993; Ohmori and Hamilton, 1997). IL-10, in addition, inhibits intracellular killing (Spellberg and Edwards, 2001). It also inhibits antigen presentation, leading to T-cell anergy (Spellberg and Edwards, 2001), and it directly inhibits human Th-cell proliferation and IL-2 production (De Waal Malefyt and al, 1993).

Additional populations of $CD4^+$ T-cells have been described as Th3 and T-regulatory 1 (Tr1) cells. Both are involved in regulation of immune responses. Th3 cells secrete transforming growth factor -β (Chen and al, 1994; Fukaura and al, 1996; McGuirk and Mills, 2002) whereas Tr1 cells secrete very high levels of IL-10 and lower levels of TGF-β (Groux and al, 1997; Mc Guirk and al, 2002; McGuirk and Mills, 2002; Mac Donald and al, 2002; McMarshall and al, 2003). The physiological role of regulatory $CD4^+$ T-cells is to control inflammatory pathology, to inhibit autoimmune diseases mediated by Th1 cells (Chen and al, 1994; Groux and al, 1997; McGuirk and Mills, 2002) and allergic diseases mediated by Th2 cells (McGuirk and Mills, 2002)(Fig.1). There is also evidence that induction of regulatory T cells may be beneficial for pathogens such as hepatitis C virus (MacDonald and al, 2002), Epstein Barr virus (Marshall and al, 2003) or *Bordetella pertussis* (McGuirk and al, 2002), as protective Th1 immunity may be too extensively inhibited.



10.5 T-helper cells and cytokines in B19 infection

To date, most knowledge on the possible role of B19 virus-specific T-cell functions in clinical manifestations such as arthropathy is based on indirect data, such as cytokine mRNA or circulating cytokines. IL-4 and -10 (Bluth and al, 2003) and IL1, IL-6 and IFN-γ (Wagner and al, 1995) mRNA expression has been detected in PBMC from recently B19 infected patients. Placentas from women with acute B19 infection during pregnancy contained higher number of $CD3^+$ T-cell with positive IL-2 staining than placentas from women with uncomplicated pregnancy (Jordan and al, 2000). Transforming growth factor β gene polymorphisms have been correlated with the occurrence of rash (Kerr and al, 2003), and cytokines tumor necrosis factor-α (TNF-α) and IFN-γ have been detected among patients with prolonged fatigue (Kerr and al, 2001), meningoencephalitis (Kerr and al, 2002b) or myocarditis (Nigro and al, 2000). In addition, circulating TNF-α has been detectable among recently infected children (Barash and al, 2003) and in adults with B19-associated haemophagocytic syndrome (Tsuda and al, 1994; Watanabe and al, 1994). Symptomatic B19 infection was found to be associated with HLA-DRB1*01,*04, *07 and HLA-B49 alleles (Kerr and al, 2002), but interestingly, the HLA-B35 allele, reported to be important in presenting a defined 9-mer NS1-epitope to CTLs (Tolfvenstam and al, 2001), was not associated with symptomatic B19 infection.

During the last ten years, *in vitro* experiments have been carried out to further characterize B19-virus specific Th-cell immunity.

NS1-specific Th-cell responses. Von Poblotzki and al. studied NS1-specific Th-cell proliferation responses among 10 remotely B19-infected adults. They used prokaryotically expressed NS1 as antigen and observed NS1-specific responses in only two healthy NS1 seropositive subjects who had extensively worked with this protein (Von Poblotzki and al, 1996).
Mitchell and al. studied remotely, as well as recently B19 infected subjects. They also used recombinantly expressed NS1. They concluded that NS1-specific lymphocyte proliferation correlated with the time of B19 infection rather than with the development of B19 arthropathy, since NS1-specific proliferation responses were found in recently infected patients (all of whom had arthralgia in the acute phase) with or without prolonged arthropathy. Interestingly, NS1-specific responses were also found among two B19-exposed individuals without clinical disease or VP2 seroconversion and among one VP2-seronegative rheumatology patient (Mitchell and al, 2001).

Th-cell responses against structural B19 proteins. By using prokaryotically expressed proteins, von Poblotzki and al. studied VP1, VP2 and VP1u specific Th-cell proliferation responses among 10 remotely B19-infected adults and found positive (SI≥ 2.0) proliferation responses with each antigen. 60% of study subjects showed the most vigorous proliferation responses with VP2 antigen, whereas with VP1 and VP1u antigens the percentages were 30% and 10%, respectively (Von Poblotzki and al, 1996).

Murai and al (1999) stated that VP1-antigen specific proliferation responses are stronger in patients with rheumatoid arthritis after B19 infection than in healthy control subjects, but they presented no data.
Corcoran and al (2000) studied B19 specific Th-cell responses among recently infected children with fifth disease and remotely infected healthy adults. They used baculovirus-expressed VP1 and VP2 as B19 antigens and the mitogen phytohemagglutinin (PHA) as a control antigen. Among remotely infected adults, IFN-γ responses with VP1 and VP2 antigens were readily detected, the latter being about three times stronger than the former. By contrast, among recently infected children the IFN-γ responses with both of the B19 antigens were equally weak, whereas stronger proliferation responses were detected with VP1 than with VP2 antigen. Th2- like cytokines IL4 and IL5 were never detected with the B19 antigens (Corcoran and al, 2000). Their conclusions were that (i) VP1u constitutes the major target for Th cells, particularly among recently infected individuals, (ii) children with recent infection have defective B19-specific IFN-γ responses and (iii) in adults, Th1 like immunity is mounted against B19 (Corcoran and al, 2000).

Corcoran and al. further studied Th-cell mediated IFN-γ responses among pregnant, seronegative or remotely B19 infected women. This study, though, lacked internal control subjects (remotely B19-infected, age-matched non-pregnant women). They suggested that B19-specific IFN-γ responses attenuate during pregnancy, as remotely infected, pregnant women showed weaker IFN-γ responses than remotely infected, non-pregnant adults in their preceding study (Corcoran and al, 2003).

T cell responses against animal parvoviruses. VP2 appear to provide important Th-cell epitopes in animal parvoviruses. VP2-specific Th-cell proliferation has been shown with rat virus (RV) (Ball-Goodrich and al, 2002) and canine parvovirus (CPV) (Rimmelzwaan and al, 1990; Langeveld and al, 1994).

11. VACCINE DEVELOPMENT

Due to the important morbidity, recombinant vaccines for the B19 virus are being developed (Bansal and al, 1993; Ballou and al, 2003). For efficient protection, strong induction of neutralizing antibodies is thought to be needed (Kurzman and al, 1989). Empty capsids consisting of both VP1 and VP2, VP1 proportion being at least two times higher than the ~ 5% of natural capsids, have fulfilled this antibody criterion in both animal (Bansal and al, 1993) and human models (Ballou and al, 2003). No literature on cellular responses against such candidate vaccine existed prior to our studies.

12. AIMS OF THIS STUDY

The specific aims of this study were:
1. Determination of subclass composition of B19-IgG in recent and remote infection.
2. Obtaining evidence that at least some kind of B19-specific Th cell immunity exists.
3. Characterization of the nature of B19-specific T helper cell immunity among recently and remotely B19-infected patients.
4. Characterization of how pregnancy and immunosuppressive medication alter B19-specific T helper cell immunity.
5. Characterization of B19-specific T helper cell immunity in patients with prolonged or relapsed symptoms after B19 infection.
6. Determination of how Th cells recognize VP1u and VP2 proteins in recent and remote infection.

13. MATERIALS AND METHODS

Patients with recent B19 infection. A) Donors of fresh peripheral blood mononuclear cells (PBMC): Sixteen recently infected, constitutionally healthy patients (designated #1-#16) were sampled 25-70 days after onset of symptoms of serologically documented B19 infection (table 4). Two patients (#11 and #12) were pregnant, and patient #11 underwent a fetal loss due to B19 infection. For comparison, PBMC were collected from patient G two months after delivery. She had acute B19 infection at gestation week 24, and delivered a healthy baby at full term. Patient L1 had prolonged postinfectious arthropathy (rash and arthralgia for > 6 months after B19 infection) (table 1).

Control subjects. PBMC were collected from 77 B19-seropositive, remotely infected subjects (32 men, 45 women, aged 20-62 years) and 47 B19-virus seronegative subjects (12 men, 35 women, aged 19-65 years) during this study. Twenty seven seropositive and 16 seronegative subjects were blood donors and the remaining fifty seropositive and thirty one seronegative were members of laboratory staff. Two staff members, who had previously shown strong B19-specific T-helper cell responses, became pregnant during this study, and both delivered at full term.

B) Donors of cryopreserved PBMC: PBMC were collected from nine recently infected patients (designated A1-A9) and stored in liquid nitrogen. PBMC from patients A1-A9 were obtained 10-90 days after onset of symptoms of serologically documented B19-infection. Cryopreserved PBMC were used for studying of Th cell responses against an unique portion of VP1 (VP1u) in recent infection, as this antigen was not in our hands at the time when fresh PBMC from recently infected subjects were obtained.

Cryopreserved control samples for recently infected patients A1-A9 were obtained from seven remotely B19 infected subjects (designated Sc1-Sc7) and five B19-seronegative subjects (designated Ni1-Ni5).

Antibody assays. B19-IgM was studied by using commercial EIA (Biotrin, Dublin, Ireland). B19-IgG was measured by EIA employing as antigen virus-like VP2 particles (Kaikkonen et al, 1999). For exclusion or verification of recent B19 infection, all samples were further studied for epitope-type specificity of VP2-IgG (Kaikkonen et al, 1999).

Antibody subclass assays. The IgG subclass measurements were carried out with four different B19 antigens: (a) a prokaryotically expressed β-galactosidase fusion protein (β–VP1) containing the unique portion of VP1 (Söderlund et al, 1992); (b) baculovirus-expressed (Brown et al, 1991) B19 capsids consisting of VP1 and VP2 (VP1/2 capsids) or (c) of VP2 alone (VP2 capsids); (d) commercially obtained baculovirus-expressed biotinylated B19 capsids consisting of VP2 alone (DAKO, Glostrup, Denmark).

Antigens a, b and c were adsorbed separately (in PBS at pH 7.5) onto polystyrene microtiter plates (Labsystems, Helsinki, Finland) and rinsed with 8 M urea followed by three 10-min washes with 0.05% Tween 20 in PBS (PBST). The sera (diluted 1:100 in PBST) were added to these antigens overnight at 4°C, followed by four rinses with PBST. Alternatively, the biotin-VP2 antigen d (in a diluent provided by the manufacturer) was added after the sera on streptavidin–coated microstrips (DAKO) whereafter the mixture was kept at 22°C for 30 min, followed by four rinses, according to the manufacturer´s

instructions. From this step onward all four antigens were treated identically. The antigen-specific IgG subclasses were quantified using monoclonal mouse antibodies: commercial clones (Zymed Laboratoires, San Francisco, CA) HP 6070 for IgG1 (ascites in PBST, working dilution 1/300) and HP 6002 for IgG2 (ascites in PBST, working dilution 1/3000); clones produced in house (Mäkelä et al, 1987; Seppälä et al, 1988) 2F5 for IgG3 (1:3000), IC2 for IgG4 (1:3000). The antibodies were applied onto the EIA plates on an orbital shaker for 2h at 22°C, rinsed and detected by peroxidase-conjugated anti mouse IgG (DAKO; 1:2000 in PBST), the enzyme activity of which was revealed by orthophenylene diamine (100 g/ml; 30 min) followed by H_2SO_4 stopping and measured at 492 nm in a Multiskan MCC/340 spectrophotometer (Labsystems). The cut-off absorbance between positive and negative for each subclass was the mean + 3 SD of the 20 seronegative control sera.

Antigens for T-lymphocyte studies.
a) Synthesis of VP1/2 capsids. Using viremic serum as template the complete genes for VP1 and VP2 were amplified by PCR using a DNA polymerase with strong 3'-5' proofreading exonuclease activity (DeepVent, New England Biolabs, Beverly, MA, USA). The PCR products were cloned into a baculovirus transfer vector p2Bac (Invitrogen, NV Leek, The Netherlands), VP2 under the polyhedrin promoter and VP1 under the p10 promoter, by standard methods. Bacteria (*E. coli* DH5α) containing the vector with the correct inserts were identified by restriction-enzyme analysis.

Spodoptera frugiperda cells (Sf-9) were co-transfected with the recombinant vector DNA and linearized baculovirus DNA (BaculoGold, PharMingen, San Diego, CA, USA). Transfection was done with lipofectin as recommended by the manufacturer (Bethesda Research Laboratoires, Life Technologies Inc, Gaithersburg, MD). Briefly, 300ng BaculoGold DNA and 2,5 g purified recombinant vector in 25 l aqueous solution were mixed with 25 l lipofectin, and were added to monolayer of $3x10^6$ Sf-9 cells. After 4 hours at 27°C the cells were rinsed carefully. Fresh growth medium (Gibco BRL Sf 900 II SFM) containing 10 % fetal calf serum was added. After 4 days the cells were harvested, and plaque purified twice (Summers and Smith, 1987). SDS-PAGE and immunoblotting showed VP1 and VP2 production [Brown et al, 1991].

b) Synthesis of VP2 capsids. The recombinant baculovirus for expression of VP2-alone capsids was constructed as described above, with the exception that the recombinant baculovirus contained only the VP2 gene, under control of the polyhedron promoter.

For preparative scale production, the cells were infected for 4 hours with the recombinant baculovirus. Growth medium (200-500ml) was added to final cell density 2 million per ml. After 3-4 days the cells were lysed by sonication in 0.1% Triton /PBS. A pellet containing cellular debris was remowed by low-speed centrifucation.

B19 capsids were purified from the supernatant by ultracentrifucation in 28% CsCl gradients (100 000 g, 48h), followed by precipitation in 40% ammonium sulphate. The protein pellet was resuspended in and dialysed against PBS. After dialysis the capsids were sterile filtered through 0.2- m filters (Anotop 10 plus; Whatman). Total protein concentration in the sterile capsid preparations was determined by the bichinchoninic acid protein assay (Pierce). Purity for B19 proteins was determined by immunoblotting with a VP2-specific murine monoclonal antibody R92F6 (a generous gift from Dr HJ O´Neill, Belfast Link Labs, Belfast, Northern Ireland) and an isotype (mouse IgG1) control C7H (a

generous gift from Dr M. Kaartinen, Haartman Institute) and SDS-PAGE with silver staining (Novex SilverXpress Silver Staining Kit; Novel Experimental Technology) and densitometry (Gel Doc 2000 Gel Documentation system with Quantity one Quantitation Software; Bio-Rad). According to densitometry of silverstained gels, our VP1/2 capsid preparations contained approximately 66% VP2 and 33% VP1, the ratio recommended for vaccine use (Bansal et al, 1993). EM with negative staining showed native-like B19 capsids (Kajigaya et al, 1989).

c) Recombinant VP1 unique portion (VP1u): The 227-aa VP1u was prokaryotically expressed and purified as described (Dorsch and al, 2001). After extensive dialysis against PBS, contaminating endotoxins were removed by passaging four times through endotoxin removal columns (Detoxi-Gel AffinityPak; Pierce, Rockford, IL). Then, the VP1u protein was sterile-filtered by using 0.2 - m filters (Anotop 10 Plus; Whatman).

d) Control antigens were sterile and preservative-free tetanus toxoid (TT), purchased commercially (National Public Health Institute, Helsinki), and *Candida albicans* antigen which was prepared in house and heat inactivated at 56°C for 30 min.

Endotoxin assays. Endotoxin content of the B19 antigens was determined by the Limulus amebocyte lysate assay (QCL-1000; Bio Whittaker), and it was <0.0015 EU/ g with VP1/2 and VP2 capsids and 0.013 EU/ g with VP1u protein (>> 1.5 EU/ g in a preparation designated VP1u^{e}, from which the endotoxin removal step was omitted).

Isolation of PBMC. Blood donors: PBMC were obtained from leukocyte enriched buffy coats by Ficoll-Paque (Pharmacia, Uppsala, Sweden) centrifugation (400g, 30 min) and were washed twice in PBS. The PBMC separations were completed within 8 h of blood withdrawal. Staff members: blood was drawn into mononuclear cell separation tubes (Vacutainer CPT, Becton Dickinson, Franklin Lakes, NJ) containing 0.45 ml sodium citrate. Cells were spun for 30 min at 1700 g, washed twice in PBS and were prepared within 2 h of sampling.

***Cryopreservation of PBMC**.* If not used immediately, PBMC were cryopreserved in 10% dimethyl sulphoxide (DMSO) and 90% fetal calf serum (FCS) and stored in liquid nitrogen. For use, the PBMC were thawed, washed twice with complete RPMI and cultured identically as fresh PBMC.

***Lymphocyte culture**.* Isolated PBMC were resuspended in complete RPMI 1640 containing 20 mM HEPES, 2 mM L-glutamine, streptomycin (100 g/ml), penicillin (100 U/ml), 50 M 2-ME and 10 % heat-inactivated human AB serum (Finnish Red Cross Blood Transfusion Service) containing B19-IgG. Manually counted PBMC (200 000/well) and the antigens were cultured in 96-well U-bottom plates (Costar, Corning Inc., Corning, NY) in a humidified incubator (37°C and 5% CO2). The B19 VP1/2 and VP2 capsids and the VP1u antigen were used in the proliferation and cytokine assays at 1.5 g/ml, whereas the control antigens tetanus toxoid and *Candida albicans* were used at 5 g/ml at 2.5 g/ml, respectively.

Proliferation assay. Manually counted PBMC and the antigens in triplicate were cultured for 6 days (37°C and 5% CO2) and pulsed for the last 16 h with 1 Ci of tritiated thymidine (specific activity 50 Ci/mmol, Nycomed Amersham, Buckinghamshire, UK). Thymidine incorporation was measured in a liquid scintillation counter (Microbeta,

Wallac Ltd, Turku, Finland). The data were expressed both as counts per minute (delta CPM) and as stimulation indices (SI), respectively: delta CPM = mean CPM (test antigen) – mean CPM (media); SI = mean CPM (test antigen) / mean CPM (media).

Cytokine assays. PBMC culture supernatants were harvested after 3 days for IFN-γ and 5 days for IL-10 and were stored at -20°C. Cytokine production was determined by using specific IFN-γ and IL-10 EIAs (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions. Background cytokine production was subtracted from total to yield antigen-specific cytokine production. The detection limits were 5 pg/ml for IFN-γ and 8pg/ml for IL10.

Depletion of CD4- and CD8-positive cells. CD4- and CD8-positive T cells were depleted from PBMC using magnetic beads coated with CD4- and CD8-specific monoclonal antibodies (Dynabeads M-450, Dynal A.S, Oslo, Norway), according to the manufacturer´s instructions. Depletion efficiency was confirmed by a flow cytometer (FACScan; Becton Dickinson).

Antibody blocking assays. Class restriction of the T cell IFN-γ and IL-10 responses were studied by using HLA class I (IgG2a, clone W6/32; Dako) and class II-specific (HLA-DR) (IgG2a, clone L243; Becton Dickinson) monoclonal antibodies at 1.5 g/mL.

Statistical methods. Proliferation and cytokine responses were statistically evaluated using the Mann-Whitney U test and the paired t-test. P values <0.05 were considered significant.

Table 4. Clinical pictures of the B19-infected patients, donors of fresh PBMC.

Subject, gender, age (years)	Days after onset of symptoms	Symptoms at onset	Other symptoms? Treatment?
#1, f, 31	30	A,R,F	
#1 follow up	155	-	
#2, f, 54	40	A,R,F	Pneumonia and pleuritis, cured by short-term prednisone treatment (20mg prednisone daily for 7 days with subsequently tapered dose. Treatment commenced 3 weeks before PBMC collection).
#2 follow up	150	A (severe) relapsed [a]	
#3, m, 52	62	R,F	
#4, f, 42	27	A,R,F	
#5, f, 33	60	R	
#6, f, 34	64	A (severe),R,F[a]	Swelling of joints during the acute phase. Extensive rash and fever present.
#7, f, 26	70	A,R,F	
#8, m, 42	54	A,F	
#9, f, 44	43	A,R,F	Oedema of lower extremities, signs of congestive heart failure in X-ray, permanent resolving by short-term furosemide treatment (20mg daily for 10 days).
#10, m, 37	30	R,F	Thrombocytopenia (peripheral destruction of platelets), 14th day of prednisolone treatment, currently 60mg daily.
#10 follow up	230	A (mild) [a]	Thrombocytopenia cured. Prednisolone tapered to 5mg every other day.
#11, f, 36	30	-	Fetal death (gestation wk19).
#11 follow up	60	-	
#12, f, 39	25	A,R,F	Symptoms during gestation week 27. Full-term pregnancy and healthy baby.
#13, f,32	32	A, R, F	.
#13,follow-up	515		
#14, f,36	55	A (severe), R	
#15,m,21	51	A (severe), R, F	
#16, f,31	48	A, R,F [a]	Rash present
G, f, 36	180	R	Symptoms during gestation wk 24. Full-term pregnancy and healthy baby.
L1 , f, 30	180	A, R, F [a]	Persisting arthritis and rash.

NOTE. f, female; m, male; A, arthralgia; R, rash; F, fever. [a] Symptoms present during the study.

14. RESULTS

(I) IgG SUBCLASSES

IgG subclasses in B19 infection

In serum pools the predominant βVP1-specific subclass was IgG1. After an initial rise in acute infection, relatively constant IgG1-EIA values were observed at all time points after infection. In contrast, IgG2-EIA levels remained below the cutoff at al time points. The reactivity of IgG3 peaked of ~ 2 weeks after infection, reaching the cutoff level in about six months. IgG4-EIA values were barely detectable during the first 1-3 months, but thereafter they rose vigorously and persisted at relatively high levels.

In the early phase (10 to 30 days after onset) 29/30 (97%) patients had detectable IgG3 and 11/30 (37%) had detectable IgG4 (at low levels). During late convalescence (205-700 days after onset) and in long-term immunity, altogether 8/62 (13%) subjects had IgG3 and 48/62 (77%) had IgG4. Similar response patterns were also seen with the baculovirus-expressed VP1/2 capsid antigen.

With the nondenatured biotin-VP2 capsid antigen, the binding kinetics of subclasses IgG1, IgG2 and IgG3 were similar to those seen with VP1u containing antigens. Because of the weakness of the IgG2 response, this subclass was not studied further. On the other hand, the IgG4 response towards VP2 differed strikingly from that towards VP1. No increase in VP2-specific IgG4 activity was detectable either in serum pools or in the individual sera. Since affinity-purified IgG4 fractions containing more βVP1-specific IgG4 than IgG1 (from a serum with high IgG4 towards βVP1) gave similar results as the nonfractionated sera, the absence of IgG4 reactivity could not be due to competition by other B19-specific subclasses. The IgG1 and IgG3 subclass responses towards denatured VP2 had kinetics similar to that of βVP1 or nondenatured biotin-VP2 for the first weeks after infection, thereafter IgG1 and IgG3 activity towards denatured VP2 declined progressively reaching background levels by the end of the first year. No rising of IgG4 binding to denatured VP2 was seen at any time point.

IgG subclasses in diagnosis

Twenty nine out of the 30 studied patients were β-VP1 IgG3 positive 10-30 days after onset of symptoms, whereas only 8/62 patients with long-term immunity had IgG3 levels above cutoff. As a marker of recent infection, this subclass had a sensitivity of 93% but a specificity of only 87%. In order to improve test specificity, the apparent complementarity of kinetics of β-VP1 IgG3 and IgG4 was used to calculate the ratios of EIA absorbances for these subclasses. An assay applying such an approach had a sensitivity of 97% and a specificity of 98% as a diagnostic test for recent infection.

(II) VP1/2 CAPSID-SPECIFIC T-HELPER CELL PROLIFERATION RESPONSES IN RECENT AND REMOTE B19-INFECTION

Proliferation responses among remotely infected and seronegative subjects
The VP1/2 capsid specific T cell responses were much stronger than the responses of the seronegative subjects ($P< .0001$). With the control antigens TT, PPD and *Candida* no statistically significant differences were found, and the same held for background proliferation.

Comparison of recently and remotely B19-infected subjects
The recently infected patients generally had stronger B19-specific proliferation responses. However, some of the strongest responders of the latter group ("top responders") had B19-specific responses comparable to the highest values seen among the recently infected subjects. These top responders also showed particularly vigorous proliferation responses with the control antigens TT and PPD.

Comparison of initial and follow-up data from recent infection
During 515 days of follow-up, the B19-specific proliferation response of patient R1 with self-limiting infection was well retained and comparable to the vigorous Th cell reactivity of patient L1 with persistent rash and arthralgia. Patients R5, with a self-limiting infection, and R6, with relapsed arthralgia, both retained T-cell reactivity at acute phase levels.

Identification of the proliferating cells
To characterize the proliferating cell population, PBMC were depleted of $CD4^+$ and $CD8^+$ T cells. CD8 depletion had little effect on B19-specific T cell proliferation, whereas CD4 depletion abrogated the responses among all groups studied. Blocking experiments using HLA-DR specific monoclonal antibodies (Mabs) reduced T cell proliferation by 74%-89%. Blocking experiments with HLA-class I specific Mabs inhibited the T cell responses by ≤ 15%.

(III) VP1/2- AND VP2-CAPSID-SPECIFIC T-HELPER CELL PROLIFERATION, INTERFERON-γ AND IL-10 RESPONSES IN REMOTE B19 INFECTION

Proliferation responses among B19-seropositive and seronegative subjects.
The VP1/2 and VP2 antigen-specific T-cell responses of the B19-seropositive subjects were much stronger than the responses of the seronegative subjects, whereas background proliferation responses and control antigen (TT)-specific responses were similar (P 0.50).

IFN-γ and IL-10 responses among B19-seropositive and seronegative subjects.
The B19-specific IFN-γ and IL-10 responses of the B19-seropositive subjects were also much stronger than the responses of the seronegative subjects ($P <0.0001$). With the control antigen TT no statistically significant differences were found (P 0.67), nor were such found for background cytokine production (P 0.58). Among the B19 seronegative subjects, IFN-

γ secretion with VP1/2 and VP2 antigens was virtually absent whereas IL-10 was occasionally detectable at low levels. No statistically significant differences were found in cytokine and proliferation responses between B19 seropositive staff members and blood donors or seronegative test subjects (data not shown).

Comparison of VP1/2 and VP2 capsid-specific proliferation, IFN-γ and IL-10 responses among B19-seropositive subjects.
The average VP1/2 and VP2 antigen-specific proliferation responses of the B19-seropositive subjects were virtually identical, and no significant differences were found when the responses were compared using the paired t-test (P= 0.38). Interestingly, IFN-γ responses were often slightly higher with VP2 antigen than with VP1/2 antigen; a difference reaching statistical significance (P = 0.042). High IFN-γ responses (200pg/ml) with VP1/2 antigen were always accompanied by high IFN-γ responses with VP2 whereas a few subjects showed high-level IL-10 responses (60pg/ml) only with VP1/2 antigen. Most test subjects, however, had similar IL-10 reactivity with both of the B19 antigens, and no statistical significance was found when individual VP1/2 vs VP2 responses were compared (P= 0.17).

IFN-γ versus IL-10 responses among B19-seropositive subjects.
With both of the B19 antigens VP1/2 and VP2, average IFN-γ (pg/ml) responses were higher than average IL-10 responses. When individual IFN-γ versus IL-10 responses with VP1/2 and VP2 were studied, most subjects had both cytokines detectable, but IFN-γ secretion was usually dominant. However, 20% of B19 seropositive subjects responded to the B19 antigens only with IL-10 secretion. Also with the control antigen TT, most subjects responded with both IFN-γ and IL-10 whereas 6% of subjects responded only with IL-10.

Identification of the IFN-γ and IL-10 secreting cells.
To characterize the cell populations secreting IFN-γ and IL10, the PBMC were depleted either of CD4- or CD8-positive T-cells using Mabs attached to magnetic beads. While CD8 depletion had little effect on the secretion of B19-specific IFN-γ and IL-10, CD4 depletion strongly reduced IFN-γ and IL-10 responses among all the subjects studied.

(IV) VP1u AND VP2 ANTIGEN-SPECIFIC T-HELPER CELL PROLIFERATION, INTERFERON-γ AND IL-10 RESPONSES IN RECENT AND REMOTE B19-INFECTION

VP1/2 and VP2 antigen-specific responses in recent versus remote B19 immunity
The VP1/2 and VP2 antigen-specific proliferation, IFN-γ and IL-10 responses of the recently infected, immunocompetent subjects C1-9 were stronger than those of the remotely infected subjects. However, statistical significance was achieved only with the proliferation and IFN-γ responses ($P \leq 0.003$), not with IL-10 (P 0.52). Interestingly, with the control antigen TT, the recently infected patients showed weaker ($P \leq 0.013$) proliferation, IFN-γ and IL-10 responses. In the corresponding background responses, no statistically significant differences (P 0.096) were found between the recently and the remotely infected subjects. The remotely infected subjects showed considerably stronger ($P \leq 0.0001$) B19-antigen specific

proliferation, IFN-γ and IL-10 responses than did the seronegative subjects, whereas the control-antigen (TT) specific responses were similar ($P \geq 0.77$).

VP1/2 versus VP2 antigen-specific proliferation, IFN-γ and IL-10 responses among recently and remotely B19-infected subjects

The mean proliferation and IL-10 responses among the recently infected subjects C1-9 were similar ($P \geq 0.38$) in comparison of the VP1/2 and VP2 antigens. The mean IFN-γ responses appeared higher with the VP1/2 capsids than with the VP2 capsids, but statistical significance was not reached ($P = 0.14$). In magnitude the B19-specific proliferation, IFN-γ and IL-10 responses varied strongly from patient to patient, giving rise to large SD's. The dominant B19-specific cytokine was usually IFN-γ, yet most patients also showed readily detectable IL-10 responses.

When VP1/2 and VP2 antigen-specific proliferation, IFN-γ and IL-10 responses within the remotely B19-infected subjects were compared, no statistically significant differences between the two B19 antigens ($P \geq 0.071$) were found, confirming our previous results (Franssila and Hedman, 2004).

VP1u versus VP2-specific IFN-γ and IL-10 responses among remotely and recently B19-infected subjects

We next determined the ability of Th cells to recognize isolated VP1u antigen. First, we used fresh PBMC from remotely infected subjects: stronger IFN-γ responses ($P = 0.015$) and IL-10 responses ($P = 0.037$) were found with VP2 capsids than with VP1u. Proliferation responses (mean cpm ± SD) were also stronger with VP2 (12282±16338) than with VP1u (328±593) ($P = 0.010$), confirming that PBMC from remotely B19-infected subjects poorly recognize VP1u.

We then compared the ability of cryopreserved PBMC from recently and remotely infected subjects to recognize the VP1u and VP2 antigens. The B19 antigen-specific responses of the recently infected patients differed strongly from the responses described above: the VP1u antigen elicited stronger IFN-γ and IL-10 responses than did the VP2 antigen. However, statistical significance was reached only with IFN-γ responses ($P = 0.017$), not with IL-10 responses ($P = 0.37$). Of note, the IFN-γ responses were at least two times stronger with VP1u than with VP2 in 7 of 9 recently infected patients.

With the remotely infected subjects, the B19 antigen-specific responses first determined with fresh PBMC were reproduced with cryopreserved PBMC, where stronger IFN-γ and IL-10 responses were detected with the VP2 antigen than with the VP1u antigen. However, statistical significance was reached only with IFN-γ responses ($P = 0.024$), not with IL-10 ($P = 0.37$).

Of note, the recently infected patients showed clearly higher IFN-γ background responses with cryopreserved PBMC than with fresh PBMC. It is likely that factors related to recent B19 infection have had a major role in this enhanced spontaneous IFN-γ secretion, whereas cryopreservation possibly had a minor role, since also the remotely infected subjects showed somewhat higher IFN-γ background responses with cryopreserved PBMC than with fres PBMC.

On the other hand, cryopreservation had a significant effect on spontaneous IL-10 secretion, as all subject groups showed clearly higher IL-10 background responses with cryopreserved PBMC than with fresh PBMC. The reasons for this increased IL-10 secretion are unknown. Possibly the high proportion (90%) of FCS in our cryopreservation media activated PBMC for IL-10 secretion upon freezing and/or thawing.

The effect of endotoxin contamination of VP1u antigen on IL-10 responses
The importance of endotoxin removal was elucidated by studying PBMC-mediated IL-10 responses with VP1u^e antigen from which endotoxins were *not* removed. Fresh PBMC were used. The IL-10 responses (mean ± SD) among 15 remotely infected seropositive and 9 seronegative subjects were as high as 428±262 and 394±211 pg/ml (P= 0.68), respectively. These high and non-specific responses were expected, as endotoxins are known to activate monocytes to produce high levels of IL-10 (De Waal Malefyt and al, 1991).

Comparison of acute-phase and convalescent-phase T-cell function
During follow-up after primary infection, the B19 specific IFN-γ responses decreased and IL-10 responses strongly increased in patient C1 who recovered without complications. Patient C2, with a preceding short-term low-dose corticosteroid course, had a very different response pattern: she showed a concomitant increase in B19-specific IFN-γ responses and disappearance of IL-10 responses during follow-up, at which time she suffered from a relapsed, severe arthralgia.

Patient C10 with long-term and (initially) high-dose corticosteroid course for B19-associated thrombocytopenia showed with the B19 antigens an initially strong IL-10 response, whereas the corresponding proliferation and IFN-γ responses were low. At follow-up, his B19-specific IFN-γ responses remained low and IL-10 responses were profoundly reduced, whereas his proliferation responses increased with both of the B19 antigens. Interestingly, patient C10 showed much stronger proliferation, IFN-γ and IL-10 responses with the control antigen TT during follow-up than during onset.

Comparison of patients with self-limiting or persistent symptoms
The B19-specific PBMC proliferation and IFN-γ responses during follow-up of patient R1 who recovered without complications, and patient L1 with rash and arthralgia persisting over 180 days after B19 infection were equally strong. By contrast, the B19-specific IL-10 response was very strong in patient R1 but minimal in patient L1.

PBMC responses in patients with B19 infection during pregnancy
Three subjects (patients C11, C12 and G) with B19 infection during pregnancy were studied. Their B19-specific PBMC proliferation, IFN-γ and, importantly, IL-10 responses were much weaker than were those of corresponding recently infected non-pregnant patients. Overall, strong B19-specific IL-10 responses were not encountered among patients with B19 infection during pregnancy. Patients C12 and G, who had symptomatic B19 infection and successful pregnancy, showed similar IFN-γ responses with the VP1/2 and VP2 antigens. Patient C11

with asymptomatic infection and fetal death, in turn, showed stronger IFN-γ responses with the VP1/2 antigen than with the VP2 antigen.

Influence of pregnancy on established Th-cell immunity

Before pregnancy, staff members S1 and S2 had shown particularly strong proliferation responses with B19 capsids. Also, their B19-specific IFN-γ and IL-10 responses were repeatedly strong and comparable to those seen among the recently infected patients C1-C9. During pregnancy, their proliferation, IFN-γ and, importantly, IL-10 responses were reduced with either B19 antigen. The mean background IL-10 response with S1 was 5 pg/ml before pregnancy; and only slight increases to 16 and 10 pg/ml were observed during gestation weeks 18 and 24, respectively. The mean background IL-10 response with S2 was 4 pg/ml before pregnancy; and a decline to 0 pg/ml was found during gestation weeks 15 and 35. The background IFN-γ responses with S1 and S2 before or during pregnancy were 0 pg/ml. After delivery, staff member S1 showed a slight increase in B19-specific IFN-γ responses and a strong increase in IL-10 responses, whereas her proliferation responses remained low.

HLA restriction of IFN-γ and IL-10 secreting cells

HLA class restriction of the IFN-γ and IL-10 responses were studied by using class I and class II-specific monoclonal antibodies. Blocking of antigen presentation via HLA class I by using monoclonal antibody W6/32 showed little, if any inhibitory effect on B19-specific IFN-γ or IL-10 responses, whereas blocking of antigen presentation via HLA class II by using monoclonal antibody L243 strongly inhibited IFN-γ and IL-10 responses among all subjects.

15. DISCUSSION

In study (**I**) the patients with recent B19 infection showed IgG1 and IgG3 restricted IgG responses towards all four B19-antigens studied. Levels of subclass IgG2 remained invariably low, as expected by the absence of polysaccharides in the structural proteins of B19 (Heegaard and Brown, 2002). The presence of virus-specific IgG3 turned out to be a sensitive marker of recent infection, as has been suggested before with other viruses (Linde and al, 1988), and the specificity of IgG3 as a marker of recent infection could be improved by using the apparent complementarity of kinetics of β-VP1 IgG3 and IgG4 by calculating ratios of EIA absorbances for these subclasses. An assay applying such an approach had a sensitivity of 97% and a specificity of 98% as a diagnostic test for recent infection.

The key findings of study (**I**) concerned IgG4 subclass, known to reflect longstanding or repeated exposure of antigen (Aalberse and al, 1983; Linde and al, 1988; Bird and al, 1989). It is therefore possible, that even after noncomplicated infections, B19 antigens persist in the human body. Another explanation for the postponed rise of B19-IgG4 could be repeated subclinical reinfection, which is easy to envision in epidemiologically active environments if the immunity is not solid to this virus. Third, and the most exciting explanation for the rise of B19-IgG4 could be a reactivation of persistent B19 infection. This hypothesis is supported by findings of persistent of B19 DNA among immunocompetent patients suffering from prolonged symptoms such as arthritis or vasculitis (see chapter 6) as well as among asymptomatic patients (see chapter 5).

However, another research group was not able to confirm the IgG4 results of study (**I**); of note, only 3 of 20 (15%) subjects in their convalescent group showed VP1-specific antibody reactivity of any IgG subclass (Corcoran and al, 2000), which strongly disagrees with VP1-IgG results published by other groups, which have found detectable IgG against linear VP1 among 84-86% of remotely infected subjects (Söderlund and al, 1992; Zuffi and al, 2001).

One purpose of study (**I**) was to obtain evidence that at least some kind of B19-specific Th cell immunity exists. At the beginning of study (**I**), attemps to measure *in vitro* T-cell responses against B19 had been unsuccesful, and B19-specific immunity was thefore postulated to be 'mainly humoral' (Kurtzman and al, 1989). By discovering B19-specific Th-cell dependent subclass (IgG1, IgG3 and IgG4) responses we become encouraged that B-19 specific Th cell responses might be directly measurable.

In study (**II**) we were the first to investigate B19-specific Th cell immunity by using VP1/2 capsids containing ~33%VP1 and 66%VP2, the ratios recommended for vaccine use (Bansal and al, 1993). Furthermore, we were the first to show that Th cell proliferation responses against B19 structural proteins in recently infected adults. We showed that B cells recognizing such a candidate vaccine-antigen receive class II-restricted help from $CD4^{+}$ lymphocytes. Previously, von Poblotzki and al (1996) had showed that remotely B19-infected subjects show HLA class II-restricted responses against prokaryotically expressed B19-antigens, and Murai and al (1999) reported (they showed no data) stronger VP1-antigen specific proliferation responses in patients with rheumatoid arthritis after B19 infection than in healthy control subjects. Subsequently, Mitchell and al (2001) concluded that NS1-specific

lymphocyte proliferation correlated with the time of B19 infection, rather than with the development of B19 arthropathy.

Strong B19-specific T cell proliferation responses were not confined to recently B19-infected patients or in patients with prolonged or relapsed arthropathy, as top responders among remotely B19-infected healthy subjects were found. These top responders had B19-specific T cell activity comparable to the B19-specific responses among recently infected patients. Since the top responders showed also vigorous control antigen-specific reactivity, their strong VP1/2-specific reactivity can be most readily explained by their good general ability to maintain T cell memory to recall antigens.

In study (**III**) we investigated how VP2-only capsids and VP1/2 capsids stimulate Th cells from remotely B19 infected subjects to proliferate, or to secrete interferon IFN-γ and interleukin IL-10. When individual IFN-γ versus IL10 responses with VP1/2 and VP2 antigens were studied, most subjects had both cytokines detectable, but IFN-γ secretion was usually dominant. The B19-antigen specific Th-cell cytokine responses in most subjects resembled those described for Th0 clones (Yssel and al, 1992). However, 20% of our B19 seropositive subjects responded to the B19 antigens only with IL10 secretion, i.e. they showed Tr1 like responses (McGuirk and Mills, 2002). We could not locate any Th cell activity within VP1u. Thus, VP1u seemed not to provide major Th epitopes for IFN-γ responses among remotely infected subjects. The first major conclusion of study (**III**) was that, whereas VP1u is known to contain important B-cell epitopes, it is VP2 that contains the epitope(s) capable of inducing vigorous Th cell proliferation, IFN-γ and IL10 responses among remotely B19-infected subjects.

Synthetic peptides of the VP1u region have been suggested for boosting of B19 immunity (Saikawa and al, 1993). However, Th cells are needed for the activation of memory B-cells into IgG secreting plasma cells (Vieira and Rajewsky, 1990; Bachmann and Zinkernagel, 1997; Ochsenbain and al, 2000), particularly if soluble proteins are used as antigens (Hebeis and al, 2004). Therefore, the second major conclusion of study (**III**) was that vaccines based on VP1u should have a fusion partner containing known Th cell epitopes, particularly if they are used as a booster of remote B19-specific B cell immunity.

In study (**IV**) Th cell-mediated, B19-antigen specific IFN-γ and IL-10 responses were measured among recently B19-infected adults. Antibody blocking experiments confirmed that the sources of the IFN-γ and IL-10 responses were $CD4^+$ Th cells. IFN-γ turned out to be the dominant B19-specific cytokine in both recent and remote infection; yet B19-specific IL-10 responses were readily detectable among asymptomatic, recently or remotely infected subjects, consistent with a role in the restoration of immune system homeostasis upon infection clearance (Spellberg and Edwards, 2001). Only one patient, C10, treated with high-dose corticosteroids, showed B19-specific Th cell responses with IL-10 as the dominant cytokine. This supports the finding that corticosteroids, when present in high doses during priming, favor generation of effector Th cells producing mainly IL-10 (Brinkmann and Kristofic, 1995).

To date, most studies on the pathogenesis of prolonged or relapsing B19 arthropathy suggest that persisting B19 infection is essential for chronic arthropathy (Saal and al, 1992; Takahashi and al, 1998; Mehraein and al, 2002; Lehmann and al, 2002). Only one study favors the view that virus persistence might not be necessary, as in predisposed individuals, the virus could trigger an autoimmune response that could be self-maintained also beyond virus clearance (Lunardi and al, 1998). We investigated two patients with relapsing or persisting symptoms. Vigorous B19-specific proliferation and IFN-γ responses were not confined within these patients, whereas they showed strikingly low IL-10 responses. Hence, if B19-specific Th cells that recognize persisting viral antigens or cross-reactive self antigens play a role in pathogenesis, their insufficient regulation via IL-10 could lead to the prolongation of symptoms.

We also determined B19-specific Th-cell function during pregnancy. In that condition, downregulation of cellular immunity is important, as the effector cells and/or Th 1 type cytokines such as IL-2, IFN-γ and TNF-α are harmful to the conceptus (Raghupathy, 2001). Elevated levels of cytokines, hormones and other molecules are likely to play critical roles in suppressing Th 1 type immunity (Raghupathy, 2001).

Pregnancy was found to attenuate both acute-phase Th-cell responses and previously-strong Th-cell memory responses against B19 structural proteins. Attenuation of B19-specific IFN-γ responses during pregnancy has been suggested also in a recent study in which internal control subjects were not included (Corcoran and al, 2003). In our study pregnancy strongly suppressed B19-specific proliferation and IFN-γ responses among both recently and remotely infected subjects; yet interestingly, also B19-specific IL-10 responses were suppressed. We did expect to see high B19-specific IL-10 responses among pregnant subjects, as earlier studies using mitogen activated PBMC have shown higher IL-10 responses among pregnant than non-pregnant women (Marzi and al, 1995; Raghupathy, 2001), In our study, however, pregnancy suppressed all aspects of B19-specific Th cell function. This suggests that pregnancy suppresses more strongly recall antigen-specific Th cell responses than mitogen-specific Th cell responses.

TT-specific responses in study (**IV**) were surprisingly low in recent infection. The reasons for this are unknown, but a mild immunosuppression accompanied with acute infection has to be considered. One possible mechanism might be direct lymphocyte infection, known to occur in some animal parvovirus infections, such as rat virus (McKisic and al, 1995). Indeed, B19 has been detected in lymphocytes (Takahashi and al, 1998; Mehraein and al, 2002). Another mechanism might be higher spontaneous secretion of immunosuppressive cytokines, such as IL-10, by PBMC of recently infected subjects.

Study (**IV**) was initiated by using VP2 capsids and VP1/2 capsids containing ~ 33% VP1, a proportion higher than the ~ 5% of the natural virus (Cotmore and al, 1986; Rosenfeld and al, 1992). As statistically significant differences in T-cell reactivity were not seen between the VP1/2 and VP2 capsids, it appears likely that with a natural B19 virus capsid, most Th cell reactivity targets epitopes within the major capsid protein VP2. With isolated VP1u antigen, the proliferation, IFN-γ and IL-10 responses were virtually absent in remote infection, whereas very strong VP1u-specific IFN-γ responses were detected in the majority of recently infected patients.

Prior to our study (**IV**), only one investigation of B19 specific cytokine responses using *in vitro* assays and recently infected patients had appeared (Corcoran and al, 2000). In this study children with fifth disease showed stronger proliferation responses with VP1 than with VP2 antigen, and weak IFN-γ responses with both B19 structural proteins. The authors concluded that VP1u constitutes the major target for Th cells, and that children with recent infection have defective B19-specific IFN-γ responses (Corcoran and al, 2000).

We furthermore showed that recently infected adults can mount strong IFN-γ responses against VP2 and, in particular, against VP1u, but Th cell immunity against the latter is not maintained in remote immunity. This was the key finding of the study (**IV**), not only as it might be useful in diagnostics of acute B19 infection, but because of an interesting opposite response pattern exists in B19-specific B-cell immunity; during late convalescence, IgG for VP2 linear epitopes disappear, whereas IgG specific for VP1u persists (Kurtzman and al, 1989; Söderlund and al, 1995b). These phenomena might furthermore be linked: as bound antibodies influence the presentation of Th cell epitopes (Simitsek and al, 1995), the VP2-IgG could favour the presentation of Th cell epitopes within VP1u, leading to IFN-γ oriented Th cell 'help' inhibiting IgG secretion (Kawano and al, 1994), and could even be cytotoxic to the B-cells specific for the primary structure of VP2 (Del Prete and al, 1991).

The reason why the strong IFN-γ responses with VP1u are confined within recent infection is currently unknown and deserves further study. One possible mechanism is extensive activation-induced cell death (AICD) in the (late) convalescent phase (Ahmed and Gray, 1996). Alternatively, the VP1u-specific Th-cells could become suppressed by Th3 cells secreting transforming growth factor β or by direct $CD4^{+}CD25^{+}$ cell contact (McGuirk and Mills, 2002).

16. CLINICAL SIGNIFICANCE

The findings with greatest clinical significe of this thesis work are:

1) The B19-IgG4 results suggest long-term persistence of B19 proteins persist in the human body. These viral proteins may be endogenously produced by persistent B19 virus, or alternatively, introduced by reinfecting exogenous B19.

2) Since only VP2-specific Th cell reactivity is well maintained, VP1u-based vaccines should contain a fusion partner containing known Th cell epitopes.

3) Strikingly low Th cell mediated IL-10 responses were found with patients with relapsed or persistent symptoms, suggesting that insufficient regulation of T cell immunity may be associated with prolongation of symptoms.

4) B19 may turn out to be a useful model for studying of the maintenance of Th cell memory in general, since Th cell reactivities against the structural proteins VP1 and VP2 are so differently maintained. Importantly, as B19 infections are also common, recently infected patients and seropositive and seronegative control subjects should be easy to find.

17. NEW PROJECTS

New important questions concerning B19 specific Th cell immunity, and its possible role in the pathogenesis of B19 associated diseases arose during this thesis work. Thus, future experiments are needed to elucidate:

(1) Are there cross-reactive, rheumatic disease-associated Th cell epitopes within VP1u and/or VP2?

(2) Do inadequate immunosuppressive cytokine responses have a role in the pathogenesis of B19 arthropathy?

(3) Are other cytokines, such as IL-4, IL-13, TNF-α and TGF-β, measurble with B19-antigens?

(4)Why do the strong VP1u-specific IFN-γ responses disappear?

(5) Do antiviral cytokines, such as IFN-γ or TNF-α have importance in preventing the reactivation of persisting B19 DNA?

(6) How do Th cells isolated from synovia or lymphoid tissues respond to B19 antigens? Are these responses different when compared with responses of peripheral blood Th cells?

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