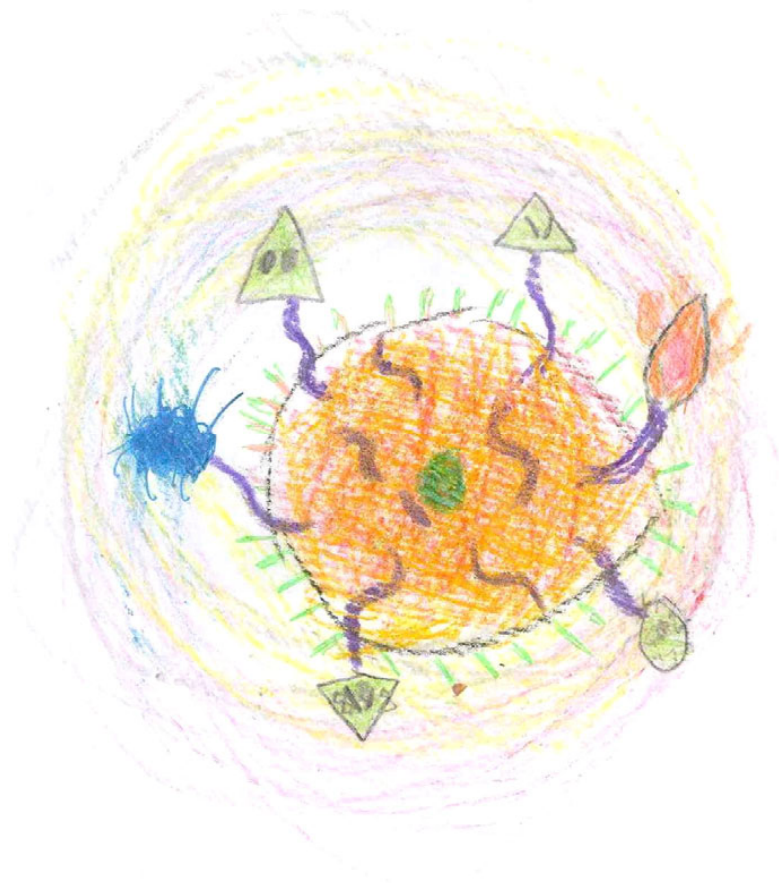


Human parvovirus B19: Tissue persistence and prevalence of prototypic and new variants



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Kati Hokynar

ACADEMIC DISSERTATION

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Abstract

Human parvovirus B19 is a minute ssDNA virus causing a wide variety of diseases, including erythema infectiosum, arthropathy, anemias, and fetal death. After primary infection, genomic DNA of B19 has been shown to persist in solid tissues of not only symptomatic but also of constitutionally healthy, immunocompetent individuals. In this thesis, the viral DNA was shown to persist as an apparently intact molecule of full length, and without persistence-specific mutations. Thus, although the mere presence of B19 DNA in tissue can not be used as a diagnostic criterion, a possible role in the pathogenesis of diseases e.g. through mRNA or protein production can not be excluded. The molecular mechanism, the host-cell type and the possible clinical significance of B19 DNA tissue persistence are yet to be elucidated.

In the beginning of this work, the B19 genomic sequence was considered highly conserved. However, new variants were found: V9 was detected in 1998 in France, in serum of a child with aplastic crisis. This variant differed from the prototypic B19 sequences by ~10 %. In 2002 we found, persisting in skin of constitutionally healthy humans, DNA of another novel B19 variant, LaLi. Genetically this variant differed from both the prototypic sequences and the variant V9 also by ~10%. Simultaneously, B19 isolates with DNA sequences similar to LaLi were introduced by two other groups, in the USA and France. Based on phylogeny, a classification scheme based on three genotypes (B19 types 1-3) was proposed.

Although the B19 virus is mainly transmitted via the respiratory route, blood and plasma-derived products contaminated with high levels of B19 DNA have also been shown to be infectious. The European Pharmacopoeia stipulates that, in Europe, from the beginning of 2004, plasma pools for manufacture must contain less than 10^4 IU/ml of B19 DNA. Quantitative PCR screening is therefore a prerequisite for restriction of the B19 DNA load and obtaining of safe plasma products. Due to the DNA sequence variation among the three B19 genotypes, however, B19 PCR methods might fail to detect the new variants. We therefore examined the suitability of the two commercially available quantitative B19 PCR tests, LightCycler-Parvovirus B19 quantification kit (Roche Diagnostics) and RealArt Parvo B19 LC PCR (Artus), for detection, quantification and differentiation of the three B19 types known, including B19 types 2 and 3. The former method was highly sensitive for detection of the B19 prototype but was not suitable for detection of types 2 and 3. The latter method detected and differentiated all three B19 virus types. However, one of the two type-3 strains was detected at a lower sensitivity.

Then, we assessed the prevalence of the three B19 virus types among Finnish blood donors, by screening pooled plasma samples derived from >140 000 blood-donor units: none of the pools contained detectable levels of B19 virus types 2 or 3. According to the

results of other groups, B19 type 2 was absent also among Danish blood-donors, and extremely rare among symptomatic European patients. B19 type 3 has been encountered endemically in Ghana and (apparently) in Brazil, and sporadic cases have been detected in France and the UK.

We next examined the biological characteristics of these virus types. The p6 promoter regions of virus types 1-3 were cloned in front of a reporter gene, the constructs were transfected into different cell lines, and the promoter activities were measured. As a result, we found that the activities of the three p6 promoters, although differing in sequence by >20%, were of equal strength, and most active in B19-permissive cells. Furthermore, the infectivity of the three B19 types was examined in two B19-permissive cell lines. RT-PCR revealed synthesis of spliced B19 mRNAs, and immunofluorescence verified the production of NS1 and VP proteins in the infected cells. These experiments suggested similar host-cell tropism and showed that the three virus types are strains of the same species, i.e. human parvovirus B19. Last but not least, the sera from subjects infected in the past either with B19 type 1 or type 2 (as evidenced by tissue persistence of the respective DNAs), revealed in VP1/2- and VP2-EIAs a 100 % cross-reactivity between virus types 1 and 2. These results, together with similar studies by others, indicate that the three B19 genotypes constitute a single serotype.

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List of original publications

This thesis is based on the following original publications:

- I Hokynar K., Brunstein J., Söderlund-Venermo M., Kiviluoto O., Partio E.K., Konttinen Y., Hedman K. Integrity and full coding sequence of B19 virus DNA persisting in human synovial tissue. *Journal of General Virology* 2000; 81, 1017-1025.
- II Hokynar, K., Söderlund-Venermo, M., Kiviluoto, O., Partio, E.K., Hedman, K. A new parvovirus genotype persistent in human skin. *Virology* 2002; 302: 224-228.
- III Hokynar, K., Norja, P., Laitinen, H., Palomäki, P., Garbarg-Chenon, A., Ranki, A., Hedman, K. and Söderlund-Venermo M. Detection and differentiation of human parvovirus variants by commercial quantitative real-time PCR tests. *Journal of Clinical Microbiology* 2004; 42:1095-1106.
- IV Ekman, A. *, Hokynar, K. *, Kakkola, L., Kantola, K., Hedman, L., Bondén, H., Gessner, M., Aberham, C., Norja, P., Miettinen, S., Hedman, K. and Söderlund-Venermo, M. Biological and immunological relation of human parvovirus B19 types 1-3. *Journal of Virology* 2007; 81:6927-35.
*equal contribution

The publications are referred to in the text by their Roman numerals.

Abbreviations

A6	DNA isolate of B19 type 2	NHLF	normal human lung fibroblasts
aa	amino acid	nm	nanometer
AAV	adeno-associated virus	NS1	non-structural protein of B19
AIDS	acquired immunodeficiency syndrome	nt	nucleotide
AMDV	aleutian mink disease virus	NTP	nucleotide triphosphate
Au	B19 type 1 isolate	ORF	open reading frame
B19	human parvovirus B19	p6	B19 promoter
BFU-E	erythroid burst-forming units	PARV4	Parvovirus 4
BM	bone marrow	PARV5	Parvovirus 5
CP	crossing point	PCR	polymerase chain reaction
bp	base pair	pi	post infection
BPV	bovine parvovirus	PLA ₂	phospholipase 2
CDS	coding sequence	PPV	porcine parvovirus
CFU-E	erythroid colony forming units	qPCR	quantitative polymerase chain reaction
CnMV	canine minute virus	RA	rheumatoid arthritis
CPV	canine parvovirus	RT-PCR	reverse-transcription PCR
D91.1	DNA isolate of B19 type 3	S/D plasma	solvent-detergent treated plasma
DNA	deoxyribonucleotide	SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
EI	erythema infectiosum	SPR3	B19 type 1-containing plasma
EIA	enzyme immuno assay	SPV	simian parvovirus
ETS	epitope-type specificity (of antibodies)	ssDNA	single-stranded DNA
FH	fulminant hepatitis	SV40	simian virus 40
FPV	feline parvovirus	T _m	melting temperature
FRET	fluorescence resonance energy transfer	V9	DNA isolate of B19 type 3
HA	hemagglutination assay	VLP	virus-like particle
HAA	hepatitis-associated aplastic anemia	VP	viral protein
HaAM	DNA isolate of B19 type 2	VP1	structural protein of B19
HBoV	human bocavirus	VP1/2	virus-like particle containing VP1 and VP2
HBV	hepatitis B-virus	VP1u	unique region of VP1 protein
HCV	hepatitis C-virus	VP2	structural protein of B19
HIV	human immunodeficiency virus	Wi	B19 type 1 isolate
HUVEC	human umbilical vein endothelial cells	Å	Ångström
IC	internal control		
ICTV	International Committee on Taxonomy of Viruses		
ICTVdb	the Universal Virus Database of the ICTV		
IF	immunofluorescence		
IgG	immunoglobulin G		
IgM	immunoglobulin M		
IM-81	B19 type 2-containing serum		
ITR	inverted terminal repeat		
IVDU	intravenous drug user		
kb	kilobase		
kDa	kilodalton		
LaLi	DNA isolate of B19 type 2		
LC-PCR	light-cycler PCR		
mRNA	messenger ribonucleic acid		
MVM	minute virus of mice		
NAN	B19 type 1-containing serum		

Review of the literature

Parvoviruses

Parvoviridae

Parvoviruses are among the smallest DNA viruses able to infect mammalian cells: the family name originates from the Latin word *parvum*, meaning small. Parvoviruses are ubiquitous among animal kingdom, infecting vertebrates as well as insects. The unique feature among viruses belonging to the family *Parvoviridae* is that their genome is a linear and single-stranded DNA molecule (Crawford *et al.*, 1969; Rose *et al.*, 1969; Tattersall, 2006). The genomic DNA molecule is usually 4-6 kb in size, and contains at both ends palindromic sequences that can be folded into hairpin structures essential for viral DNA replication. These terminal sequences can either be identical or different from each other (Tattersall, 2006).

Parvoviruses are, in general, structurally very simple, consisting of the DNA genome that is packed in a protein shell. Virions of all parvoviruses lack lipids, and thus are resistant to inactivation by organic solvents. None of the viral proteins are known to be glycosylated (Muzyczka and Berns. 2001; Tattersall, 2006). The three-dimensional structures of the virions have been determined for several parvoviruses, such as canine parvovirus (CPV) (Tsao *et al.*, 1991), adeno-associated virus 2 (AAV2) (Xie *et al.*, 2002), minute virus of mice (MVM) (Agbandje-McKenna *et al.*, 1998; Kontou *et al.*, 2005), feline panleukopenia virus (FPV) (Agbandje *et al.*, 1993), porcine parvovirus (PPV) (Simpson *et al.*, 2002) and human parvovirus B19 (Agbandje *et al.*, 1991; Agbandje *et al.*, 1994; Kaufmann *et al.*, 2004). The capsid shell is icosahedral, non-enveloped and only ~ 18-26 nm in diameter. It is composed of 60 capsid protein particles arranged with T=1 symmetry, usually the smallest of the VP proteins (VP1-4, depending on the virus species) being the major component. The viral proteins are alternative forms of the same gene product, predominantly differing in the N-terminus, and are translated from alternatively spliced mRNAs or result from posttranslational cleavage (Muzyczka and Berns. 2001; Tattersall, 2006). The main structural motif is an eight-stranded anti-parallel β -barrell, which is a highly conserved structure among most viral capsid structures (Rossmann and Johnson, 1989). The insertions between the strands of the β -barrell form large loops on the capsid surface, and differ between distinct parvovirus species (Muzyczka and Berns. 2001).

Parvovirinae

The *Parvoviridae* family is further divided into two sub-families on the basis of host range: those infecting vertebrates comprise the *Parvovirinae* subfamily, and those infecting insects and other arthropods compose the *Densovirinae* subfamily (ICTVdB - The Universal Virus Database, version 4.; Tattersall *et al.* 2005). Viruses of the former family each infect a single type of vertebrate host. Members of the latter group are not discussed further within this thesis.

The genome organisation of all parvoviruses is similar, and of all members of the *Parvovirinae*, monosense (Tattersall, 2006). The region between the terminal palindromes contains two open reading frames: one half contains the ORF encoding the non-structural proteins required for DNA replication, and the other half contains the ORF encoding the structural proteins of the capsid (Astell *et al.*, 1983; Ozawa *et al.*, 1987; Srivastava *et al.*, 1983). In some parvoviruses, other minor ORFs have been detected. Some parvoviruses preferentially encapsidate ssDNA of negative polarity (the strand that is complementary to mRNA), e.g. MVM, (Crawford *et al.*, 1969), while others may encapsidate ssDNA molecules of either polarities, e.g. AAV (Berns and Adler, 1972; Rose *et al.*, 1969). Depending on the virus, there might be one, two or three promoters for mRNA transcription. Further, some parvoviruses are capable of autonomous replication, while others depend on co-infection of a helper-virus. According to these structural and biological properties, and subsequently supplemented by computer-based phylogenetic analysis, members of the *Parvovirinae* subfamily are further divided into five genera: *Parvovirus*, *Erythrovirus*, *Dependovirus*, *Amdovirus* and *Bocavirus* (ICTVdB - The Universal Virus Database, version 4.; Tattersall *et al.* 2005; Tattersall, 2006).

Parvovirus

The type species of the *Parvovirus* genus is the minute virus of mice (MVM). The majority of the species within the *Parvovirus* genus encapsidate the negative-strand DNA genome (except LuIII where both polarities are encapsidated in equimolar amounts) (Bates *et al.*, 1984; Muzyczka and Berns. 2001). The hairpin structures at both ends of the genome are different from each other in sequence, length, and in predicted structure (Astell *et al.*, 1983). Transcription is initiated by two promoters, at map unit ~4 and ~40, and terminated in one polyadenylation site located near the 5' end (Pintel *et al.*, 1983).

Parvoviruses are autonomously replicating viruses that do not require aid from a helper-virus. Because of the limited coding capacity, however, the major requirement is that the host cell must undergo the S phase, in order to accomplish a productive parvovirus infection. Therefore, the host-cell-range of parvoviruses is narrow.

Dependovirus

The Dependoviruses encapsidate both negative- and positive-strand DNA in equivalent amounts (Berns and Adler, 1972; Rose *et al.*, 1969). The hairpin structures at both ends of the genome are identical, each containing inverted terminal repeats of 145 nt, of which the distal 125 nts form a palindromic sequence (Berns and Hauswirth. 1984; Lusby *et al.*, 1980). Transcription of dependoviruses appears to be initiated from three promoters located at map units ~5, ~19 and ~40, and terminated in one polyadenylation site near the 5' end (Green and Roeder, 1980; Lusby and Berns, 1982).

The helper-virus dependent parvoviruses were originally grouped together to form the *Dependovirus* genus. It has been shown that for efficient replication, these viruses need simultaneous co-infection of another virus, usually adeno or herpes viruses. Indeed, the dependoviruses were initially discovered as contaminants of purified adenovirus stocks, and they were therefore named adeno-associated viruses (AAV) (Atchison *et al.*, 1965). Infection without helper virus has been shown to result in a persistent latent infection. During such latent-phase of infection, at least AAV2 has been shown to integrate site-specifically into the 13.4q-qter on the host's chromosome 19 (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Kotin *et al.*, 1992; Samulski *et al.*, 1991). In some *in vitro* conditions (some chemical carcinogens and UV irradiation), however, AAV has been shown to replicate independently, without the aid of helperviruses (Yakobson *et al.*, 1987). For example, helper-virus free AAV replication has been observed in raft cultures of epithelial cells (Meyers *et al.*, 2000). After phylogenetic analysis, the autonomously replicating goose and duck parvoviruses are also placed within the dependovirus genus (ICTVdB - The Universal Virus Database, version 4.).

Amdovirus and Bocavirus

By phylogenetic analysis, two new genera, *Amdovirus* and *Bocavirus*, within the subfamily of *Parvovirinae* were recently created (ICTVdB - The Universal Virus Database, version 4.). The *Amdovirus* genus contains only one species, the Aleutian mink disease virus (AMDV), while the *Bocavirus* genus contains two species, bovine parvovirus (BPV) and canine minute virus (CnMV) (hence the name *bovine* and *canine*). All members are autonomously replicating viruses that were earlier classified in the *Parvovirus* genus.

The AMDV genome is a negative strand DNA of 4.8 kb containing different palindromic sequences at each end (Alexandersen *et al.*, 1988; Bloom *et al.*, 1980; Hahn *et al.*, 1983). The major distinguishing feature of AMDV is the VP1 N-terminus, which is reasonably shorter than those of other parvoviruses, and, in contrast to that of most other parvoviruses, lacks a phospholipase A₂ enzymatic core (Tattersall *et al.* 2005).

The genome of bocaviruses is slightly larger than that of AMDV, ~5.5 kb, and has different sequences at the two genomic ends (Chen *et al.*, 1986; Schwartz *et al.*, 2002), and, unlike any other parvoviruses, encodes a nuclear phosphoprotein NP1 with unknown function(s) (Chen *et al.*, 1986; Lederman *et al.*, 1984; Schwartz *et al.*, 2002; Tattersall *et al.* 2005).

Erythrovirus

Human parvovirus B19 is the type species of *erythrovirus* genus. It was found in 1974 in serum of an asymptomatic blood donor while screening for hepatitis B virus surface antigen (Cossart *et al.*, 1975). Electron microscopy revealed the presence of ~23 nm particles resembling parvoviruses. The original sample was coded “number 19 in panel B”, whereby the virus was subsequently named B19. Characterisation of the genome showed that it was a single-stranded DNA of ~5.6 kb in length, and the complementary strands were shown to be encapsidated in separate virions. After complete sequencing of the genome, it was definitively established that it was a parvovirus, and B19 was officially accepted as a member of the *Parvoviridae* by the International committee of Taxonomy of Viruses (ICTV) in 1985 (Brown, 2006). However, the B19 genome contains some unique features: the terminal repeats are identical and considerably longer than those of the other parvoviruses (Deiss *et al.*, 1990), and there is only a single promoter (Doerig *et al.*, 1987) but two transcription termination (polyadenylation) sites (Ozawa *et al.*, 1987). Eventually, phylogenetic analysis showed that B19 did not cluster either with species within the *Parvovirus* or *Dependovirus* genera. Therefore a new genus was created and, because of the extreme tropism of B19 for the erythroid lineage, it was named *Erythrovirus* (Tattersall, 2006). At first, B19 was the sole member of the new genus, but subsequently, related viruses were isolated in cynomolgus monkeys (simian parvovirus, SPV) (O'Sullivan *et al.*, 1994), in Manchurian chipmunks (chipmunk parvovirus) (Yoo *et al.*, 1999), and rhesus- and pig-tailed macaques (pig-tailed and macacue parvoviruses) (Green *et al.*, 2000), and are now classified as erythroviruses (ICTVdB - The Universal Virus Database, version 4.).

New human parvoviruses

For a long time B19 was considered the only human pathogen of its family. Recently, a new human parvovirus was found in respiratory tract samples. By sequencing this virus was shown to be related to the species within the genus *Bocavirus*, and was thus named human bocavirus (HBoV) (Allander *et al.*, 2005). Subsequently HBoV has been shown to be prevalent in respiratory tract samples of small children with severe lower respiratory

tract disease in several laboratories around the world (Bastien *et al.*, 2007; Kesebir *et al.*, 2006; Ma *et al.*, 2006; Manning *et al.*, 2006; Sloots *et al.*, 2006), and occasionally also in adults (Kupfer *et al.*, 2006; Maggi *et al.*, 2007). The virus has been associated with a variety of respiratory conditions, e.g. fever, cough, bronchiolitis, pneumonia and wheezing, and thus seems to be another human pathogen among parvoviruses. However, in many cases co-infections with other respiratory viruses have been observed, complicating the determination of the role of HBoV in the pathogenicity in those subjects (Mahy, 2006).

Another new virus was discovered in plasma of an HIV negative, HBV positive intravenous drug user (IVDU) with fatigue, night sweats, pharyngitis, neck stiffness, vomiting, diarrhea, arthralgia and confusions (Jones *et al.*, 2005). The newly found virus resembled parvoviruses by DNA sequence, and was named parvovirus 4 (PARV4). Phylogenetic analysis indicated that PARV4 was not related closely to any of the previously known parvovirus groups, but situated closest to the erythro- and dependoviruses. Subsequently, PARV4 and a related variant PARV5 have been found in pooled plasma used for the manufacture of plasma products (Fryer *et al.*, 2006). In a recent study, PARV4 and PARV5 were detected in serum of HCV-positive IVDUs, but not in non-IVDUs (Fryer *et al.*, 2007). Although this might point to transmission via blood or plasma, as has been shown for B19, little is known about the role of these viruses in human disease.

Table 1: Classification of the family *Parvoviridae* (ICTVdB - The Universal Virus Database, version 4.; Tattersall *et al.* 2005).

Subfamily	Genus	Type species
<i>Densovirinae</i>	<i>Brevidensovirus</i>	<i>Aedes aegypti densovirus</i>
	<i>Densovirus</i>	<i>Junonia coenia densovirus</i>
	<i>Iteravirus</i>	<i>Bombyx mori densovirus</i>
	<i>Pefudensovirus</i>	<i>Periplaneta fuliginosa densovirus</i>
<i>Parvovirinae</i>	<i>Dependovirus</i>	<i>Adeno-associated virus 1</i>
		<i>Adeno-associated virus 2</i>
		<i>Adeno-associated virus 3</i>
		<i>Adeno-associated virus 4</i>
		<i>Adeno-associated virus 5</i>
		<i>Avian adeno-associated virus</i>
		<i>Bovine adeno-associated virus</i>
		<i>Goose parvovirus</i>
	<i>Duck parvovirus</i>	
	<i>Erythrovirus</i>	<i>Human parvovirus B19</i> (Strains: A6, Au, LaLi, V9, Wi)
		<i>Pig-tailed macaque parvovirus</i>
		<i>Rhesus macaque parvovirus</i>
		<i>Simian parvovirus</i>
	<i>Parvovirus</i>	<i>Feline panleukopenia virus/ Canine parvovirus H-1 virus Kilham rat virus Minute virus of mice Porcine parvovirus</i>
	<i>Amdovirus</i>	<i>Aleutian mink disease virus</i>
	<i>Bocavirus</i>	<i>Bovine parvovirus Canine minute virus</i>

Human parvovirus B19

Molecular biology

Virion

The B19 virion is simple, composed of only two proteins and a linear, single stranded DNA molecule. Unlike in most of the other autonomous parvoviruses, genomes of either negative or positive sense are encapsidated in equal amounts (Summers *et al.*, 1983). The non-enveloped capsid is only ~22 nm in diameter, and has a molecular weight of $\sim 5.6 \times 10^6$ da, of which ~80% consists of protein and ~20% of DNA. The buoyant density in a cesium chloride gradient has been measured to be ~1.43 g/ml (Clewley, 1984). The simple structure and the absence of a lipid envelope make B19 extremely difficult to inactivate: the virus is stable at 56°C for 60 minutes, it is not susceptible to pH (stable within the pH range 3-9), and resists lipid solvents. It can be inactivated by formalin, β -propiolactone and oxidizing agents (Muzyczka and Berns. 2001).

B19 particles are icosahedrons (20-sided) and made up of 60 copies of the capsid proteins VP1 and VP2, arranged in T=1 symmetry. VP2 is the major capsid protein of 58 kDa, comprising approximately 96 % of the virion structure (Ozawa and Young, 1987). VP1 is the minor capsid protein comprising about 4 % of the virion structure (~3 copies / capsid). The structural distribution of VP1 in the mature B19 capsid is not known. Based on its amino acid sequence, VP1 is identical with the major capsid component VP2, except that it contains 227 additional aa at the aminoterminal (= the VP1 unique region, VP1u). This unique region has been reported to be located, at least in part, outside the mature virus capsid (Kawase *et al.*, 1995; Rosenfeld *et al.*, 1992). Recent results, however, suggest that VP1u becomes exposed on the capsid surface only after receptor attachment (Ros *et al.*, 2006).

B19 can not be produced in continuous cell lines in high yields, and therefore the structure of the native B19 capsid has been difficult to study. However, small amounts (in Chinese hamster ovary cells) of capsid structures have been observed to self-assemble from the viral protein components (VP2 alone or VP2 and VP1 together) in the absence of the B19 genome (Kajigaya *et al.*, 1989). Empty B19 capsids have been produced in high yields in a baculovirus expression system (Brown *et al.*, 1991; Kajigaya *et al.*, 1991), and the structure of the recombinant VP2 capsid has been determined by X-ray crystallography, with a resolution of ~ 3.5 Å (Kaufmann *et al.*, 2004). The VP2 capsid was

shown to contain the common eight-stranded antiparallel β -barrel motif, but to differ from the other autonomous parvoviruses in surface topology.

Genome

The B19 genome of one isolate (Wi), was first characterized in 1984 (Cotmore and Tattersall, 1984), and two years later, a second isolate (Au) was described (Shade *et al.*, 1986). The B19 genome was shown to be a linear single-stranded DNA molecule of 5596 nucleotides (nt). After successful cloning of the genomic ends, they were shown to contain identical inverted terminal repeats (ITR) of ~380 nt that are self-complementary and able to form double-stranded hairpin loops, and consist mainly of GC-pairs (Astell and Blundell, 1989; Deiss *et al.*, 1990; Zhi *et al.*, 2004). The distal ~365 nt of the ITRs are imperfect palindromes, with a few base asymmetries, leading to two distinct configurations of which one is the reverse-complement of the other, and which are referred to as flip and flop. The few unpaired nucleotides form “bubbles” in the double stranded hairpin structure. The size and the positions of these bubbles seem to be conserved among different B19 isolates (Deiss *et al.*, 1990; Zhi *et al.*, 2004). The ITRs are used as primers for the complementary strand synthesis during replication, and by experiments with clones containing full-length or truncated forms of ITRs, it has been shown that the hairpin structure is essential for B19 replication (Zhi *et al.*, 2004). For adeno-associated virus 2 (AAV2), the ITRs, resembling those of the B19 virus, have also been reported to be essential for encapsidation and integration into the hosts chromosome (Wang *et al.*, 1996).

Replication mechanism

B19 genome replication takes place in the host cell nucleus. Because of the small size and restricted coding capacity of the genome, parvoviruses do not possess their own replication machinery but rely completely on that of the host cell. The unique feature of parvoviruses is the single stranded, linear DNA genome. Because DNA polymerases synthesize only in 5'-to 3'- direction and need a basepaired primer, copying of the extreme 3'-end of a linear DNA molecule is not simple. Therefore parvoviruses use a mechanism called “rolling-hairpin replication” (Cotmore and Tattersall, 2006). The replication mechanism in those parvoviruses containing distinct ITRs at the two genomic ends (e.g. MVM) is more complex than in those containing identical ITRs (e.g. AAV). Since the B19 genome resembles that of AAV in that the terminal sequences are identical, B19 probably uses a genome replication mechanism similar to that of AAV (Fig. 1).

AAV replication (Berns and Hauswirth, 1984) is initiated by using the 3'-terminal hairpin as primer for complementary strand synthesis. Elongation begins at the 3'-OH group of the folded hairpin structure. As a polymerase completes the synthesis of the complementary strand, a duplex form of the parental strand is formed, which is covalently linked at one end. Then, a single strand cut is created exactly opposite of the original 3'-parental terminus. This nicking is performed by homodimers of the viral NS1 protein, and occurs via a nucleolytic transesterification reaction that liberates a 3'-nucleotide to further prime the polymerase. The hairpin structure is then melted, and the original 3'-end of the parental strand now forms the 5'-end of the progeny strand. The parental strand is then repaired, resulting in displacement and inversion of the original (parental) 3'-terminal sequence.

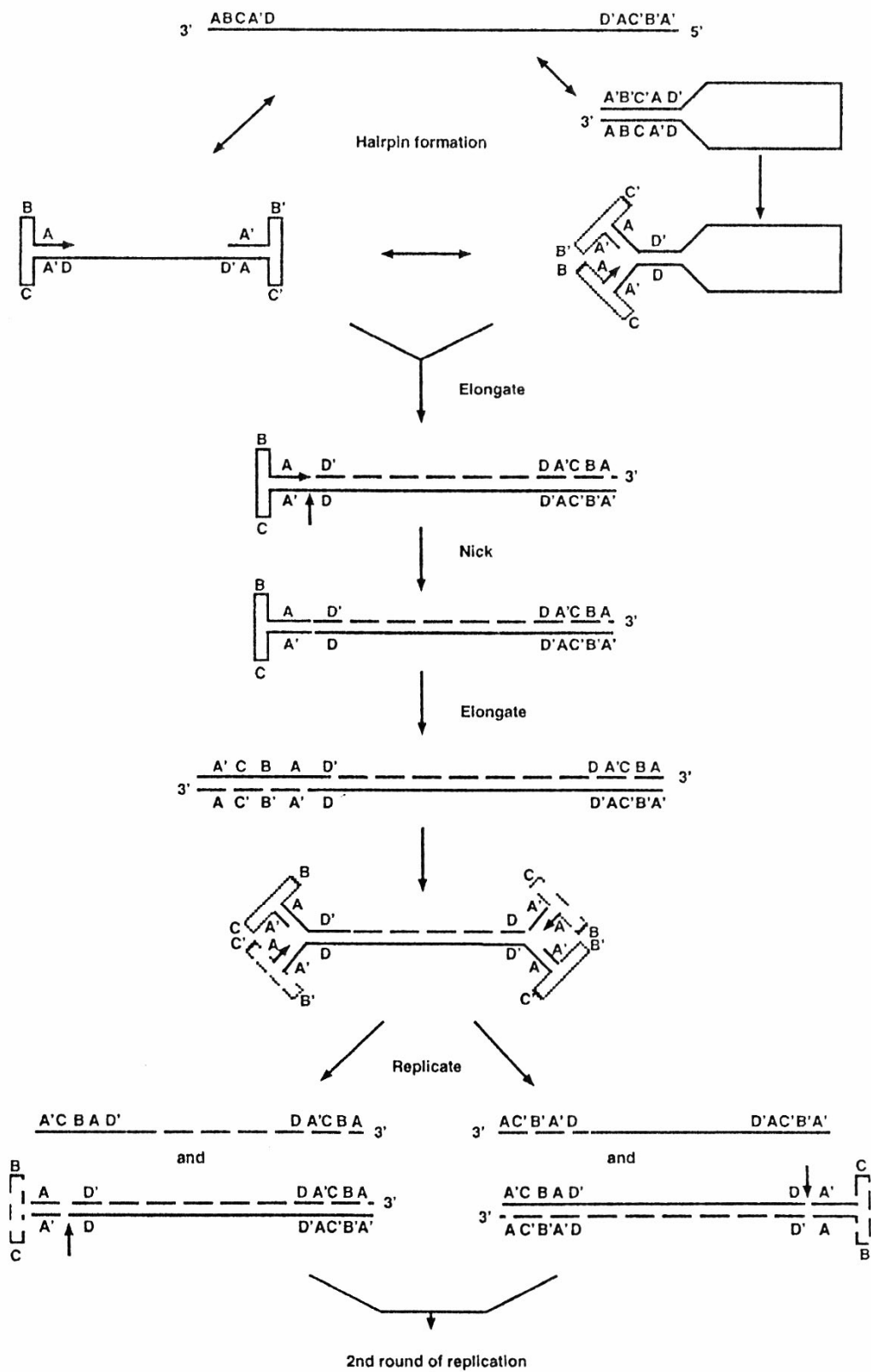


Figure 1: Replication mechanism of AAV, modified from (Berns and Hauswirth, 1984). From Soderlund (1996), with permission.

Transcription

In the B19 genome, open reading frames are present only on the negative polarity strand, with arrangement common to all parvoviruses: the 3' side of the genome encodes the non-structural protein NS1 and the 5' side encodes the two capsid proteins VP1 and VP2 (Cotmore *et al.*, 1986; Ozawa *et al.*, 1987; Ozawa *et al.*, 1988b). As with other parvoviruses, the two structural proteins of B19, VP1 and VP2, are encoded by the same reading frame so that the gene for VP2 is completely included in that of VP1 (Ozawa *et al.*, 1988b). The VP1 gene contains additional 681 nucleotides that encode the VP1 unique region of 227 amino acids in the aminotermus. The capsid protein transcripts are produced by alternative splicing. Two additional genes for small non-structural proteins, the 7.5 and 11 kDa proteins, are present in the center and at the 5' end of the genome (Cotmore *et al.*, 1986; Luo and Astell, 1993; Ozawa *et al.*, 1987; Ozawa *et al.*, 1988b; St Amand *et al.*, 1991; St Amand and Astell, 1993).

The only active promoter, p6, is located at the 3'-palindrome at map unit 6 and thus regulates the synthesis of all nine transcripts (Doerig *et al.*, 1987). According to the Au reference sequence, nucleotides 258-321 have been shown to be necessary for in vitro transcriptional activity in HeLa cells (Blundell *et al.*, 1987). In the same study, the translational start site (ATG) was recognized at nucleotide 350-351, downstream from the TATA-box. Although several TATA-elements are located in the middle of the genome, no other active B19 promoters have been recognized (Brown, 2006; Liu *et al.*, 1991a; Ozawa *et al.*, 1987; Shade *et al.*, 1986).

The p6 promoter strength has been shown to be regulated by cellular factors binding to the upstream enhancer elements (Figure 2) (Blundell and Astell, 1989; Liu *et al.*, 1991b, Raab *et al.* 2001). Nucleotides 100-190 and 233-298 have been shown to be particularly important for p6 activity (Gareus *et al.*, 1998). Transcription regulation is a highly complicated and not fully understood event, in which a number of cellular factors have been shown to be involved: the two GC-rich elements tandemly present upstream of the TATA-box have been shown to be important through binding of the cellular transactivating factors Sp1 and Sp3 (Blundell and Astell, 1989; Raab *et al.*, 2001). Three strong binding sites for another transcriptional regulator, YY1 (Shimomura *et al.*, 1993), and a core motif for Ets family proteins have also been determined (Vassias *et al.*, 1998). More recently, Raab *et al.* demonstrated binding of the Oct-1 protein to an octamer motif (Raab *et al.*, 2001). They also observed interaction of Sp3 with one of these boxes, and suggested that the ratio of bound Sp1 and Sp3 might be involved in the regulation of p6 activity.

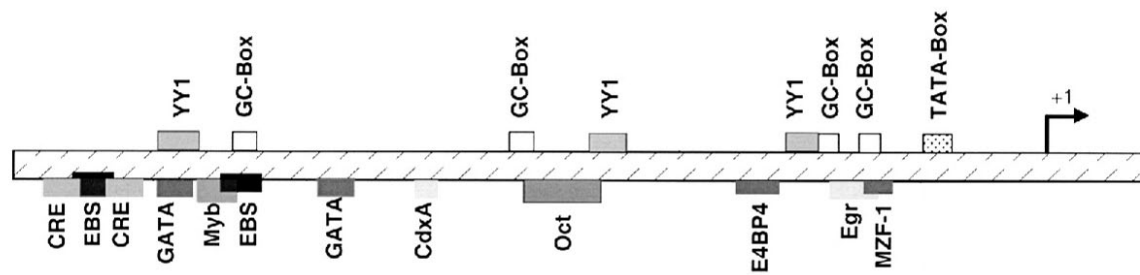


Figure 2. Binding sites for cellular factors on the B19 p6 promoter. From Raab *et al.* (2001), with permission.

In addition to the cellular factors, the viral NS1 protein has been shown to take part in regulation of the p6 promoter activity (Doerig *et al.*, 1990). For NS1 transactivation, nucleotides 100-160 and the two GC boxes adjacent to the TATA-box have been shown to be essential (Gareus *et al.*, 1998). NS1 has been suggested to bind to DNA directly, since it has been shown that oligonucleotides containing nucleotides 126-162 could be immunoprecipitated with anti-NS1 antibodies, as well as through cellular factors (Brown, 2006; Raab *et al.*, 2002).

The p6 promoter activity was initially shown not to be restricted to B19 permissive cells (Liu *et al.*, 1991a). However, by transfection of expression vectors, carrying the p6 promoter and the upstream enhancer elements upstream of a reporter gene (luciferase of *Pothonus pyralis*), into different cell lines, the p6 transcriptional activity was shown to be higher in permissive cell lines than in cell lines non-permissive for B19 (Gareus *et al.*, 1998).

All nine B19 transcripts (Figure 3.) contain a short 5' leader sequence of ~60 bases (Blundell *et al.*, 1987; Doerig *et al.*, 1990; Ozawa *et al.*, 1987; St Amand *et al.*, 1991), corresponding to nucleotides 350-406 according to the Au-reference sequence (Shade *et al.*, 1986). The corresponding region has been shown to be important for transcriptional activity (Gareus *et al.*, 1998). All but one of the transcripts contain large introns (Ozawa *et al.*, 1987). The single non-spliced transcript encodes the non-structural protein (NS1), and the other eight transcripts, produced by differential splicing events, encode the two structural proteins VP1 and VP2, and the two smaller proteins of 7.5 kDa and the 11 kDa, of incompletely known functions. Two separate polyadenylation sites are used, one in the middle of the genome, (pA)p, for the NS1- and 7.5 kDa- transcripts, and another in the 5' end for the transcripts encoding the structural and 11 kDa proteins. Polyadenylation at the (pA)p site prevents the RNA polymerase from including the VP1/2 ORF in the transcript. Therefore, polyadenylation at this site must be incomplete: indeed, polyadenylation at the

(pA)_p uses a non-consensus cleavage and polyadenylation specificity factor-binding hexanucleotide AUUAAA, to allow for efficient readthrough of p6-generated pre-mRNAs into the VP1/2 coding region. Both upstream and downstream cis-acting elements and an adjacent AAUAAC motif are involved in controlling the relative expression rates of the non-structural and structural genes, which has been suggested to play a role in B19 tropism (Liu *et al.*, 1992; Yoto *et al.*, 2006). In cells non-permissive for B19 replication, NS1 transcripts have been shown to predominate, while in B19 permissive cells the capsid transcripts predominate (Leruez *et al.*, 1994). Blocking of NS1 protein production in a B19 permissive cell line UT-7 has been shown to abolish capsid protein mRNA transcription (Shimomura *et al.*, 1993).

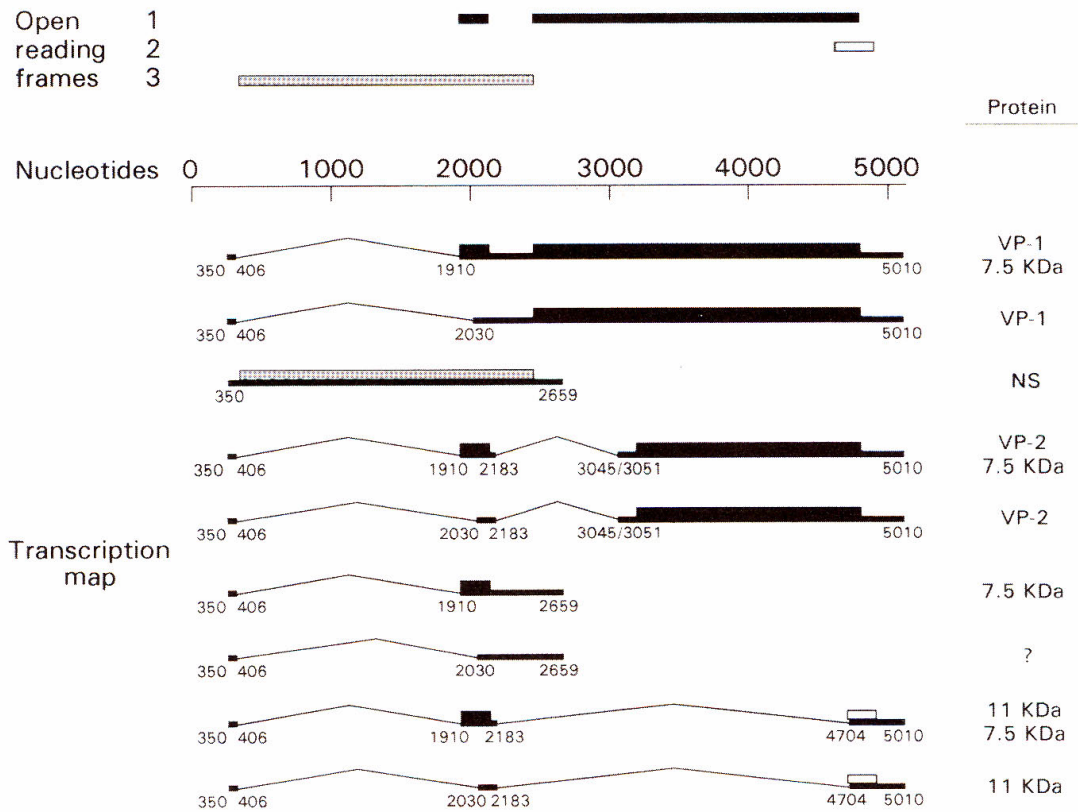


Figure 3: Transcription map for human parvovirus B19. From Brown (2006), reproduced by permission of Edward Arnold (Publishers) Ltd.

Proteins

Structural proteins: The two structural proteins of B19 are VP1 (84 kDa) and VP2 (58 kDa). VP2 is the major capsid component, and mediates receptor binding (Brown *et al.*, 1993). In the infected cells both capsid proteins are localized in the cytoplasm and also in the nucleus, where it was suggested to be transported by aid of a non-conventional nuclear localization signal at the carboxyl sequence of VP2 (Pillet *et al.*, 2003). Both VP1 and VP2 have been shown to contain epitopes for neutralizing antibodies (Kajigaya *et al.*, 1991; Sato *et al.*, 1991). The unique region of VP1 contains a conserved secreted phospholipase A₂, which has been identified in several members of the *Parvoviridae* (Zadori *et al.*, 2001). The PLA₂ motifs have been shown to be functional in B19 (Dorsch *et al.*, 2002) and other parvoviruses (Zadori *et al.*, 2001), and are probably required for escape from late endosomes during viral trafficking to the nucleus after receptor mediated entry (Suikkanen *et al.*, 2003; Zadori *et al.*, 2001).

Non-structural protein NS1: The major non-structural protein of B19 virus, NS1, is of 671 aa in size and with a molecular weight of 77 kDa. NS1 has multiple functions. As described, it acts as a transcription transactivator both by binding to the p6 promoter DNA directly as well as via cellular transcription factors (Raab *et al.*, 2002). The motifs associated with single-strand nicking activities (Ding *et al.*, 2002), and the region containing the predicted ATPase / helicase domains and nucleoside triphosphate binding (NTP) domains (Moffatt *et al.*, 1998; Momoeda *et al.*, 1994b) are relatively conserved among parvoviruses and are assumed to function during viral replication.

NS1 is located in the nucleus of the infected cell, (Ozawa and Young, 1987), and has been shown to be cytotoxic and to block cellular proliferation (Ozawa *et al.*, 1988a; Srivastava *et al.*, 1990). By mutation analysis, the NTP-binding domain of NS1 has been shown to be important for cytotoxicity (Moffatt *et al.*, 1998; Momoeda *et al.*, 1994b), which in erythroid lineage cells and liver-derived cells is likely accomplished via induction of apoptosis (Moffatt *et al.*, 1998; Poole *et al.*, 2004; Poole *et al.*, 2006; Sol *et al.*, 1999).

Small proteins: In addition to the two main open reading frames (ORF) encoding the non-structural and capsid proteins, the B19 genome contains additional small ORFs, on the far right side of the genome, and in the middle (Ozawa *et al.*, 1987). Two of these ORFs (in the middle and on the right) contain an in-frame ATG codon, and produce small proteins of 7.5 and 11 kDa (Luo and Astell, 1993; St Amand *et al.*, 1991; St Amand and Astell, 1993). Although the exact roles of these proteins are yet not known, in studies using an infectious B19 clone, the 11 kDa protein participated in regulating the relative production rate of the B19 capsid proteins (Zhi *et al.*, 2006). However, the third potential small ORF, in the middle of the genome, has not been shown to produce protein (Brown, 2006).

Cell tropism

The B19 virus shows extreme tropism for the erythrocyte precursors CFU-E and BFU-E in bone marrow (Ozawa *et al.*, 1986; Srivastava and Lu, 1988), but has also been found in fetal myocardium and liver (Naides and Weiner, 1989). B19 can be cultured in primary erythroid progenitor cells derived from bone marrow (Ozawa *et al.*, 1986), fetal liver (Brown *et al.*, 1991; Yaegashi *et al.*, 1989) and umbilical cord blood (Sosa *et al.*, 1992; Srivastava *et al.*, 1992). B19 has been propagated also in a few cell lines such as the human megakaryocytic leukemia cell line MB-02 (Munshi *et al.*, 1993), the human erythroblastoid cell line UT7/Epo (Shimomura *et al.*, 1992), and the erythroid leukemia cell lines KU812Ep6 (Miyagawa *et al.*, 1999) and JK-1 (Takahashi *et al.*, 1993), although

the yields of progeny viruses in these cell lines are low. In addition, low-level replication has recently been reported also in a non-erythroid cell line U932 (Munakata *et al.*, 2006). Several features are associated with the B19 host-cell range (supporting productive infection), such as the cellular receptor, the blood-group P antigen (Brown *et al.*, 1993) and co-receptors Ku80 (Munakata *et al.*, 2005) and $\alpha 5\beta 1$ integrin (Weigel-Kelley *et al.*, 2003), intracellular transcription factors (Gareus *et al.*, 1998; Raab *et al.*, 2001; Vassias *et al.*, 1998), and the relative production rate of the structural and non-structural proteins in the infected cells (Liu *et al.*, 1992). The levels of protein types are possibly regulated via atypical mRNA splicing (Brunstein *et al.*, 2000), impaired ribosome loading of structural gene transcripts (Liu *et al.*, 1992; Pallier *et al.*, 1997), polyadenylation sites (Yoto *et al.*, 2006), or the 11-kDa protein function (Zhi *et al.*, 2006).

Receptor and co-receptors

The receptor for B19 is the blood group P antigen or globoside (globotetraosylceramide GalNAc(β 1-3)Gal(α 1-4)Gal(β 1-4)GlcCer) (Brown *et al.*, 1993). As other autonomously replicating parvoviruses, B19 agglutinates red cells of primate origin. As hemagglutination was observed also by using baculovirus-produced recombinant VP2 capsids, it was suggested to be mediated by the viral VP2 protein (Brown and Cohen, 1992). Hemagglutination, as well as infectivity of B19 virus in a hematopoietic colony assay, was inhibited by purified globoside (Brown *et al.*, 1993; Kaufmann *et al.*, 2005). In later studies, however, no binding of membrane-associated globoside to B19 capsids were observed (Kaufmann *et al.*, 2005).

The erythrocytes of subjects of the rare blood group p phenotype lack the P antigen (frequency ~ 1 in 200 000). In infection studies, bone marrow cells from patients of p phenotype were resistant to B19 infection (Brown *et al.*, 1994), and the P antigen was thus regarded as the cellular receptor for B19 virus. Globoside has been shown to be expressed on erythrocytes, platelets, granulocytes, the lung, fetal heart, synovium, liver, kidney, endothelium, and vascular smooth muscle (Cooling *et al.*, 1995).

It has been suggested that P antigen by itself might not be sufficient for successful infection by B19 virus, because i) mature red cells abundantly express P antigen on their surface, but lack nuclei, a prerequisite for B19 replication and ii) a number of nonerythroid cells express P antigen, yet are nonpermissive for B19 infection. Indeed, subsequent studies have shown that the expression level of P antigen does not correlate with B19 binding, and suggest that the P antigen is necessary for binding but not sufficient for entry of the virus into cells (Weigel-Kelley *et al.*, 2001).

With a recombinant B19 vector (Ponnazhagan *et al.*, 1998), cell-surface binding was detected in all cell types expressing globoside on the plasma membrane, which also in this

study was not sufficient for B19 entry. Expression of marker genes carried by the B19 vector, indicating viral entry and nuclear transport of the recombinant genome, could be seen in two cell lines of epitheloid origin, 293 and HeLa, and also in two human primary cell lines (human umbilical vein endothelial cells, HUVEC, and normal human lung fibroblasts, NHLF), but not in the human erythroleukemia cell lines HEL and K562, despite globoside on their surface. Subsequently, B19 entry was shown to be mediated by $\alpha 5\beta 1$ -integrins in their high-affinity conformation (Weigel-Kelley *et al.*, 2003), regulated in a cell-type specific manner (Weigel-Kelley *et al.*, 2006). Recently, expression of Ku80 on transfected HeLa cells was shown to enhance entry of B19, suggesting that Ku80 mediates efficient B19 entry in cooperation with globoside and probably with $\alpha 5\beta 1$ -integrins (Munakata *et al.*, 2005).

Clinical aspects

Transmission

The B19 virus has been detected in respiratory secretions of viremic patients, and the major route for B19-virus transmission is generally thought to be via aerosol droplets and contaminated surfaces. In one study, B19 was administered intranasally into voluntary healthy adults, who subsequently became viremic and seroconverted (Anderson *et al.*, 1985). During pregnancy, the virus can be transmitted from the infected mother to the fetus (Brown *et al.*, 1984). The B19 receptor is abundant in the human placenta in early gestation, which might provide a pathway for the virus (Jordan and DeLoia, 1999).

Transmission also occurs via blood or plasma products of various kinds (Siegl and Cassinotti, 1998). High seroconversion rates and some cases of symptomatic illness have been due to blood products prepared from B19-containing plasma pools, and rarely by single-donor blood components (Cohen *et al.*, 1997; Jordan *et al.*, 1998; Mortimer *et al.*, 1983; Yee *et al.*, 1995; Zanella *et al.*, 1995). As a non-enveloped virus with small genomic size, B19 is resistant to ordinary physicochemical factors, including the solvent / detergent (S/D) and heat treatments. Because of its minute size, the pathogen is also relatively difficult to remove by filtration (Siegl and Cassinotti, 1998), although it is, at least in part, removable using small pore size filters (Parkkinen *et al.*, 2006; Terpstra *et al.*, 2006).

The plasma pools used for manufacture, in addition to B19 DNA, also contain antibodies. However, in S/D plasma safety studies (Brown *et al.*, 2001), the recipients were shown to seroconvert due to pools containing B19 DNA in high titers (10^7 IU/ml). They also became B19-DNA positive, verifying virus transmission. By contrast,

transfusion of B19 DNA in concentrations below 10^4 IU/ml is not considered to result in detectable seroconversion, with some exceptions (Blumel *et al.*, 2002). Consequently, according to the revised European Pharmacopoeia (2004), plasma pools used for human anti-D immunoglobulin production, must not (after January 1st 2004) contain more than 10^4 IU/ml of B19 DNA. In order to identify such high-titer units, a quantitative DNA detection method is required.

Disease associations

B19 infection is common worldwide. As documented by B19 specific immunoglobulin G (IgG) antibodies in serum, 5-15 % of young children, 60 % of adults, and 85 % of the geriatric population have been infected with this virus (Anderson *et al.*, 1986; Cohen and Buckley, 1988). Seasonal variation is seen, B19 infection occurring mainly during late winter or early spring (Bremner and Cohen, 1994; Enders *et al.*, 2007; Heegaard and Brown, 2002). Although the B19 virus is connected with a wide spectrum of disease, large proportion of infections remains subclinical, both in children and in adults (Heegaard and Brown, 2002). In some cases symptoms are nonspecific and indistinguishable from common cold, and some of the clinical manifestations are mild and self-limited. In patients with shortened red cell survival, in the immunocompromised, or in pregnant women, however, the infection may be severe. Variation in host genes has been shown to be related with B19 associated symptoms (Kerr, 2005).

Erythema infectiosum: In 1983, B19 was shown to be the cause of the childhood rash erythema infectiosum (EI), also called “slapped cheek” or fifth disease (Anderson *et al.*, 1984). The connection was later confirmed by experimentally infecting volunteers with the virus (Anderson *et al.*, 1985). In the immunocompetent host, EI is the most common manifestation of B19 infection, and usually appears after viremia (approximately 18 days after infection), simultaneously with appearance of B19 specific IgG antibodies (Heegaard and Brown, 2002). EI is characterized by transient or recurrent rash typically beginning on cheeks, and subsequently spreading to the trunk and limbs. Fluctuations of intensity may be caused by environmental factors such as exposure to sunlight or heat, or physical activity (Naides, 1993). B19 induced rash may be difficult to distinguish clinically from that caused by other viruses. Usually EI is transient and requires no treatment.

Anemia: B19 is erythrotropic and infects the red cell precursors in bone marrow. In subjects with shortened red cell survival, the infection may lead to aplastic crisis (Pattison *et al.*, 1981). In healthy subjects this condition is transient, but in the immunocompromised (e.g. subjects with HIV infection, congenital immunodeficiency, or

those receiving immunosuppressive therapy) the anemia may become chronic (Kurtzman *et al.*, 1989; Kurtzman *et al.*, 1987; Kurtzman *et al.*, 1988).

Arthritis and arthralgia: The association between B19 infection and acute arthralgia or arthritis was established in 1985 (Reid *et al.*, 1985; White *et al.*, 1985). In children with EI the incidence of arthralgia is ~10 % or less, while in adults, arthralgia and arthritis are the most common manifestations, affecting 60 % of women and 30 % of men (Heegaard and Brown, 2002). The onset of joint symptoms coincides with the appearance of B19 specific antibodies, and is therefore presumably immunologically mediated. Arthritis is often symmetrical affecting preferentially the small joints of hands, wrists and knees (Reid *et al.*, 1985; White *et al.*, 1985). In some cases arthropathy may become chronic, and even fulfill the criteria of rheumatoid arthritis (RA) (Naides *et al.*, 1990; White *et al.*, 1985). RA is a chronic, systemic inflammatory connective tissue disorder of unknown causative mechanisms, and no specific diagnostic tests are available. The American College of Rheumatology criteria for classification of rheumatoid arthritis are used to diagnose and classify this disease (Smith and Arnett, 1991).

B19 infection and pregnancy: About 40 % of women of child bearing age do not have B19 specific antibodies, and are thus at risk of acute B19 infection and subsequent transmission of the virus to the fetus. In case of maternal infection, diverse estimations have been reported on the rate of vertical transmission. Fetal infection may be asymptomatic, but has also been associated with fetal anemia, spontaneous abortion, hydrops and death (Brown *et al.*, 1984; Enders *et al.*, 2006). Fetal death with or without hydrops have been observed when maternal infection occurs in early gestation, in approximately 11 % among those infected within the first 22 weeks (Enders *et al.*, 2004; Miller *et al.*, 1998), but in some reports also later in pregnancy (Norbeck *et al.*, 2002). Most fetal complications (hydrops and death) occur within 12 weeks following maternal B19 infection (Enders *et al.*, 2004; Enders *et al.*, 2006; Miller *et al.*, 1998; Yaegashi, 2000).

Others: Other proposed disease associations include myocarditis and dilated cardiomyopathy (Bultmann *et al.*, 2003; Enders *et al.*, 1998; Heegaard *et al.*, 1998; Kuhl *et al.*, 2005a; Kuhl *et al.*, 2005b), pneumonia (Beske *et al.*, 2007; Janner *et al.*, 1994), nephritis (Diaz and Collazos, 2000; Nakazawa *et al.*, 2000), hepatitis and fulminant liver failure (Hillingso *et al.*, 1998; Karetnyi *et al.*, 1999; Langnas *et al.*, 1995; Yoto *et al.*, 1996), neurological disorders including meningoencephalitis, cerebellar ataxia, seizure and stroke (Barah *et al.*, 2001; Barah *et al.*, 2003), and several auto-immune-like diseases such as the gloves and socks syndrome, Hashimoto's thyroiditis, Wegener's granulomatosis and lupus-like syndromes (Lehmann *et al.*, 2003; Nikkari *et al.*, 1994).

Treatment

No specific antiviral drug is available against B19 infection. Usually in the immunocompetent host, treatment is not required, but patients with arthralgia may sometimes be treated with anti-inflammatory drugs. In immunodeficient patients or in subjects with increased turnover of red blood cells, chronic anemia or transient aplastic crisis may be treated with erythrocyte transfusions and intravenous immunoglobulin (IVIG) containing neutralizing antibodies. However, even after IVIG treatment viral clearance is not always complete. Also in the case of fetal hydrops and / or anemia, intrauterine erythrocyte transfusions have been shown to reduce the mortality rate substantially (Broliden *et al.*, 2006; Frickhofen *et al.*, 1990; Hedman *et al.* 2006; Heegaard and Brown, 2002; Kurtzman *et al.*, 1989).

Diagnosis

Due to its extreme cell tropism, B19 virus is difficult to culture in continuous cell lines and thus there are in general no virus isolation methods to be used in diagnostics. Morphological markers (no / low level of mature erythroid precursors, presence of giant pronormoblasts) in BM aspirates or peripheral blood are suggestive for acute B19 infection, but alone are not sufficient for diagnosis (Heegaard and Brown, 2002). Numerous methods have been published for direct detection of B19 virus components: B19 virus in serum can be detected by EM and hemagglutination (Brown and Cohen, 1992; Cossart *et al.*, 1975; Curry *et al.*, 2006); antigens can be detected with monoclonal antibodies by EIA or immunoblot (Manaresi *et al.*, 1999; Schwarz *et al.*, 1988); viral nucleic acid in serum can be detected by direct hybridization e.g. in dot blot format, which is sensitive enough to detect acutely infected subjects with high viral loads, or by PCR, which is far more sensitive and can therefore detect also low-level viremias (Clewley, 1989; Koch and Adler, 1990; Koch *et al.*, 1990). Serological methods are available for detection of antibodies specific for B19 (Broliden *et al.*, 2006; Cohen *et al.*, 1983; Corcoran and Doyle, 2004; Enders *et al.*, 2006; Kaikkonen *et al.*, 1999; Soderlund *et al.*, 1995a; Soderlund *et al.*, 1995b; Soderlund *et al.*, 1997a).

Modern diagnostics of B19 infection is usually based on measurement of B19 IgG and IgM antibodies in blood, and / or detection of B19 DNA by PCR (Broliden *et al.*, 2006). Acute B19 infection is usually diagnosed by the presence of B19-specific immunoglobulin M. Experimental B19 infection of healthy adult volunteers showed, during 5-12 days after exposure, transient viremia, which declined rapidly with the appearance of specific IgM antibodies during the second week after inoculation (Anderson *et al.*, 1985). IgM starts to decline during the second month of clinical onset, but may persist for several months (Anderson *et al.*, 1986; Anderson *et al.*, 1985; Hedman *et al.* 2006). IgG appears by the

end of the third week. IgG specific for VP1 and for conformational epitopes of VP2 persist for life, while IgG recognizing linear epitopes of VP2 are expressed only during the acute phase of B19 infection (Kaikkonen *et al.*, 1999; Soderlund *et al.*, 1995b; Soderlund, 1996). In addition, measurement of IgG avidity and epitope-type specificity can be used for identifying primary infection (Enders *et al.*, 2006; Kaikkonen *et al.*, 1999; Kaikkonen *et al.*, 2001; Soderlund *et al.*, 1995a; Soderlund *et al.*, 1995b).

In diagnosis of the immunocompromized, PCR is often preferential (or complementary) to serology. Positive PCR findings in blood or BM may indicate acute infection. However, detectable levels of B19 DNA have been shown to remain in BM and serum for extended time periods, even in healthy immunocompetent individuals (see below). Quantitative PCR might be of use in distinguishing acute from remote infections; however this issue is under ongoing study (de Haan *et al.*, 2007; Enders *et al.*, 2006; Enders *et al.*, 2007).

B19 DNA in blood and bone marrow

In immunosuppressed individuals unable to produce neutralizing antibodies, B19 persists in blood or in bone marrow (BM), with or without anemia (Kurtzman *et al.*, 1987; Kurtzman *et al.*, 1988; LaMonte *et al.*, 2004). B19 DNA has also been shown to persist in BM of immunocompetent patients with recurrent or chronic arthropathies (Foto *et al.*, 1993; Sasaki *et al.*, 1995) and, to a lesser extent, in healthy subjects (Cassinotti *et al.*, 1997; Heegaard and Brown, 2002). Indeed, recently, Lundqvist *et al.* found among 50 rheumatic subjects, B19 DNA in 0% of blood but in 26% of BM samples (Lundqvist *et al.*, 2005), in contrast to a prevalence in BM of up to 9 % among healthy B19 seropositive individuals as reported earlier (Cassinotti *et al.*, 1997; Heegaard and Brown, 2002) and of 4% among seropositive patients with hematological disorders (Lundqvist *et al.*, 1999). However, this difference might be due to different detections methods, since the patients and healthy controls were not studied in parallel within the same study (Lundqvist *et al.*, 2005).

However, even in the immunocompetent subjects' blood, viral clearance is often slower than previously believed (Musiani *et al.*, 1995). Lindblom *et al.* assessed, more recently, the kinetics of viral clearance after acute B19 infection in 5 immunocompetent subjects with typical symptoms. By quantitative PCR, a rapid decrease in the viral load was observed coincidentally with development of IgG antibodies and resolution of clinical symptoms. However, the DNA levels remained detectable in 4/5 subjects through the 128-week follow-up period (Lindblom *et al.*, 2005). Similarly, in studies among pregnant women, Enders *et al.* observed a drastic decrease in viral loads during the first two weeks of infection (Enders *et al.*, 2006). The DNA levels did not differ between symptomatic and

asymptomatic patients, and the symptoms were mainly self-limited despite persistence of B19 DNAemia. In one patient, however, who maintained arthropathy for 1.5 years, elevated DNA levels ($\sim 10^5$ geq/ml) in serum were seen for >322 days. In this study, B19 DNA was detected for ≥ 5 months in 20/22 patients (Enders *et al.*, 2006). By qualitative and quantitative real-time PCRs, Candotti *et al.* (Candotti *et al.*, 2004) detected a low-level persistence of B19 DNA in blood of ~ 1 % of immunocompetent and nonsymptomatic blood donors, despite the presence of B19-specific IgG antibodies. Similar figures have been reported for pregnant women (Lefrere *et al.*, 2005). In multitransfused (immunocompetent) individuals, the B19-DNA prevalence can be higher and persist, together with B19-IgG antibodies, for up to 5 years (Lefrere *et al.*, 2005).

B19 DNA in tissue

Because B19 often causes transient and sometimes chronic arthropathy, it has been considered a potential etiologic agent for rheumatoid arthritis. B19 DNA has been detected in several studies in the synovial membranes and synovial fluid of patients with arthritis (Cassinotti *et al.*, 1998; Dijkmans *et al.*, 1988; Franssila and Hedman, 2006; Kerr *et al.*, 1995; Nikkari *et al.*, 1995; Pallier *et al.*, 1997; Saal *et al.*, 1992; Soderlund-Venermo *et al.*, 2002; Zakrzewska *et al.*, 2001).

In studies by Söderlund *et al.*, B19 DNA was detected by nested PCR, in the synovia of constitutionally healthy, immunocompetent young adults with joint trauma, and with serological evidence of pre-existing immunity (Soderlund *et al.*, 1997b). This finding showed that mere detection of B19 DNA in the synovial tissue can not be used as a diagnostic marker of chronic arthritis, although it did not exclude the possibility of B19 playing a role in the pathology of these diseases, e.g. through mRNA and protein production.

Also other disease associations of B19 have been sought by detection of viral DNA in corresponding tissues. B19 DNA and capsid proteins were detected in skin of a patient with EI (Schwarz *et al.*, 1994), and the presence of viral components therein was concluded to suggest a direct role of B19 virus in the formation of the exanthematous rash in erythema infection. Later B19 DNA was detected also in skin biopsies derived from subjects with chronic urticaria (Vuorinen *et al.*, 2002). In the latter study, as a control group, skin biopsies from healthy adults were studied in parallel for B19 DNA. The results showed that also a large proportion of healthy controls carried B19 DNA in skin. This work thus verified the phenomenon observed by Söderlund *et al.* (Soderlund *et al.*, 1997b) and expanded it by suggesting that B19 DNA persistence is not restricted to synovial tissue. Subsequently, in addition to skin and synovium, persistence of the B19 genome has been detected in various tissue types, e.g. muscle, testis, liver, tonsils, brain, salivary gland

and heart, (Baskan *et al.*, 2007; Chevrel *et al.*, 2000; De Stefano *et al.*, 2003; Diss *et al.*, 1999; Eis-Hubinger *et al.*, 2001; Gray *et al.*, 1998; Hobbs, 2006; Lotze *et al.*, 2004; Manning *et al.*, 2007; Norja *et al.*, 2006). The B19 genomes persisting in synovial tissue and skin are characterized further in this thesis (I and II).

Sequence variation

At the onset of our studies, the B19 DNA sequence was considered extremely stable with a variation of only ~1-2 %. The near-full-length nucleotide sequence was available from only two blood-derived isolates Wi (Blundell *et al.*, 1987), from an asymptomatic donor in England, and Au (Shade *et al.*, 1986), from a child with aplastic crisis in the USA.

A number of studies focused on finding the possible correlation between sequence variations within the B19 genome with different outcomes of infection. In investigations using restriction site polymorphism (RSP) (Mori *et al.*, 1987; Morinet *et al.*, 1986; Umene and Nunoue, 1990; Umene and Nunoue, 1991), B19 isolates were assorted into several genome types according to the restriction pattern. Partial sequences of the VP1/2 genes i) from sequential isolates from an infected individual or ii) from a single community outbreak or iii) from within the same household were essentially identical (Erdman *et al.*, 1996), while isolates from distinct epidemiological settings showed slightly higher variation. Although some geographical clustering could be seen, genetic differences were minor, and no disease associations were detected.

Comparison of sequences from serum samples of patients with acute or persistent B19 infection revealed a higher degree of variation in patients with persistent infection than in those with acute infection, both at DNA and amino acid level (Hemauer *et al.*, 1996). This, in one individual, was suggested to be due to continuous replication. Most variation was located within the VP1 unique region and, however, remained ≤ 4 %. Because important neutralizing epitopes are localized within this region, other studies focused on this area, with similar results (Haseyama *et al.*, 1998). Expressed VP1u-proteins with this kind of low-level variation showed similar affinity for human monoclonal antibodies, also comparable with recombinant VP1/2 capsids produced in a baculovirus system (Dorsch *et al.*, 2001).

In 1998, a new B19 variant was found in France, in blood from a child with severe anemia (Nguyen *et al.*, 1998; Nguyen *et al.*, 1999). By sequencing, this new variant, V9, differed by more than 10 % from the earlier recognized B19 isolates, demonstrating that the B19 sequences could be much more divergent than previously thought.

Aims of this study

- i) to assess whether B19 DNA persists in human tissues as an intact molecule or as fragmented
- ii) to determine whether the synovium or skin-derived B19 viruses are prototypic, or (persistence related?) variants
- iii) to examine the suitability of the two (commercially available) real-time PCR tests for detection, quantification and differentiation of all known B19 virus types.
- iv) to assess the prevalence of B19 virus types among Finnish blood donors.
- v) to compare the biological and immunological characteristics of the newly found virus types

Materials and methods

Characterization of B19 DNA persisting in synovial tissue and skin (I & II)

The first aim of this study was to characterize B19 DNA persisting in human synovial tissue (I) and skin (II).

Samples

Synovial tissue: Samples of synovial tissue were obtained during arthroscopy from 30 constitutionally healthy adults (age span 18-53 years, mean 21) with joint trauma or exertion (I).

Skin: Biopsies of skin were obtained from 34 subjects; 20 had inflammatory B19-nonrelated dermatological lesions (Group 1) and 14 were healthy members of hospital staff (Group 2). The range of birth year of these subjects was 1913-1991 (mean 1957).

Sera: Sera were obtained from all tissue donors for antibody testing. B19-IgG antibodies were measured (I) by a commercial EIA (Dako, Glostrup, Denmark) or (II) by in-house EIAs, and B19-IgM antibodies by the EIA of Biotrin (Dublin, Ireland). For further evidence of the time of B19 primary infection, the sera of subjects examined in depth for persisting B19 DNA were studied for epitope-type-specificity (ETS) of VP2-IgG (Soderlund *et al.*, 1995b) as described recently (Kaikkonen *et al.*, 1999).

DNA extraction

DNA was purified by proteinase K digestion (0.45% NP-40, 0.45% Tween, 2.5 mM MgCl₂, 50 mM KCl, 1% gelatine in 10 mM Tris, pH 8.3) over night at 55°C followed by 10 min at 95°C to inactivate the enzyme (group 1 skin samples), or by proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation (synovial tissue samples and group 2 skin samples).

PCRs

All the synovial tissue and skin samples were first screened for B19 DNA with a nested PCR (VP1-PCR) amplifying a 1066 bp region in the middle of the genome. This method is described in Soderlund *et al.* (1997b). Of all positive samples, the persisting B19 DNA was further characterized with two additional nested PCRs, NS1- and VP2-PCR, amplifying the NS1 gene and the VP2 gene, respectively. All the three PCRs (primer sequences are shown in Table 2) and the corresponding hybridization probes were

designed according to the sequence of the Au isolate (Shade *et al.*, 1986). The second round products of all three PCRs were detected by agarose gel electrophoresis and ethidium bromide staining. In addition, the products were transferred to a nylon membrane and hybridized with a digoxigenin-labelled probe specific for the corresponding genomic region. Optimal reaction conditions for each primer set were determined using the control DNA, extracted from the viremic serum NAN, as template.

Semi-quantification

To assess whether B19 DNA persists as an intact molecule or is fragmented, we examined, by semi-quantification, whether or not all three genomic regions could be detected in equal amounts. The sensitivities of the NS1 and VP2-PCR methods were equalized to the level of the VP1 PCR, which was shown to detect one target molecule / reaction (Soderlund *et al.*, 1997b). The detection sensitivities of all three PCRs were within one logarithmic unit. For semi-quantification of the different B19 genomic regions, DNA suspensions were diluted serially in 10-fold steps, and each dilution was studied by the nested VP1-PCR. The last dilution giving a positive signal by ethidium bromide staining (in this thesis referred to as end point titer) was then used as a template in the NS1- and VP2-PCRs.

Duplex-PCR

To support our theory for the genomic intactness, of synovial DNA preparations diluted to end point, both the VP2 and NS protein-coding sequences were amplified simultaneously in one tube (I). The first PCR round utilized the outer primer pairs of the VP2 and NS reactions. The product was purified with High Pure PCR Product Purification Kit (Boehringer Mannheim, Mannheim, Germany) and transferred to the second reaction tube. Primers for the nested reaction were NSifwd, NSirev, rt1 and VP2irev (table 2). The resulting amplicons were 439 and 639 nucleotides in size, respectively, and they were separated electrophoretically on a 1 % TAE-agarose gel and were Southern blotted. Hybridization was done simultaneously with NS1 and VP2 probes.

Sequencing and phylogenetic analysis

Synovial isolates (I): In order to assess whether the synovial B19 is of a unique genotype or involves persistence-specific mutations that could be causally related to tissue tropism, the protein-coding region (constituting ~97 % of the whole B19 genome) was amplified and sequenced. These experiments were first conducted with synovial tissue of four individuals (I); two subjects represented acute-phase infection and two subjects represented long-term carriership. B19 DNA purified from these samples was amplified to five partly overlapping amplicons of ~1000 bp, which together covered the whole protein

coding region of the genome (I). Sequencing was done at the Institute of Biotechnology, University of Helsinki. The sequences of B19 DNA isolated from synovia recently or remotely after infection were compared with each other and with blood-derived B19 sequences from GenBank. Phylogenetic analyses were performed of the four synovial tissue-derived B19 sequences together with others from GenBank by using a 346-nt region corresponding to viral nt. 2246 through 2789. Sequence alignments were done by using CLUSTAL W version 1.75, and subsequent analyses were done by using the PHYLIP package (Thompson *et al.*, 1994).

Dermal isolates (II): While determining the molecular characteristics of the skin-derived B19 DNA (II), we observed that most of the amplicons from the NS1- and VP2-PCRs did not hybridize or hybridized only weakly. These amplicons were therefore (partly) sequenced from several samples, either directly or after cloning (TA-cloning, Novagen Inc., Darmstadt, Germany). In addition to the three B19 primer sets (VP1, NS1 and VP2 PCRs), others (Table 2), prepared according to the blood-derived reference sequence Au or the current dermal B19 DNA, were used for amplification and sequencing to gain a near full-length sequence from one dermal isolate, LaLi. This was from a subject representing long-term carriership according to serological data. DNA sequences were obtained with ABI PRISM (Perkin-Elmer, Foster City, CA) at the sequencing core facility of the Haartman Institute, University of Helsinki, Finland. Again, sequences of the new variant, LaLi, were compared with the prototypic dermal and synovial sequences, as well with others found in GenBank. A phylogenetic analysis was performed by using the full-length protein-coding sequence of the dermal isolate LaLi together with 13 B19 sequences from GenBank (including 4 B19 DNA isolates persisting in synovial membranes and a Simian parvovirus sequence).

Table 2. Sequence data for primers used in this study.

PCR test	Primer	Sequence
VP1 PCR	p6	GGAGAATCATTGTGTCGGAAG
	p3	CTTCTGCAGAATTAAGTGAAGTC
	p8	TGTGCTTACCTGTCTGGATTG
	p5	AGG CTT GTG TAA GTC TTC AC
	p1	ATGAGTAAAGAAAAGTGGCAAATGG
NS1 PCR	NSofwd	ATGGAGCTATTTAGAGGGGTG
	NSorev	TTTGCAATCCAGACAGGTAAGC
	NSifwd	ACTGGTTGTGTGAAAACAGAGTG
	NSirev	TTTTCTGCTACATCATTAATGG
	NSpfwd	AGAGGATAAGTGGAAACTAGTTG
	NSprev	TACTGGAACACTTTTAGCAATGG
VP2 PCR	VP2ofwd	TGACTTCAGTTAATTCTGCAGAAG
	VP2orev	TGG GTG CAC ACG GCT TTT GG
	VP2ifwd	CTAGAATATCCTTACGCCCTGG
	VP2irev	GTGGCTGATGCAAACCCCATC
	VP2pfwd	CCATTTCTCATGGTCAGACCAC
	VP2prev	CCATACAGAACCCACCATTAGG
Sequencing	rt1	AATTTAGAGGGCTGCAGTCAAC
	NSs fwd	GCAATGGCCATTGCTAAAAGTG
	p9	CTTTAGGTATAGCCAAGTGG
	rts rev	ATGTGTCAGGAACCCCTAAGC
K71 PCR	ofwd	TTTACTGAAGACAAATGGAAGT
	orev	CACTGGGACAGTTTTGGCAATA
	ifwd	AGTGGATTTCAATCAATATACA
	irev	TCATAATTTTGGCATAATAATAG
VP1/2 cloning	KK1	CTCAGAAAACGGGATCCCGATGACTTCAGTTAATTCTG
	KK2	CGGGATCCCGTTACAATGGGTGCACACGGCT
	KK3	GTGAAGATCTTCATGAGTAAAGAAAAGTGGTAAATG
	KK4	GGAATTCCTTACAATGGGTGCACACGGCTTT
Promoter cloning	NheIfwd	TTTGCTAGCTAGTTGCACGTCAACCCC
	B19RNArev	TGCATAAGGCGGGAAAAAGCCC
	Prom5fwd	TGTCCGCCATCTGAACCG
	Xhorev	TTTCTCGAGGTTAGTAAGTTAAAAGTTAGTATAAATACCTGTTAG
	XhoD91.1rev	TTTCTCGAGGTTAGTAAGTTAAAAGTTAGTATAAATACCTGTTAG

Detection methods for prototypic and variant B19 (II & III)

Qualitative PCRs

While VP1-PCR was able to detect (in addition to the prototypic B19 DNA) LaLi-like B19 DNA, results of NS1- and VP2-PCRs on genotype 2-containing samples were contradictory. Therefore, to identify genotype 2 strains from the VP1-positive samples, primers specific for genotype 2 DNA were designed according to the sequence of the LaLi DNA-isolate, AY044266 (II). To gain absolute specificity, this new PCR (K71-PCR) was conducted in a nested format. After 2x40 cycles and electrophoresis, the PCR products were detected by ethidium-bromide staining.

Quantitative PCRs

We compared the performances of two LightCycler-based qPCR assays for B19-DNA: qPCR-1 (Parvovirus B19 Quantification Kit by Roche), and qPCR-2 (RealArt™ Parvo B19 LC PCR by Artus) (III). The principle in both assays is basically the same. Each provides B19-specific primers and two hybridization probes labelled with fluorescent molecules. Hybridization leads to fluorescence resonance energy transfer (FRET) between the two fluorophores, and the emitted light is measured by the LightCycler instrument. Real-time (during amplification) monitoring of fluorescence intensities, relative to external standards of known target concentrations, allows for quantification of the accumulating product. To monitor the efficiencies of the nucleic acid extraction and the PCR process, internal controls (IC) are amplified with the same primers as the target, but hybridized with probes carrying different fluorophores.

The raw data created by either qPCR test were analyzed with LightCycler Software version 3.5 (Roche). Crossing points and calculated concentrations were obtained by the second derivative maximum method together with proportional base-line adjustment. This method calculates the fractional cycle number of the Crossing Point (CP) value of each sample automatically and thus makes the method independent of user-born influences. A previously generated color compensation file (Roche) was activated during the LightCycler run to reduce flow-through signal from other channels.

Evaluation of the sensitivities of the two qPCRs for B19 genotypes 1-3

Primer sequences for both qPCR-1 and qPCR-2 assays were considered confidential by the manufacturers. Therefore, we needed to map the approximate target regions for each PCR assay in order to select, among plasmids containing parts of genotype 2, those containing the appropriate target sequence (Figure 4, Table 3). For this purpose, the amplicons of B19 genotype 1 produced by qPCR-1 and qPCR-2 were analyzed on agaroses gel, transferred to a nylon membrane and hybridized with probes specific for two different genomic regions, a 359-bp fragment of the NS1 gene (used for hybridizing

products of the NS1-PCR) and a 354-bp fragment of the VP1 gene (used for hybridizing the products of the VP1-PCR). Hybridization of the qPCR-1 amplicon gave a positive result with the NS probe and a negative result with the VP1 probe. As the amplicon size of qPCR-1 is ~170-180 bp and the overlapping area (covered by the NS probe) of the inserts in plasmids pLaLi1 and pLaLi2 is 223 nucleotides, both of these plasmids probably contain the target sequence of qPCR-1, and can be used as genotype 2 template in qPCR-1. For evaluation, we chose pLaLi2, and to be sure, joined the three areas together by cloning (plasmid pLaLi6). Southern hybridization of the qPCR-2 amplicon gave a positive result with the VP1 probe and a negative result with the NS probe. As the VP1 probe covers the VP1 unique region included in plasmid pLaLi3, this plasmid was chosen to be used as a genotype-2 template sequence in qPCR-2.

The plasmid constructs carrying B19-virus DNA of the different genotypes were used for evaluation of the two qPCR methods. DNA concentration of the genotype 1-containing plasmid, pB19-Lit, was first measured by the validated (Hokynar et al, unpublished data) qPCR-1 test. The DNA concentrations of all plasmids were then measured spectrophotometrically and converted into plasmid copy numbers / μl . The plasmid preparations were equalized to contain 10^9 copies / μl each. To confirm the concentrations, serial dilutions of the equalized plasmid purifications were dot-blotted onto a nylon membrane and hybridized with a digoxigenin-labelled probe against the plasmid backbone.

In order to determine the sensitivity of the two B19 qPCR tests, qPCR-1 and qPCR-2, ten-fold dilutions were used of the plasmid constructs that contained the DNA inserts from each of the three genotypes. With both qPCRs, each dilution was tested in 1 to 3 replicates. The PCR reactions were performed as recommended by the manufacturer. In every PCR run, water was included as a negative control. The WHO International Standard for Parvovirus B19 DNA nucleic acid assays (NIBSC 99/800) (Saldanha *et al.*, 2002) was used as a positive control. Plasmids Litmus29, pSTBlue-1 and pcDNA2.1 without insert were studied for background by the plasmid backbone.

Differentiation of the B19 genotypes by melting curve analysis

The ability of qPCR-1 and qPCR-2 to differentiate the amplicons obtained from DNA of different B19 genotypes was examined by melting curve analysis (segment 1: 95 °C, hold time 15 s, slope 20°C/s, acquisition mode none. Segment 2: 40°C, hold time 15 s, slope 20 °C/s, acquisition mode none. Segment 3: 80 °C, hold time 0 s, slope 0.1 °C/s, acquisition mode continuous). Melting points were determined with serial dilutions of DNA from each genotype alone, as well as of mixtures of DNA of two or three genotypes in equal amounts.

Quantitative PCR of B19 DNA in clinical samples

To further examine the suitability of the PCR kits for identification of the three genotypes under natural conditions, clinical samples previously shown to contain genotypes 1 (4 skin biopsies) (II), 2 (6 skin biopsies) (II) or 3 (2 serum samples) (Nguyen *et al.*, 1999; Servant *et al.*, 2002) were analyzed. DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. N.B., as pointed out by the manufacturer, phenol-based extraction is not recommended for qPCR-2. Therefore, to detect possible DNA polymerase inhibitors in these preparations, internal control (IC) provided in each kit was added to the master mix (0.5 μ l / reaction) and detected on channel F3. Two dilutions of each sample were studied in 1 to 4 replicates.

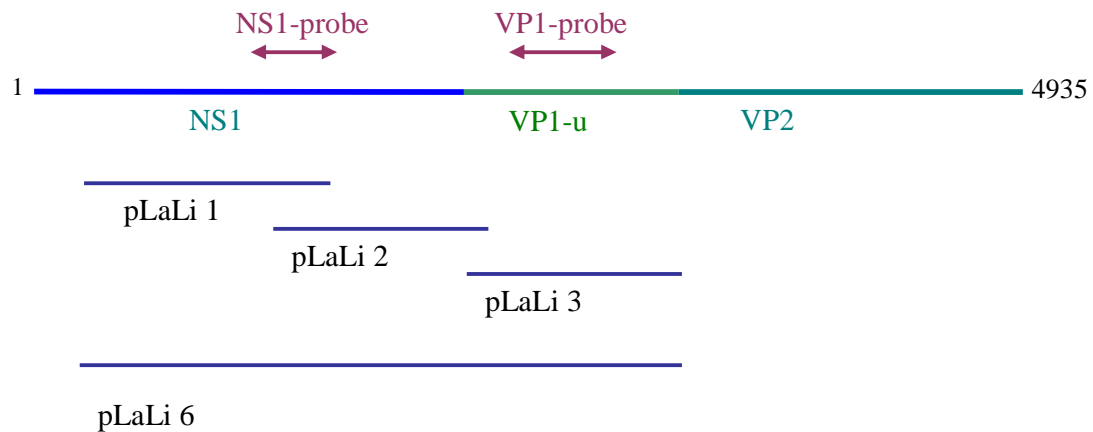


Figure 4. Schematic illustration of the positions (on the LaLi-sequence) of the NS1- and VP1-probes, and the inserts of plasmids pLaLi1, pLaLi2, pLaLi3 and pLaLi6, used for qPCR evaluation.

Table 3. *Plasmid constructs used in qPCR evaluation.*

	Genotype	Source	Insert	GenBank no	Plasmid backbone
pB19-Lit	1	Serum	1-5300	AY504945	Litmus29 (New England Biolabs, Beverly, USA)
pLaLi1	2	Skin	105-1312	AY044266	Litmus29
pLaLi2	2	Skin	1092-2332	AY044266	Litmus29
pLaLi3	2	Skin	2058-3121	AY044266	pSTBlue-1 (Novagen, Madison Wisconsin, USA)
pLaLi6	2	Skin	105-3121	AY044266	pSTBlue-1
pV9-C22*	3	Serum / BM	1-5028	AJ249437	a modified plasmid pcDNA2.1 (Invitrogen Life Technologies, Paisley, UK)
pD91.1-C10*	3	Serum	1-5028	AY083234	pcDNA3.1HisB (Invitrogen)

*) Plasmids pV9-C22 and pD91.1-C10 were kindly donated by A. Garbarg-Chenon. Empty plasmid (with no insert) pSTBlue-1, Litmus29 and pcDNA2.1 were used as negative controls.

Prevalence of variant B19 (II & III)

Plasma pools: All blood donations collected at the Finnish Red Cross Blood Transfusion Service since January 2002 have been pre-screened with qPCR-1 for genotype 1 DNA as EDTA-plasma maxipools of 480 units each. Nucleic acid eluates of 292 such maxipools, altogether representing 140 160 blood donation units, were randomly selected and studied with qPCR-2.

Of maxi pools collected during 2002-3, 11 single donations with high-titer B19 DNA (determined by qPCR-1) were available for genotyping. Since phenol-based extraction is not recommended for qPCR-2, one of the recommended methods, QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany) was used in parallel. The DNA extracts were studied with qPCR-2, and the B19 genotype was determined by melting curve analysis. To detect possible DNA polymerase inhibitors in these preparations, internal control (IC) provided in each kit was added to the master mix (0.5 µl / reaction) and detected on channel F3. For

comparison, both DNA extracts were additionally studied by the conventional VP1-PCR and K71-PCR assays.

Tissue samples: To determine the prevalence of B19 type 2 DNA among the synovial tissue and skin, all the tissue samples that had earlier been examined by the qualitative NS1-, VP1- and VP2-PCRs were studied further with the genotype 2-specific K71-PCR. B19 type 3 was not examined.

Biological and immunological relations of B19 genotypes 1-3 (IV)

Assay for measuring promoter activities

Construction of the promoter-luciferase vectors: The full-length p6 promoter regions (nt. 195-623 according to the NAN reference sequence, GenBank accession number AY504945) were amplified by PCR from viremic serum NAN (genotype 1), from skin sample LaLi (genotype 2), and from viremic serum D91.1 (genotype 3) (Servant *et al.*, 2002). The p6 regions of genotypes 2 and 3 were amplified by PCR in two steps. In step one, two overlapping PCR amplicons of each genotype were produced: one with primers NheIfwd (modified from (Gareus *et al.*, 1998)) and B19RNArev (table 2), and the other with primer prom5fwd and xhorev for genotype 2, and prom5fwd and xhoD91.1rev for genotype 3. The two PCR products were excised and purified from the agarose gel with a gel-extraction kit (Qiagen and Sigma, St. Louis, USA). In step 2, the purified products were combined by using as template the two overlapping products in a PCR reaction performed with the primers NheIfwd and xhorev (genotype 2) or NheIfwd and xhoD91.1rev (genotype 3). The final products were subsequently integrated into NheI and XhoI cloning sites of the plasmid pGL3basic containing a firefly (*Photinus pyralis*) luciferase as reporter (Promega Corporation, Madison, WI, USA). Inserts of the three promoter-luciferase constructs were sequenced at the core facility of the Haartman Institute (University of Helsinki, Finland) by the ABI PRISM technique.

Transfections: For measurement of promoter strengths of the three virus types, the three p6 promoter constructs were transfected into several cell lines permissive and non-permissive for B19. As an internal reference, production of a second type of luciferase derived from *Renilla reniformis* was achieved by co-transfecting a plasmid pRL-TK (Promega) to the cells together with the promoter-luciferase constructs. Cell-lines and transfections are described in table 4.

Measurements: The cells were collected one day post transfection, and a 20- μ l sample of the lysate was used for quantification of the luciferase activity. Each sample was assayed in duplicate. The assays for the *Photinus* and *Renilla* luciferase activities were performed sequentially with a Dual-Luciferase Reporter assay system (Promega) as recommended by the manufacturer. The expression of the test and the control reporters were measured by a Digene DCR-1 luminometer (MG Instruments Inc., Hamden, CT, USA) in relative luminescence units (RLU) during a period of ten seconds for each luciferase. Experimental RLU was normalized to the activity of the control RLU to minimize inter-reaction variability caused by differences in cell viability or transfection efficiency.

Controls: In all experiments, the vector pGL3control, which contains the firefly luciferase gene under control of the simian virus 40 (SV40) promoter and enhancer sequences, was used as positive control. To compare the activities of the different p6-promoter constructs within the different cell lines, the activity of the SV40 promoter was set at 1 in all cell types. The RLU of each virus type (normalized against the Renilla standard) was divided with the normalized RLU of the pGL3control. Water and empty vector pGL3basic were used as negative controls. All transfection experiments were performed at least three times.

Table 4. Cell-lines and transfection methods used.

Cell line	Cell type	Maintained in:	Transfection:
KU812Ep6	Erythroid leukemia cells	RPMI 1640, 10% FBS, 6 U/ml erythropoietin	Electroporation: 350 V, 960 μ F; 2 μ g reporter construct, 0.2 μ g pRL-TK
UT7/Epo-S1	Erythroblastoid cells	IMDM, 10% FBS, 2 U/ml erythropoietin	Electroporation: 350 V, 960 μ F; 2 μ g reporter construct, 0.2 μ g pRL-TK
MB-02	Megakaryocytic leukemia cells	RPMI 1640, 10% human serum, 200 U/ml GM-CSF	Electroporation: 300 V, 960 μ F; 2 μ g reporter construct, 0.2 μ g pRL-TK
U937	Monocytic cells	RPMI 1640, 10% FBS, 10 mM HEPES, 1mM sodium pyruvate, 4.5 mg/ml glucose, 1.5 mg/ml sodium carbonate, 4U/ml erythropoietin, 25 ng/ml hSCF	Electroporation: 350 V, 960 μ F; 2 μ g reporter construct, 0.2 μ g pRL-TK
HUH-7	Hepatocytes	MEM	Lipofectamine 2000; 1.6 μ g reporter, 0.16 μ g pRL-TK
HaCaT	Keratinocytes	DMEM, 10% FBS	Lipofectamine 2000; 2.0 μ g reporter, 0.2 μ g pRL-TK
HeLa	Cervical epithelial cells	MEM	Lipofectamine 2000; 0.8 μ g reporter, 0.08 μ g pRL-TK
HeLa/pGRE HeLa/pGRE.NS	HeLa-cells expressing the B19 type-1 NS1 protein when induced by 1mM dexamethasone	MEM, 300 μ g/ml hygromycin B	Lipofectamine 2000; 0.8 μ g reporter, 0.08 μ g pRL-TK

All media contained penicillin, streptomycin and L-glutamine, and the cells were grown in 5% CO₂ at 37°C.

Recombinant expression and purification of virus type 1 and 2 capsids (IV)

A baculovirus expression method (Brown *et al.*, 1991) was used for production of recombinant capsids of B19 type 1 and 2. The synthesis of B19 type 1 recombinant capsids has been reported before (Franssila *et al.*, 2001). Virus-like particles (VLPs) of B19 virus type 2, composed either of capsid protein VP2 alone or of proteins VP1 and VP2 together, were synthesized with a similar protocol. The VP2 and VP1 genes of virus type 2 were amplified by PCR with primers KK1-KK4 (Table 2) using as template a virus type-2 clone (LaLi) in a pSTBlue-1 vector (Novagen, Madison, WI). The amplified VP1 and VP2 genes were cloned into the baculovirus transfer vector pAcuW51 (Pharmingen, San Diego, CA, USA) under the control of the p10 and polyhedrin promoters, respectively. The recombinant VP2 and VP1/2 plasmids together with linearized baculovirus DNA (Baculogold; Pharmingen) were co-transfected into Sf9 cells with Fugene6™ transfection reagent (Roche Diagnostics, Indianapolis, IN, USA). The production of viral proteins was determined by SDS-PAGE and Western-blotting, and the formation of VLPs was verified by EM. The recombinant VP2 or VP1/2 capsids of B19 types 1 and 2 were produced in large scale in High Five™ cells and were purified with 28 % (w/w) CsCl-gradient ultracentrifugation (100 000 x g for 48 h at +4°C) followed by precipitation with 40 % ammonium sulfate.

Hemagglutination assay

In order to detect and compare the quantities of the viral particles in the viremic serum samples SPR3 (genotype 1), IM-81 (genotype 2) and D91.1 (genotype 3), a hemagglutination assay (HA) was performed (Brown and Cohen, 1992). Recombinant capsids, produced with the baculovirus system, were used to set up the HA: human erythrocytes (obtained from FRC-BTS) were washed three times and suspended (10 % v/v) in PBS. The suspension was further diluted 1:20 in HA-buffer (NaCl 8 g/l, 0.2 g/l KCl, 5 g/l dextrose and 0.2% BSA in 0.05M PBS, pH 5.8), and 50 µl of the suspension was added to each well on a 96-well plate. Serial three-fold dilutions of viremic sera were prepared in HA-buffer, and 50 µl of each dilution was added to the plates and incubated for two hours at 4°C.

Infectivity assay

KU812Ep6 or UT7 /EpoS1 cells were suspended in media containing the viremic sera of types 1, 2 or 3. After incubation for 2 h at 37°C (KU812Ep6 cells) or 2.5 h on ice (UT7/Epo-S1 cells), fresh medium was added to obtain a final density of $0,2 \times 10^6$ cells/ml. Infection was monitored on day 3 with immunofluorescence (IF), as described in IV, and RT-PCR, as described in Brunstein *et al.* (2000).

Enzyme immunoassays

Four different EIAs, using as antigen biotinylated VP2 or VP1/2 capsids of B19 types 1 or 2, were set up (as described in (Kaikkonen *et al.*, 1999)) for comparison of antibody activities of B19 type 1- or type 2- infected subjects. The optimal concentrations of each antigen preparation (40 ng/well for type 1 VP2, 80 ng/well for type 1 VP1/2, 80 ng/well for type 2 VP2 and 100 ng/well for type 2 VP1/2) were first determined by end-point titration. The four antigens were then tested with dilutions of the WHO International standard for Anti-Parvovirus B19 serum IgG (NIBSC 93/724) (Ferguson *et al.*, 1997). In addition, all antigens were tested with acute-phase (low avidity of IgG, acute epitope-type specificity) and past-immunity (high avidity of IgG, non-acute epitope-type specificity) human serum pools (Kaikkonen *et al.*, 1999; Soderlund *et al.*, 1995a; Soderlund *et al.*, 1995b).

To examine the extent of IgG cross-reactivity between virus types 1 and 2, three groups of sera were studied: group 1 contained the sera of 24 subjects persistently carrying B19 DNA of type 1 in skin and/or synovial tissue; group 2 contained the sera of 25 subjects carrying B19 DNA of type 2; and group 3 contained the sera of 13 B19-IgG-negative subjects. The group-3 IgG-negative samples were used to set the EIA cut-offs (mean + 3 x SD).

The subjects in groups 1-3 were healthy members of hospital or laboratory staff, or patients with trauma or other B19-nonrelated disease, and either skin or synovial tissue samples derived from these subjects had been previously examined for B19 DNA persistence (I, II). All the sera had been studied for B19-IgG and IgM antibodies by in-house (Kaikkonen *et al.*, 1999) and commercial (Biotrin, Dublin, Ireland) EIAs, respectively, and for VP2-IgG epitope-type specificity (Kaikkonen *et al.*, 1999; Soderlund *et al.*, 1995b) and VP1-IgG avidity (Soderlund *et al.*, 1995a), for verification of long-term immunity.

Results

B19 DNA in tissues (I & II)

VP1 PCR

Samples of synovial tissue (I) and skin (II) were screened for B19 DNA by amplifying a fragment in the middle of the genome, covering the junction of NS1 and VP genes (VP1 PCR). Blood samples taken from the same patients were studied for B19 antibodies (Table 5).

In study I we had both synovial tissue and serum from 12 B19-seronegative and 18 B19-seropositive subjects. Of the latter group, 16 had B19-IgG but no B19-IgM, and 2 patients had both B19-IgG and IgM. Among the 18 B19-seropositive patients, 12/18 (67 %) had B19 DNA in synovia, whereas all the seronegative subjects were negative for synovial B19 DNA. In study II we used skin and serum samples from 15 B19-seronegative and from 19 B19-seropositive subjects with past immunity (IgG+ with non-acute epitope-type specificity and IgM-). Among the 19 seropositive subjects 14/19 (74%) were VP1-DNA positive in skin, whereas all the seronegative subjects were B19 VP1-DNA negative by PCR in skin. Thus, every patient with B19 DNA in synovium or skin had been infected with the virus.

Of the B19 DNA-positive synovia we chose randomly for further examination four cases representing long-term carriage of B19-DNA (positive IgG, negative IgM, “non-acute” ETS patterns) and for comparison both 2 cases with serological evidence for recent B19 infection (positive IgG, positive IgM, “acute” ETS patterns). The six samples chosen for further study were titrated serially in 10-fold steps for VP1-PCR positivity. Of the skin samples, all 14 B19-DNA positive samples were studied further.

NS1 and VP2 PCR

Synovial samples (I): In study I, to assess whether the tissues contain the entire B19 coding region, the last dilutions of synovial DNA preparations giving a positive VP1 signal (“end-point titers”) were used as a template in PCR assays for the VP2 and NS1 genes. The entire protein coding area of the B19 genome could be found in all the 6 samples. With 5 of these samples, the last dilution that was positive in VP1-PCR gave a positive result also in VP2- and NS1-PCR assays. With the one sample positive in VP1 PCR in dilutions up to 1:10 000, the NS1 and VP2 regions could be detected in dilutions up to 1:1000. To assess whether the DNA is intact or fragmented, both ends of the coding region were co-amplified in end-point diluted templates. Both the NS1 and the VP2 regions were simultaneously detectable in all four samples studied.

Skin samples (II): In study II, among the VP1-positive dermal samples only 5/14 gave the expected positive results in the B19 NS1- and VP2- PCRs, whereas the remaining 9/14 samples showed poorly reproducible amplicons or weak hybridization signals, when using standard B19 probes, even with undiluted DNA preparations (Table 5.).

Table 5. Results of antibody testing and B19 specific PCRs.

	B19 serology	VP1-PCR	NS1-PCR	VP2-PCR	Number
Synovia	IgG+, IgM-	+	+	+	10
		-	-	-	6
	IgG+, IgM+	+	+	+	2
	IgG-, IgM-	-	ND	ND	12
				total	30
Skin	IgG+, IgM-	+	+	+	5
		+	-	-	9
	-	-	-	5	
	IgG-, IgM-	-	ND	ND	15
				total	34

Analysis of the B19 genomes in tissue

Synovial tissue samples (I): Of the 4 synovial tissue samples (I), nearly complete sequences were obtained. In all, ~97 % of the B19 coding region (of 4355 nucleotides with the Au strain, corresponding to a coding sequence of 4362 nucleotides) was sequenced from each of the 4 isolates (Kati 1-Kati 4). The synovial sequences were compared with the blood-derived reference sequences Au (M13178) and Wi (M24682). Both in our patients with recent infection and in our subjects with past immunity, the sequence identity relative to the Au reference was >99%. Altogether, our synovial sequences differed from either reference (Au or Wi) by 27 conserved (occurring in every subject studied) nucleotide changes. Yet, all our conserved changes relative to either reference strain were found to agree with the other reference strain. Of the 9 conserved changes relative to Au, 5 were within the NS gene and 4 within the VP1/2 gene. While 6 of those mutations were silent, 3 changed an amino acid; nt 692 converted threonine into

isoleucine, nt 3809 converted serine into threonine, and nt 3182 converted serine into proline. Conversely, our synovial sequences differed from the Wi reference in 18 conserved changes. For all those 18 nucleotides our sequences were identical with the Au reference. The Au and Wi references differ from each other by 35 nucleotides within the region sequenced here. For the 8 nucleotides beyond those 27 described above, all our sequences individually followed either the Au or the Wi reference.

We next examined our sequence data for evidence of nonconserved mutations that might functionally inactivate the B19 proteins. Throughout our samples, nonsilent mutations were rare, comprising <25 % of all nucleotide differences. No stop-codons, frame-shifts, insertions or deletions were found.

Skin samples (II): The weakly hybridising PCR products from 4 subjects were sequenced directly, and 2 subjects' DNA preparations underwent several additional PCRs for cloning and sequencing. From one B19 DNA isolate we sequenced nucleotides 144-4763 (LaLi, AY044266), and from the other (HaAM) nucleotides 144-1510 and 2134-4763 (GenBank AY044267 and AY044268, respectively) (numbering according to the Au reference; M13178).

Within the overall coding region, LaLi-sequence differed from Au by 10.8%. Divergence within the different parts of the protein coding region of LaLi, in comparison with the B19 Au, Wi (M24682) and V9 (AJ249437) sequences, at DNA and amino acid levels are shown in table 6. The two isolates (LaLi and HaAM) differed from each other by only 0.3%.

The most striking nucleotide variation was seen between the non-coding regions, where the sequence of LaLi differed from the Au reference by 26.5 %. This region covers partly the p6 promoter area. In addition to extensive nucleotide variation, alignment of the p6 regions of B19 types 1-3 revealed an 8 nt deletion, comprising majority of one of the two tandemly arranged GC-boxes upstream of the TATA-box (Figure 6). Since these changes might alter the promoter function, the p6 promoters of the three B19 genotypes were later amplified, cloned and sequenced in full length (IV), and their activities were measured (see Biological relations of B19 types 1-3).

Table 6. Sequence divergences (%) between the different B19 genotypes (the prototypic B19 genotype represented by isolates Au and Wi, genotype 2 represented by isolate LaLi, and genotype 3 represented by V9).

1) Divergences were obtained by the Distances (with no corrections) program of the Wisconsin Package of GCG.

2) CDS stands for the total protein coding sequence (nucleotides 436-4789).

DNA ¹⁾	non-cds ²⁾	cds ²⁾	NS1	VP1/2	VP2	uVP1
LaLi vs Au	26,5	11,8	14,3	9,7	11,9	4,7
LaLi vs V9	17,2	9,3	8,1	10,3	10,9	8,8
V9 vs Au	30,7	13,9	15	12,9	14,5	9,1
Au vs Wi	1,6	0,8	0,7	0,9	1	0,6

Protein	NS1	VP1/2	VP2	uVP1
LaLi vs Au	6,2	2,4	1,5	4,5
LaLi vs V9	3	2,8	1,3	6,4
V9 vs Au	6,1	3,3	1,8	6,9
Au vs Wi	0,7	0,6	0,4	1,3

```

Type 2 AAGC--GCTGGCCCAGAGCCAACCCTAATTCCGGAAGTCCC GCCCACCGGAAGTGACGTC
type 3 AAGC--GCTGGCCCAGAGCCAACCCTAATTCCGGAAGTCCC GCCCACCGGAAGTGACGTC
type 1 AAGCAAGCTGGCCCAGAGCCAACCCTAATTCCGGAAGTCCC GCCCACCGGAAGTGACGTC

type 2 ACAGGAAATGACGTACAGGAAATGACGTAGTTGTCCGCCATCTTGTACCGGAAGTCCCG
type 3 ACAGGAAATGACGTACAGGAAATGACGTAACTGTCCGCCATCTTGAACCGGAAGTCCCG
type 1 ACAGGAAATGACGTACAGGAAATGACGTAATTGTCCGCCATCTTGTACCGGAAGTCCCG

type 2 CCTACCGCGGGCGACCGGCGGCATCTGATTTGGTGTCTTCTTTT--AAATTTTCGCGGGC
type 3 CCTACCGCGGGCGACCGGCGGCATCTGATTTGGTGTCTTCTTTTAAAAATTTTGGCGGG
type 1 CCTACCGCGGGCGACCGGCGGCATCTGATTTGGTGTCTTCTTTT---AAATTTTAGCGGG

type 2 TTTTTTCCCGCCTTATGCAAATAACCTGCCATGTTTGATGCTTTATTTTAATTTGATTGG
type 3 CTTTTTCCCGCCTTATGCAAATGAACAGCCATGTTTGGTGTATTATTTTAATTTTATTGG
type 1 CTTTTTCCCGCCTTATGCAAATGGGCAGCCATTTTAAGTGTTTTACTATAATTTTATTGG

type 2 ACAAAACTTAACGGTTTTTATGGGCGGAGTTACGCATGGTATATAAGCAG
type 3 ACACAGGCCTAACGGTTATTATAGGCGGAGTTACGGACAGTATATAAGCAG
type 1 TTAGTTTTGTAACGGTTAAAATGGGCGGAGCGTAGGCGGGGATTACAGTATATATAGCAC

type 2 ATGAATTCTGTACAACCTTCTTTCCTGGCTGCTTTTTACTGGAATAAATTGCTGTTATT
type 3 CTGAGTTTCGTGACACTTCTTCTTCTGGTTGCTTCTTACTGGAACCTCACTTCTGTTATT
type 1 GGAAGTGTGCGAGCTCTTCTTCTTCTGGGCTGCTTTTTCTTGG-ACTTCTTCTGCTTTTTT

type 2 TGCCTGCTAATTAACAGGTATTTATACTAACTTTTAATTTACTAACatg
type 3 TGCCTGCTAACTAACAGGTATTTATACTAACTTTTAACTTACTAACatg
type 1 TGTGAGCTAACTAACAGGTATTTATACTACTTGTTAACATACTAACatg

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Figure 5. Alignment of the cloned *p6* promoter regions of B19 type 1 (NAN), type 2 (LaLi) and type 3 (D91.1), by Clustal W (1.82). Binding sites for cellular factors (*Sp1*, *Sp3*, *YY1*, *MZF1*, *E4BP4*, *Oct-1* and *ETS*-proteins), and the TATA-box are marked with boxes as shown in Gareus *et al.* 1998 and Servant *et al.* 2002.

Seeking additional evidence of genetic clustering and abnormal variation of the dermal B19 isolates, the LaLi sequence was aligned with 13 B19 sequences from GenBank, including the blood-derived reference Au, and the four synovial B19 sequences (I), the variant V9 (Nguyen *et al.*, 1999); and a Simian parvovirus (Brown *et al.*, 1995). A phylogenetic tree based on these data was constructed (Figure 2 in II). The B19 isolates from blood and synovial tissue formed a condensed cluster except for the two variants LaLi and V9 that remained outside the B19 group, and apart from each other.

Methods for detection of the B19 variants (II & III)

Development of genotype 2-specific PCR

A B19 type 2-specific PCR (K71-PCR) was designed according to the newly found LaLi-sequence (II). All the skin and synovial tissue samples were studied again, this time with K71-PCR. DNA of B19 type 2 was detected from the 9 skin samples that gave unclear results with VP2- and NS1-PCRs, but not in those 5 samples that were clearly positive with all the three B19 PCRs designed according to the B19 prototype sequence. The 6 samples from B19-seropositive but VP1-PCR negative subjects as well as the 15 B19-seronegative subjects remained negative with K71-PCR. Additionally, 16 synovial samples previously tested positive for the conventional NS1-, VP1-, and VP2-PCRs, were studied for genotype 2 DNA: all were negative. The VP1-PCR was thus shown to detect both the prototype (genotype 1) and the new variant, LaLi (genotype 2), whereas K71-PCR was shown to be specific for the LaLi-variant representing genotype 2.

Table 7. PCR results of the skin samples. All the samples that gave positive results in VP1-, NS1- and VP2-PCRs remained negative by the B19 type 2-specific K71-PCR.

B19 serology	VP1-PCR	NS1-PCR	VP2-PCR	K71-PCR	Number
IgG+, IgM-	+	+	+	-	5
	+	-	-	+	9
	-	-	-	-	5
IgG-, IgM-	-	-	-	-	15
				total	34

Construction of plasmids for qPCR evaluation

Several plasmid constructs carrying B19-virus DNA of the different genotypes were used for evaluation of the two commercially available B19 qPCR methods. The concentrations of plasmid purifications were equalized and verified by dot-blotting serial (1:2) dilutions of each preparation onto a nylon membrane and hybridizing with a digoxigenin-labelled probe against the plasmid backbone (Figure 6).

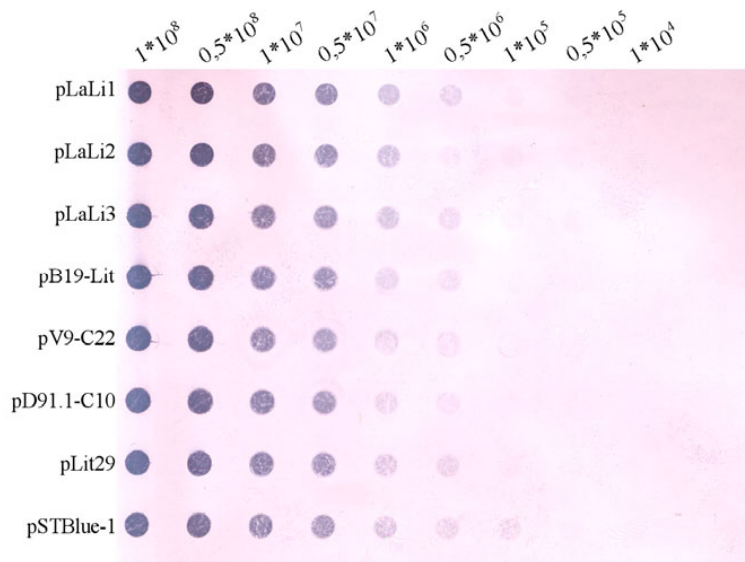


Figure 6. Dot-blot of serially diluted plasmid preparations.

The LightCycler-Parvovirus B19 quantification kit (Roche Diagnostics), qPCR-1, was highly sensitive for genotype 1 DNA detection. When tested with plasmid constructs, a positive signal was obtained even with a theoretical load of 0.5 copy / reaction. Quantification of genotype 1 was accurate down to 50 copies / reaction. However, this method barely recognized genotype 2: in two out of four parallel runs, a positive signal was obtained only with >5 million copies of pLaLi1 / reaction. Higher dilutions remained negative in every run. The qPCR-1 assay was tested further with plasmid pLaLi2 and plasmid pLaLi6 containing the full NS region, and the results were similar. Of the two genotype-3 isolates, only V9 was recognized, with a sensitivity of approximately 1 log lower than for genotype 1. The signal for the genotype-3 isolate D91.1 remained negative even with 5×10^9 copies / reaction. Melting curve analysis of qPCR-1 products showed the same two peaks (63.27 °C and 69.5 °C) for all amplicons regardless of genotype (data not shown). Thus, the three B19 genotypes could not be differentiated from each other by melting-curve analysis after qPCR-1.

To examine the suitability of the qPCR tests for identification of the three genotypes under natural condition, clinical samples previously shown to contain genotypes 1 (4 skin biopsies), 2 (6 skin biopsies) or 3 (2 serum samples), were analyzed. Because of the low B19-DNA level in these samples and because of the presence of inhibitory compounds in the phenol-chloroform extracted DNA preparations, as indicated by the absence of an internal control (IC) signal in some of the replicates, both undiluted and 1:10-diluted samples needed to be tested. qPCR-1 recognized 2/2 skin samples containing B19 DNA of genotype 1. All 5 skin samples containing genotype 2 and both serum samples containing

the two different subtypes of genotype 3 remained negative, even though the IC gave a positive signal indicating absence of PCR inhibition.

The RealArt Parvo B19 LC PCR (Artus GmbH), qPCR-2, recognized genotypes 1, 2 and isolate V9 of genotype 3 with equal sensitivity. All three were detected in copy numbers down to 5 / reaction. However, the detection sensitivity for the genotype 3 isolate D91.1 was ~ 3 log lower, with a detection limit of 5000 copies / reaction.

Melting-curve analysis distinguished all three genotypes, although T_m variation among samples of each genotype was relatively high. The T_m for genotype 1, genotype 2, and both subtypes of genotype 3 were 67.57 (66.53 – 68.45), 65.34 (64.72 – 65.77) and 60.69 (59.94 – 61.34) °C, respectively. However, a mixture of all three genotypes resulted in only two peaks characteristic of genotypes 1 and 3. If two genotypes were present in one sample, genotypes 1 and 2 showed only one peak (characteristic of genotype 1) and were not distinguished by this method; whereas both other genotype combinations, 1 and 3 as well as 2 and 3, showed the correct two peaks. The corresponding melting points were independent of DNA load. The WHO International Standard (99/800) that was used as a positive control, showed a single melting peak of 67.5, which is characteristic of genotype 1.

In agreement with the results obtained for plasmid templates, qPCR-2 recognized all genotypes in clinical samples: genotype 1 in 4/4 skin samples, genotype 2 in 6/6 skin samples, and genotype 3 subtype D91.1 in 1/1 serum sample. As with qPCR-1, the serum containing isolate V9 of genotype 3 remained negative even though the internal control was positive. All the three genotypes in the clinical samples could be differentiated by melting curve analysis.

Prevalence of B19 types 1-3

In blood: During January 2002 and April 2003, 857 maxipools were pre-screened for B19 DNA by qPCR-1 at the Finnish Red Cross Blood Service. Within the 857 pools, 580 (67.7 %) were negative for B19 DNA. Of the 277 positive pools, 13 (1.6 % of total pools) contained a high concentration ($>10^4$ IU/ml) of B19 DNA, and were excluded from the production pools (Fig. 7).

CP-value log IU/ml	NEG	34,01 < 3	34,01 - 30,75 3 - 4	30,75 - 27,38 4 - 5	27,38 > 5	Total
POOLS	580	245	19	3	10	857
%	67,7	28,6	2,2	0,4	1,2	100 %

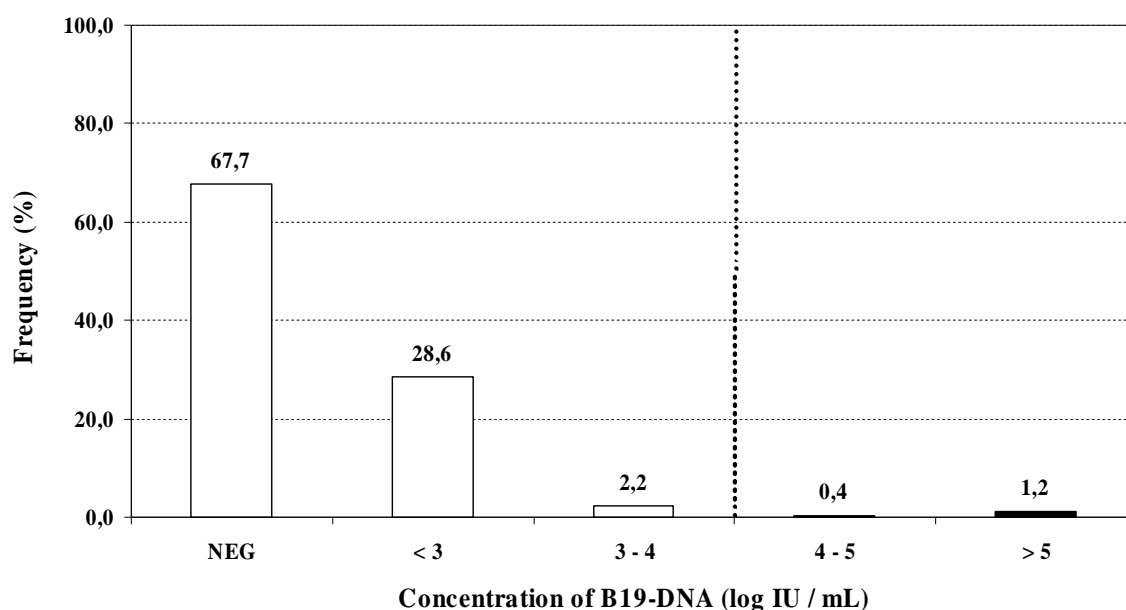


Figure 7. Results of pre-screening of maxipools for B19 DNA with qPCR-1 (Roche).

Of the 857 pre-screened pools, 292 were randomly selected and studied with qPCR-2. The samples could be divided in 4 groups: 13/292 (4.5 %) were qPCR-positive with both tests (sample group 1), 3/292 (1.0 %) were qPCR-1 negative but qPCR-2 positive (group 2), and 51/292 (17.5 %) were qPCR-1 positive but qPCR-2 negative (group 3). Majority of the maxipools, 225/292 (77.1 %), were negative with both qPCRs (group 4). To verify the qPCR-2 genotype identification in each maxipool, samples positive with qPCR-2 were studied further with the conventional PCRs, VP1-PCR and the genotype 2-specific K71-PCR (Table 2. in III).

Within the 13 samples in group 1, seven were positive by VP1-PCR and all were negative by K71-PCR. By melting curve analysis following qPCR-2, most samples (9/13) gave results corresponding to genotype 1. However, four samples gave melting points of ~63.6 °C, i.e. intermediary between the melting points characteristic for genotypes 2 and 3. Within this group the viral loads – as determined by the qPCR-1 CP value – were over 10^5 IU/ml in one sample, 10^4 - 10^5 IU/ml in 2 samples, 10^3 - 10^4 in four samples, and $<10^3$ IU/ml in six samples. The six VP1 PCR-negative samples were those from the last category.

The three qPCR-1-negative samples in Group 2 gave a weak positive signal (barely showing a curve) with qPCR-2, and were completely negative by VP1-PCR and K71-PCR. The melting point for one of these three samples was positioned at ~63 °C like the four samples in group 1, and another sample showed a melting point typical for genotype 1 (68 °C). No melting point could be observed for the third sample. The five samples of groups 1 and 2 with unusual melting points were retested with qPCR-2 with reproducible results. The qPCR-2 test gave concentration values for seven more qPCR-1-negative samples, which were however considered negative due to the lack of any visible amplification (or melting) curves.

Among the 51 samples of group 3 (positive by qPCR-1 alone) the B19-DNA loads were low, $<10^3$ IU/ml in 50 samples and 10^3 - 10^4 IU/ml in one sample. All samples had been pre-screened with qPCR-1 soon after collection (non-frozen), and had been stored frozen at -20°C (with two thawings) before qPCR-2 testing. The reason for the negative results of qPCR-2 in group 3 might thus be degradation of low-level B19 DNA.

Of the 13 (single) blood donations excluded from manufacture due to excessive B19-DNA levels by qPCR-1, 11 were still available for testing, and were found positive by qPCR-2. The QIAamp DNA Mini Kit -purified samples showed a DNA concentration slightly (≤ 1 log) higher than the same samples extracted by phenol-chloroform. When the phenol-chloroform-extracted samples were diluted 1:100 in PCR-grade water, the melting points for all samples except one were characteristic for genotype 1, with an average T_m of 67.41 °C (67.23 – 67.51). With undiluted samples the melting points were unusually low, 64.9 °C (61.81-65.97). However, the melting point for one sample (#7) remained between the positions of genotypes 2 and 3 (T_m ~63 °C) even after dilution. When DNA of these same samples was extracted with QIAamp DNA Mini Kit, all showed melting points typical for genotype 1 even undiluted, except plasma #7, which gave the unusual melting point of 63 °C with this extraction method as well. By sequencing, this sample was subsequently shown to contain B19 type 1.

In tissue: All the skin (N=34) samples, and synovial tissue samples, previously tested positive by NS1-, VP1- and VP2-PCRs (N=16), were studied with NS1-gene primers specific for the new genotype (K71). DNA of B19 type 2 was detected from skin in 9/19 B19-seropositive and in 0/15 B19-seronegative subjects (table 7.). All in all, in skin, B19

type 2 DNA was more prevalent (47 %) than the prototypic B19 DNA (26 %). In synovium, prototypic B19 DNA was detected in 67 % of the seropositive samples, while type 2 DNA was absent.

Biological and immunological relations of genotypes 1-3 (IV)

Promoter activity

To examine the impact of the >20% sequence diversity within the p6 promoter region on the biological activity or cell tropism of the variant viruses, the p6 promoter strengths of all three virus types was measured in a variety of cell types, in proportion to the strength of the SV40 promoter. In concordance with earlier studies (Gareus *et al.*, 1998), the B19 promoters were most active in the permissive cells. In all other cell types the promoters of the three B19 genotypes were of similar strength, except in HaCaT cells, in which the type 1 promoter was ~3 fold stronger than those of genotypes 2 and 3. The difference was reproducible (Figure 1 in IV).

To examine whether the NS1 protein of B19 type 1 can enhance the activity of p6 in all the three B19 types, the promoter-luciferase constructs were transfected to HeLa/pGRE.NS cells induced with dexamethasone to produce the genotype 1 NS1 protein (Leruez-Ville *et al.*, 1997). The expression of NS1 protein was verified by IF. Uninduced HeLa/pGRE.NS cells, and cells containing the native pGRE-vector prior to (HeLa/pGRE) and following (HeLa/pGRE IND) induction, served as negative controls. The promoter activities of the three virus types in the presence of NS1 were 3 – 4 times stronger than in the absence of NS1, with no significant difference between the three virus types (Figure 2 in IV).

Expression of recombinant VP2 and VP1/2 virus-like particles of genotype 2

Sf9 cells were infected with recombinant baculoviruses containing the B19 virus type 2 gene(s) for either VP2 alone or VP1/2 together. With both constructs, SDS-PAGE and subsequent Western-blotting verified the production of VP1 and VP2 proteins. EM of cell lysates showed icosahedral, parvovirus-like particles with a diameter of ~23 nm (Figure 8). Both of these VLPs of virus type 2 hemagglutinated human red blood cells and were thus used, together with those of type 1, to set up EIA-tests for IgG cross-reactivity studies.

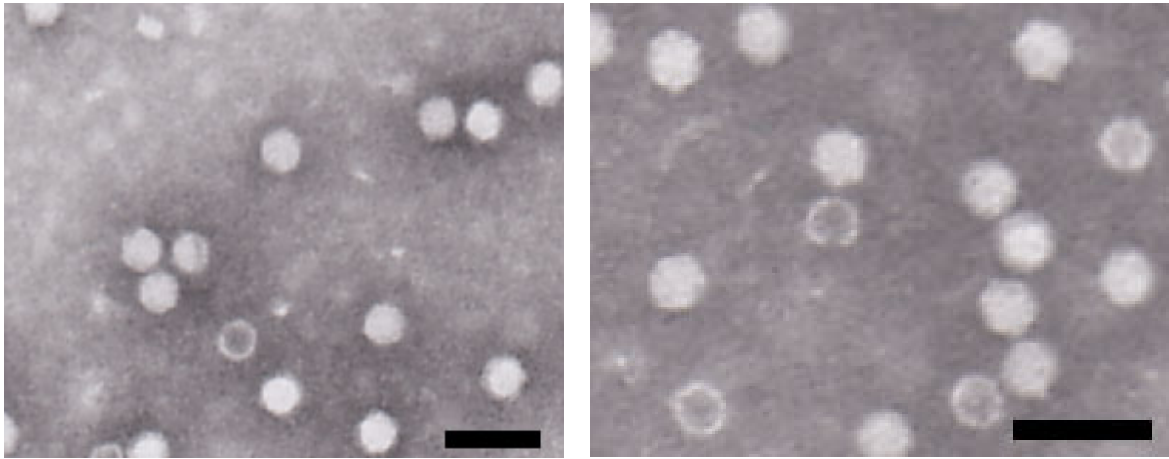


Figure 8. EM of baculovirus-expressed, purified B19 type-2 VP2 (left) and VP1/2 (right) capsids. Scale bar 50 nm.

Hemagglutination activity and infectivity of virus types 1-3

To determine the viability of the three B19 genotypes, two myeloid cell lines, KU812Ep6 and UT7/Epo-S1 were infected with virus-type 1, 2 or 3 -containing plasma / sera. These sera (SPR3, IM-81 and D91.1, respectively) were known to contain B19 DNA, and were examined further with an hemagglutination assay (HA) to make sure they also contain viral particles. All samples did hemagglutinate the human erythrocytes at +4°C, pH 5.8. With the virus type-1 positive serum (SPR3), hemagglutination was observed with DNA concentrations down to 4.7×10^7 IU/ml; with virus type 2 (IM-81) down to 1.7×10^6 IU/ml; and with virus type 3 (D91.1) down to 5.5×10^5 IU/ml (Figure 3 in IV). The corresponding HA-titers were 14400, 14400 and 8700, respectively.

All three genotypes were able to infect the two cell lines (KU812Ep6 and UT7/Epo-S1). IF showed both NS1 and VP proteins in KU812Ep6 infected with virus types 1-3, and in UT7/Epo-S1 cells infected with virus types 1 and 2. In UT7/Epo-S1 cells infected with virus type 3, IF verified only capsid protein production due to the scarcity of type 3 serum (Figure 4 in IV). RT-PCR showed the synthesis of spliced messenger-RNA in both cell types infected with any of the three B19 types.

Enzyme immunoassays

To examine the immunological relation of B19 virus types 1 and 2, four IgG-EIAs, using as antigen VP2 or VP1/2 capsids of either virus type, were set up. Sera from three groups of subjects were examined with the four EIAs: group 1 contained sera from subjects carrying in their tissues B19 type 1 DNA (n=24), group 2 contained sera from subjects carrying in their tissues type 2 DNA (n=25), and group 3 contained sera from B19 IgG

negative subjects (n=13). Cross-reactivity of 100 % was observed between genotypes 1 and 2. The mean absorbance values for the three serum groups are shown in table 8. With one sample (a type 2 serum), the type 2 VP2 EIA absorbance remained below threshold. The overall absorbances with this antigen remained lower than with the other three antigens, irrespective of the antigen concentration or of the genotype specificity of the serum used. All the antigens were equally reactive with the acute-infection and the past-immunity serum pools.

Table 8. IgG reactivity with VP2 or VP1/2 capsids of sera from subjects with B19 DNA of types 1 or 2, in tissue, or from seronegative subjects. The range of absorbance values obtained with individual serum samples and the calculated mean value for each group of samples are shown.

Sera (dilution 1:200)	Absorbance	ANTIGEN			
		TYPE1		TYPE2	
		VP2 40 ng/well	VP1/2 80 ng/well	VP2** 80 ng/well	VP1/2 100 ng/well
B19 type 1 DNA-carriers n=24	mean range	2,650 0.867 - 3.838	2,698 0.837 - 3.756	1,038 0.830 - 1.616	2,189 0.822 - 3.032
B19 type 2 DNA-carriers n=25	mean range	2,588 0.451 - 3.343	2,613 0.456 - 3.539	1,049 0.134* - 1.358	2,142 0.351 - 2.686
NIBSC (1:200)***	mean range	2,620 2.430 - 2.748	2,827 2.710 - 2.895	0,971 0.843 - 1.060	2,110 1.870 - 2.134
B19 IgG-negatives n=13	mean SD	0,034 0,028	0,041 0,029	0,045 0,040	0,039 0,036
	cut-off (mean +3SD)	0,119	0,128	0,166	0,146
Acute serum pool		2,858	2,945	1,066	2,271
Past-immunity serum pool		2,892	3,222	1,018	2,385

*One sample showed borderline reactivity.

**The overall reactivity was equally low for both genotypes.

***International standard for B19 IgG.

Discussion

Newly found B19 types (II)

For a long time, the B19 DNA sequence was considered extremely stable with a variation of only ~1-2 %. In 1998, a new B19 variant was found in France, in blood from a child with severe anemia (Nguyen *et al.*, 1998; Nguyen *et al.*, 1999). Samples of blood and bone marrow were tested for B19 DNA, but the results were peculiar: a PCR amplicon of appropriate size could clearly be seen on agarose gel after ethidium bromide staining but it gave only a faint signal when hybridized with a B19 specific probe. To determine whether this weak hybridization occurred due to mutations within the probe recognition region, the PCR products were sequenced and several nucleotide substitutions were seen. Phylogenetic analysis placed this sequence outside the B19 group, although when compared to B19 sequences in GenBank, it resembled B19 in FASTA analysis. This variant, V9, differed from the previously sequenced, prototypic B19 isolates by >10%, demonstrating that the B19 virus genome is much more divergent than previously recognized.

In order to determine the molecular characteristics of the B19 virus DNA persisting in synovial tissue (I) and skin (II), we used the three nested PCR reactions, VP1-, NS1- and VP2-PCR, which all were based on the two, blood-derived, nearly full-length reference sequences Au and Wi. While in synovial tissue all VP1-PCR-positive samples were positive also with the two additional PCRs, to our surprise many skin samples were positive by the VP1 PCR alone. Analysis of the other genomic regions revealed a novel B19 genotype. Comparative analysis by BLAST or FASTA of one dermal isolate, LaLi, with sequences stored in GenBank or EMBL database showed this new genotype to be B19-related but different from the blood-derived prototypic B19 sequences and from the previously published variant V9. At the DNA level, the protein coding sequences differed by 10.8% from the Au isolate and by 8.6% from V9. Simian parvovirus (SPV), the most B19-related of the animal parvoviruses, differed from the LaLi isolate by more than 40%.

Simultaneously, with our discovery of variant LaLi in the skin of a healthy adult (II), B19 isolates of a corresponding DNA sequence were reported by two other groups (Nguyen *et al.*, 2002; Servant *et al.*, 2002). The variant A6 was discovered in the USA, and was by DNA sequence distinct from both B19 prototype and V9, but 98 % similar to LaLi (Nguyen *et al.*, 2002). In France, 11 variants were found, two of which in phylogenetic analysis clustered with LaLi, and the others together with V9, although the V9-like cluster showed a greater divergence (5.3 % between isolates V9 and D91.1) than was seen among the prototypic or type 2 sequences (Servant *et al.*, 2002). Based on phylogeny, three genotypes of B19 were thus proposed; genotype 1 corresponding to the

prototype Au-like strains; genotype 2 corresponding to LaLi-like strains; and genotype 3 corresponding to V9-like strains. Some divergence has been detected also within these genotypes. The genotype-3 cluster of Servant *et al.* showed a divergence of 5.3 %, exemplified by the DNA isolates V9 and D91.1 (Servant *et al.*, 2002). These two genetically distinct subtypes were later identified also among 13 near-full-length genotype-3 sequences from Ghana, Europe and Brazil, which were named subtypes 3a (V9-like) and 3b (D91.1-like) (Parsyan *et al.*, 2007). Recently, in Vietnam two subtypes have been detected also within genotype 1, with a sequence variation of 5-6 % (Toan *et al.*, 2006).

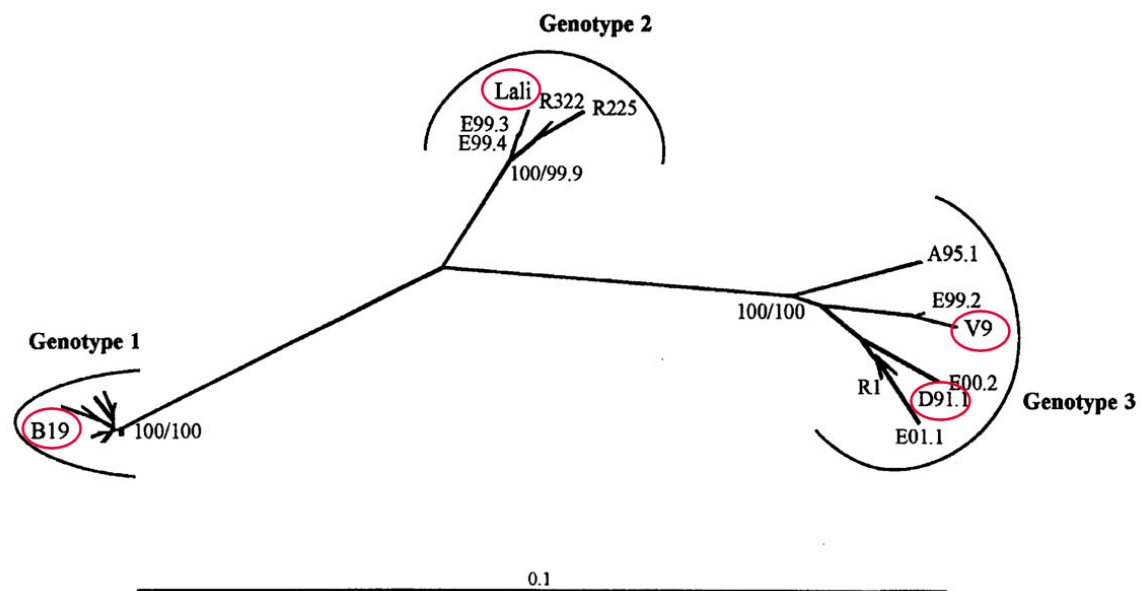


Figure 9. A phylogenetic tree showing the three B19 genotypes. From Servant *et al.* (2002), with permission.

Biological properties of B19 types 1-3 (IV)

When comparing the DNA sequence of the three B19 types, the gene encoding the NS1 protein is more divergent than the genes encoding the two capsid proteins VP1 and VP2. The divergence is smallest within the unique region of VP1. At the amino acid level, again the NS1 proteins of B19 types 2 and 3 differ most, by 6 %. The VP1 unique regions of B19 types 2 and 3 differ, at the aa level, by 4.4% and 6.6% compared to the Au isolate, while the major capsid protein VP2 shows only ~1 % difference. However, a single point mutation in a viral coding region can alter host-cell tropism or might lead to organ-specific persistence. The host range determinants of the non-human parvoviruses minute virus of mice (MVM) and the feline and canine parvoviruses (FPV; CPV) have been characterized

in detail. Only 2 amino acids of the capsid region determine the lymphoid-cell tropism of the MVM variant MVMi and the fibroblast tropism of MVMp (Ball-Goodrich and Tattersall, 1992). Likewise, a change of only a few amino acids yields canine tropism for FPV (Chang *et al.*, 1992).

The most striking variation is seen within the non-coding region containing the p6 promoter, which differs by >20% (Figure 5). By transient transfection experiments, p6 of B19 type 1 was shown to be stronger in cell lines permissive for B19 than in cell lines non-permissive for B19 (Gareus *et al.*, 1998). With recombinant AAV clones, B19 p6 has been shown to confer autonomous replication competence and erythroid specificity to AAV (Wang *et al.*, 1995). The promoter strength is regulated by the viral NS1 protein and by cellular factors (Blundell and Astell, 1989; Liu *et al.*, 1991b; Momoeda *et al.*, 1994a; Raab *et al.*, 2001; Raab *et al.*, 2002; Vassias *et al.*, 1998). Thus, the extensive divergence seen within the promoters of the three B19 types might alter the extreme tropism for erythroid cells. However, as shown in table 9, besides the B19 prototype, also both of the B19 variants have been associated with anemia or an aplastic crisis, indicating erythroid tropism.

In the first infectivity studies of the virus type 2 (isolate A6), RNA transcription could not be detected with RT-PCR in UT7/Epo-S1 cells (Nguyen *et al.*, 2002). In contrast, in KU812Ep6 cell culture mRNA expression of types 1 and 2 showed similar kinetics (Blumel *et al.*, 2005). The p6 promoter activity of virus type 3 had not been studied. Therefore, we compared the p6 activities of B19 types 1-3. Our results showed that the promoters of all three virus types were of similar strength (IV) and, in concordance with earlier studies with the B19 prototype (Gareus *et al.*, 1998), were most active in cell lines permissive for B19 type 1 infection (IV). The NS1 protein of virus type 1, a known trans-activator of B19 type 1 promoter (Doerig *et al.*, 1990; Gareus *et al.*, 1998), activated the promoters of all three types equally.

The self-assembly of structural proteins into capsids had been shown for genotypes 1 (Brown *et al.*, 1991; Franssila *et al.*, 2001) and 3 (Heegaard *et al.*, 2002b). We demonstrated the same feature for genotype 2 with the capsid proteins VP2 and VP1/2 expressed in the baculovirus system (IV). Electron micrographs revealed icosahedral particles with the same diameter (~23 nm) as with virus type 1 (Cossart *et al.*, 1975), which furthermore were able to hemagglutinate human erythrocytes. In the same HA conditions, the type 1-3 viremic sera were shown, besides DNA, to contain virus particles with red cell-surface binding activity.

To further compare the biological characteristics and cell tropism of the three B19 types, we infected two cell lines permissive for B19 type 1 with B19 DNA positive sera. In infectivity experiments, all three virus types induced transcription and maturation (splicing) of mRNAs and the synthesis of both NS1 and VP proteins in two B19 type 1-

permissive cell lines. Thus, the results showed that all three B19 types were biologically active viruses, and suggested similar cell-tropism.

Methods for detection of B19 types 2 and 3

Diagnosis of B19 infection relies on serology and the detection of viral DNA. Several PCR methods for B19 detection have been published, but as the recent findings of the new B19 types illustrate, the genome variation among the B19 virus is far more extensive than previously believed. Due to excessive DNA sequence divergence, most B19 PCR methods are unlikely to detect these new B19 variants or putative additional genotypes. Clearly, new methods for detection and differentiation of the new B19 virus variants are needed. Similarly, the possible immunological cross-reactivity of the three B19 types needed to be elucidated, to see whether antibodies against one genotype protects from infection of another, and whether the available serological methods, designed for diagnostics of the prototypic B19, could also be used to detect antibodies elicited by the new variants.

Immunological cross-reactivity of B19 types 1-3 (IV)

Studies on the antigenic relation between B19 types 1 and 3 have shown a high degree of cross-reactivity. Heegaard *et al.* produced, with the baculovirus system, V9-like recombinant capsids, and screened a panel of 270 clinical samples for the presence of IgM and IgG by EIA. The results were compared to those obtained with a commercial B19 VP2 assay, and revealed a 100 % cross-reactivity between B19 types 1 and 3 (Heegaard *et al.*, 2002a). The 270 samples, however, presumably did not contain antibodies against B19 type 3, because of the rarity of type 3 infections in Europe. Candotti *et al.* screened (with a commercial type-1 VP2-based EIA) samples collected from areas where genotypes 1 (UK) or 3 (Ghana) are endemic (Candotti *et al.*, 2004), and confirmed the high level of cross-reactivity between genotype 1- and genotype 3-specific antibodies. However, they found a small number of B19 DNA-positive samples containing immune complexes that were undetected by the genotype 1 VP2 antigen, and thus suggested a decreased reactivity of genotype 3 antibodies with genotype 1 antigen (Candotti *et al.*, 2004; Parsyan *et al.*, 2006). Parsyan *et al.* assessed the ability of genotype 1 or 3 VP2 or VP1/2 capsids to detect antibodies (Parsyan *et al.*, 2006). They reported a high degree of cross-reactivity between antigens of B19 types 1 and 3 in samples from UK blood donors and Ghanaian child populations exposed to B19 types 1 (99.3 %) or 3 (98 %), and in this respect confirmed and extended the findings of Heegaard *et al.*

In vitro neutralization tests using sera from subjects infected with B19 type 1 have shown inhibition of in-vitro infection of type 2 (Blumel *et al.*, 2005). However, due to a general lack of methods to distinguish type-2 IgG-positive sera, it has not been possible to test if sera from subjects infected with type 2 would react with antigens of type 1, or whether type 2 antigens are recognized equally well by type-1 sera. Because of our unique material, sera from subjects infected in the past with either B19 type 1 or type 2 (as evidenced by persistence of the respective DNAs in tissue), and because of our recombinant VLPs (VP2 and VP1/2 capsids) of both virus types, we were able to address this question. Our results revealed a 100 % cross-reactivity in VP1/2- and VP2-EIAs between virus types 1 and 2 (IV). Such knowledge of cross-reactivity is relevant both for diagnosis and vaccine development, as well as for the definition of the taxonomic status of these human erythrovirus variants (Tattersall, 2006). Official classification of ICTV now places Au (genotype 1), LaLi and A6 (genotype 2), as well as V9 and D91.1 (genotype 3) as strains of the B19 species (ICTVdB - The Universal Virus Database, version 4.), which according to the immunological studies of ours and others, seem to constitute a single serotype.

Qualitative PCR methods

For detection and differentiation of B19 types 1-3, two methods have been published (Heegaard *et al.*, 2001; Servant *et al.*, 2002). Servant *et al.* (2002) performed PCR with primers allowing for simultaneous amplification of the B19 prototype (genotype 1), LaLi-like sequences (genotype 2) and V9-like sequences (genotype 3), and used restriction fragment length polymorphism to identify variant types: the PCR product is digested with *MfeI*, for which a recognition site is found in the amplicon of B19 type 1 but is altered in types 2 or 3. The definitive result is obtained by sequencing. Heegaard *et al.* used a nested PCR method, where first-round primers recognize both genotypes 1 and 3, and for differentiation, distinct nested primers are used (Heegaard *et al.*, 2001).

In our studies, three nested-PCRs (VP1-, VP2-, and NS1-PCRs) were used to examine B19 DNA persisting in synovial tissue and skin. Near-full-length genome of five synovial isolates positive with all the three PCRs was sequenced, and shown to be of B19 type 1 (I). However, most of the skin-derived isolates were positive with VP1-PCR alone. Sequencing of two of such dermal isolates revealed genomic DNA of B19 type 2. Thus, although our three PCR methods detected prototypic B19 DNA in equal sensitivity (I), which was with plasmid clones determined to be 15 copies per reaction (Norja *et al.*, 2006), only the VP1-PCR could reliably detect also B19 type 2. To distinguish type 2 from the prototypic B19 DNA, a nested PCR (K71-PCR) was then designed according to the LaLi-sequence, and was shown to be specific for B19 genotype 2 (II). By using

plasmid constructs, its detection sensitivity was shown to be 1.5 copies per reaction (Norja *et al.*, 2006).

qPCR methods for detection of B19 genotypes 1-3 (III)

While the B19 virus has been known for many years, its transmission to recipients of plasma derivatives despite attempted inactivation has recently reinforced the necessity of plasma pool screening. All the methods mentioned above can reliably be used for qualitative detection and/or differentiation of the B19 genotypes but are labourious and time-consuming and therefore restrict the size of material studied. After the estimation of a “safe level” of B19 virus DNA after manufacture (European Pharmacopoeia 2004; Brown *et al.*, 2001) most plasma pool manufacturers have begun to quantify B19 DNA by nucleic-acid testing to ensure a viral load of $<10^4$ IU/ml.

Real-time PCR performs the amplification and detection steps simultaneously in one tube and when used together with external standards, provide quantitative data. Two quantitative PCR methods are commercially available and are used for plasma pool screening. We compared the performance of those two quantitative real-time PCR methods with plasmids containing DNA of B19 types 1, 2 or 3. In our studies using cloned DNA of either genotype, qPCR-1 was slightly more sensitive than qPCR-2 in detection and quantification of B19 genotype 1. In detection and quantification of B19-genotypes 2 and 3, however, qPCR-2 performed better: it detected all three genotypes, although subtype 3b (represented by isolate D91.1) with lower sensitivity. On the contrary, qPCR-1 did not detect genotype 2, and detected only subtype 3a (represented by isolate V9) of genotype 3. Similar studies using plasmid constructs were conducted by Baylis *et al.* (2004), and their results were in concordance with ours: qPCR-1 detected genotype 1 only, whereas qPCR-2 detected all genotypes, except subtype 3b with reduced sensitivity. Additionally, Braham *et al.* (2004) evaluated qPCR-1 for diagnostic testing and compared it to a nested PCR and hybridization assays for detection of B19 DNA in clinical specimens. Among 228 samples, only two sera collected within two weeks from the same renal transplant patient gave negative results with qPCR-1, although they had earlier been shown to contain B19 DNA with a nested PCR method. Sequencing of the amplicons and phylogenetic analysis showed that these samples contained B19 type 2 DNA (Cohen *et al.*, 2006). In our studies (III), qPCR-2 detected all three genotypes in clinical samples, and identified them correctly by melting curve analysis. This method in principle allows for differentiation of specific from non-specific PCR products such as primer-dimers or mispriming products but can also be used for mutation analysis. Thereby, rapid detection and differentiation of the three genotypes in a single reaction without labourious

restriction enzyme analyzes or electrophoretic procedures make qPCR-2 a valuable tool for rapid genotyping.

Occurrences of B19 types 2 and 3

Even though an increasing number of studies have addressed the role of the B19-virus prototype (genotype 1) in transfusion of blood and plasma derivatives, knowledge of the frequency and magnitude of the variant genotypes was scanty. A main reason has been the lack of suitable tools for the simultaneous detection and distinction of all genotypes. With a qualitative PCR method for detection and differentiation of B19 types 1 and 3, Heegaard *et al.* examined 100 sera containing B19 IgM, and 50 plasma pools, each from 2000 Danish blood donors, without detection of V9 (Heegaard *et al.*, 2001). The same result came from 190 bone marrow samples of healthy subjects (Heegaard *et al.*, 2002a). To evaluate the genotype circulation (in France) and frequency among patients of different entities, Servant *et al.* screened and genotyped (by PCR, RE-analysis and sequencing) 1084 clinical samples from various sources (Servant *et al.*, 2002). Indeed, LaLi-like sequences were found in only 2 sera and V9-like sequences in only 9 samples (5 serum, 3 bone marrow, 1 blood), including subtype D91.1 which was detected in serum. With the same method, Nguyen *et al.* (2002) examined 149 sera and 18 bone marrows collected in the USA. Of the 29/149 PCR-positive sera, only one (A6) was of genotype 2, and all the others of genotype 1. No variants were detected in bone marrow. Upon re-examination of the Danish blood-donor plasma pools (Heegaard *et al.*, 2002a) and additional pools (altogether representing 120 000 single donations) only genotype 1 was found.

By a quantitative PCR method, the prevalences of genotypes 1, 2 and 3 among Finnish blood donors was examined (III). None of the 140 160 blood donations studied contained B19 genotypes 2 or 3. This result is in concordance with those of Heegaard *et al.*, and suggests that viremic infections by the “new” genotypes, at present, are extremely rare. Confirmatory evidence came from recent studies by Norja *et al.* (2006): altogether 1640 serum samples, collected during 1983-1997 in Finland from patients with rash, fever or various other constitutional symptoms, also including patients with serologically confirmed erythema infectiosum, contained exclusively B19 type 1. Similarly, among 1639 clinical samples received for parvovirus diagnosis in the UK (228 during 2001-2002 (Braham *et al.*, 2004) and 1411 during 2003-2005 (Cohen *et al.*, 2006)), only one case of B19 type 2 and one of type 3 were detected (Cohen *et al.*, 2006). In Germany, virus type 2 was detected in a renal transplant recipient (Liefeldt *et al.*, 2005), in a pregnant woman (Enders *et al.*, 2006), and in a single plasma donation (Blumel *et al.*, 2005). Nevertheless, B19 type 2 has been detected in coagulation factor concentrates, albeit in lower frequency (2.5 %) than B19 type 1 (46.5 %) (Schneider *et al.*, 2004). Candotti *et al.* screened for B19

DNA by consensus nested- and quantitative-PCRs, blood donations from the United Kingdom and three countries of sub-Saharan Africa (Candotti *et al.*, 2004): among plasma samples from the UK, South Africa and Malawi, 0.55 – 1.25 % contained B19 DNA ($42 - 1 \times 10^6$ IU/ml), all of type 1. Interestingly, among Ghanaian plasma samples 1.3 % were positive for B19 DNA ($158 - 1.35 \times 10^5$ IU/ml), and all findings were of type 3 (Candotti *et al.*, 2004). Similarly, among pregnant women in Ghana, the prevalence of B19 DNA was 1.8 %, of which 94 % were of genotype 3 ($46 - 3.63 \times 10^6$ IU/ml) (Candotti *et al.*, 2006). Among Ghanaian children, the prevalence of B19 DNA was 11.5 %, all of type 3 (Parsyan *et al.*, 2006). Of the viremic children, ~35 % were positive for B19 IgM, indicating acute infection. In Brazil, among 69 bone marrow samples collected from patients with B19 type 1-related symptoms, ~17 % contained B19 DNA; 5 of type 1; 1 of type 2; and 6 of type 3 (Sanabani *et al.*, 2006). Of these patients, however, no serological results were available to determine the time of the primary infection. In Vietnam, B19 DNA was detected in 24 % of 399 plasma samples collected from HBV-infected patients. Among these subjects, B19 type 1 predominated, type 2 was detected in 4 % of the samples, while type 3 was absent (Toan *et al.*, 2006). Taken together, viremic occurrence of B19 type 2 has been observed only very sporadically. B19 type 3 appears to occur endemically in Ghana and in Brazil, but only occasionally in Europe (Candotti *et al.*, 2004).

Tissue persistence of the prototypic and recently found B19 variants

The first aim of this thesis work was to examine the integrity of the synovial B19 DNA molecules in the acute-phase of infection and in subjects representing long-term persistence. Since no quantitative PCRs were available, this study was conducted with three distinct, qualitative PCRs (NS1, VP1, VP2), amplifying different genomic regions and together covering the complete protein coding sequence. These PCRs were shown to be of similar sensitivity. By serially diluting the samples, we showed that all parts of the protein-coding region could be detected at the same, limiting template dilution, suggesting that all genomic regions in the particular sample are present in equimolar amounts. In addition, the fact that the three amplicons are relatively long (≥ 1000 bp) and that the two terminal regions of the coding area could be amplified simultaneously in the highly diluted samples gave further support to the hypothesis that the DNA molecules are retained in synovium unfragmented. The B19 genome has at both termini long palindromic repeats (Astell and Blundell, 1989; Deiss *et al.*, 1990; Shade *et al.*, 1986) consisting mainly of GC base pairs, which together with their hairpin secondary structure provide extremely difficult targets for PCR detection. We did not examine those noncoding termini.

However, with an intact full-length coding region present in synovia with no nt substitutions altering the reading frame (stop-codons, insertions or deletions), viral mRNA and protein could potentially be produced.

The recently infected individuals and those infected in the past resembled each other also in the extent of variability of the synovial protein-coding regions of the B19 genome, albeit none of the sequences were identical. Similarly, within the p6 promoter regions from 13 synovial tissue samples of hemophiliac patients with chronic synovitis, and 12 acute-phase serum samples from patients with different symptoms (Zakrzewska *et al.*, 2001), the rates of genomic variability between persistent or acute-phase isolates were not significantly different.

By sequence analysis, the B19 coding regions that we initially detected in synovium closely resembled those of two reference strains derived from blood, Au (Shade *et al.*, 1986) and Wi (Blundell *et al.*, 1987), both of B19 type 1. All the conserved (i.e. found in all our synovial samples studied) nucleotide changes relative to either reference strain were found to agree with the other reference. This implied that the genome persistence, as a phenomenon, in synovial tissue is not related to specific mutations or strain variants.

In skin samples from constitutionally healthy B19 seropositive adults (N=19), virus type 2 was more prevalent (47%) than the conventional virus type (26%) (II). In sharp contrast to our preliminary studies, none of the 30 synovial tissue samples studied for comparison, contained DNA of virus type 2. This led to the presumption of a skin predilection for genotype 2. The tissue distribution of the B19 variants has been addressed in subsequent studies: Wong *et al.* examined livers from patients with fulminant hepatitis (FH), hepatitis-associated aplastic anemia (HAA), and patients with hepatitis B (HBV) or C (HCV) infection (Wong *et al.*, 2003). They found B19 DNA in all patient groups; genotype 2 in 5/30 HBV/HCV patients, and genotype 3 in 1/30 HBV/HCV patients, and in 1/23 FH patients. They also performed RT-PCR to detect B19 transcripts, with negative results. In studies by Norja *et al.*, B19 DNA was examined in 523 solid tissue biopsies (synovial tissue, skin, tonsil and liver) collected in Finland or Germany from constitutionally healthy individuals or from patients with B19 non-related symptoms (Norja *et al.*, 2006). Both virus types 1 and 2 were found in all tissue types, albeit type 2 at a lower frequency. In this study, B19 type 3 was not detected. Demographic analysis showed the occurrence of virus type 1 DNA in subjects of all age groups, in contrast to virus type 2 DNA, which only occurred in those borne before year 1973. Taken together, the results suggest that B19 types 1 and 2 circulated to an equal extent in northern and central Europe until the 1960's, after which type 2 seems to have disappeared from wide circulation. That the prevalence of persisting B19 DNA was not diminished among the elderly, points to a storage mechanism of life-long capacity both for the B19 type 2 and the prototype (Norja *et al.*, 2006). Comparable persistence of B19 virus type 3 in solid tissues of healthy (or diseased) individuals remains to be demonstrated.

It is still unclear which cell type(s) harbour the tissue-persisting B19 DNA. B19 could be attached on the surface of follicular dendritic cells or might be carried inside macrophages. However, life-long tissue persistence, apparently without fragmentation, in actively hydrolytic cells would seem unlikely.

In addition to erythrocytes, globoside or the blood-group P antigen, the main cellular receptor for B19 (Brown *et al.*, 1993) and other glycosphingolipids capable of B19 virus binding, have been detected at several sites and in several cell types (Cooling *et al.*, 1995; Weigel-Kelley *et al.*, 2001). Globoside seems to be necessary for B19 attachment, although its expression level does not correlate with the efficiency of viral binding (Weigel-Kelley *et al.*, 2001). Weigel-Kelley *et al.* also showed with a recombinant B19 vector system (Ponnazhagan *et al.*, 1998), that in all cell types expressing globoside on the plasma membrane, cell-surface binding of B19 occurs but that it is not sufficient for B19 entry (Weigel-Kelley *et al.*, 2001). Expression of marker genes carried by the B19 vector, indicating viral entry and nuclear transport of the recombinant genome, was seen in two cell lines of epitheloid origin, 293 and HeLa, and also in two human primary cell lines (human umbilical vein endothelial cells, HUVEC, and normal human lung fibroblasts, NHLF), but not in the human erythroleukemia cell lines HEL and K562, despite the presence of globoside on their surface. Subsequently, B19 entry has been shown to be mediated by $\alpha 5\beta 1$ integrins in high-affinity conformation (Weigel-Kelley *et al.*, 2003) and regulated in a cell-type specific manner (Weigel-Kelley *et al.*, 2006). Recently, expression of Ku80 on transfected HeLa cells was shown to enhance the entry of B19, suggesting that Ku80 mediates efficient B19 entry in cooperation with globoside and probably with $\alpha 5\beta 1$ integrins (Munakata *et al.*, 2005).

The presence of globoside has been shown for primary human synovial fibroblasts (Ray *et al.*, 2001); however, even high concentrations of the B19 virus could not productively infect cultured human fibroblast-like synoviocytes (Lu *et al.*, 2006). However, B19 viremic serum has been reported to induce fibroblast invasiveness (Ray *et al.*, 2001), to activate synoviocytes and to increase their migration in vitro, apparently by the phospholipase 2 (PLA₂) activity of the VP1u region (Lu *et al.*, 2006). The VP1u of B19 types 2 and 3 differ from the prototype in 11 and 16 amino acids, respectively. Two of the aa substitutions are located in the PLA₂-domains of type 2, and one of type 3, while several are clustered in the VP1u amino terminal region. Whether these substitutions alter the PLA₂ function of B19 types 2 and 3 and the tissue tropism or pathogenicity remains to be determined.

In primary hepatocytes and HepG2 cells inoculated with the B19 virus, Poole *et al.* detected production of NS1, but not VP transcripts or proteins (Poole *et al.*, 2004). The absence of VP transcripts demonstrated that the infection in hepatocytes was restricted, with no production of progeny virions. However, NS1 was produced and translocated into the nucleus, and induced apoptosis in these cells (Poole *et al.*, 2006).

B19 DNA was detectable in skin fibroblasts (but not in keratinocytes) cultured from a systemic sclerosis patient, and remained so for several *in vitro* passages (Ferri *et al.*, 2002). B19 mRNA by *in situ* RT-PCR has been seen in endothelial cells, in the surrounding mononuclear cells, and in fibroblasts of skin biopsies from patients with a spectrum of connective tissue diseases (Magro *et al.*, 2002; Magro *et al.*, 2004). Zakrzewska *et al.* claimed low-level B19 infectivity of human dermal fibroblasts and human umbilical vein endothelial cells, as evidenced by detection of equal levels of NS1- and VP1- mRNAs (Zakrzewska *et al.*, 2005). However, RNA transcription is not necessarily followed by the production of viral proteins and replication (Gallinella *et al.*, 2000; Pallier *et al.*, 1997). No direct evidence of active replication was observed (Zakrzewska *et al.*, 2005). Taken together, these studies show that the B19 virus might attach to and be internalized into cells that do not support B19 replication.

Mechanisms of persistence

If not actively replicating, how could viral DNA be maintained in human tissues for almost a century? The DNA might be integrated into human chromosomes or stored as episomes, or it could even be encapsidated, eg. stay attached on the surface of follicular dendritic cells or perhaps carried inside macrophages as full virions, in accordance with our results suggesting intactness of the persisting DNA molecule.

Another human parvovirus, AAV2, integrates site-specifically, by a non-homologous recombination mechanism, into chromosome 19, from where it can be released by aid of a helper virus or other external stimuli (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Kotin *et al.*, 1992). The ability to integrate site-specifically into synthetic cellular episomes has been demonstrated also for minute virus of mice (MVM) (Corsini *et al.*, 1997). Kerr *et al.* found several short regions (17-26 nucleotides) of sequence identity between B19 and the human genome (Kerr and Boschetti, 2006), which could possibly be involved in promoting the B19 DNA persistence, e.g. through homologous recombination, or regulatory effects on host transcription, possibly accomplished via mRNA splicing or RNA interference. However, no clear evidence yet exists for B19 virus chromosomal integration.

It is not known whether B19 could in certain conditions, such as stress or immunosuppression, be released from latency. Reports of putative endogenous re-activations of B19 virus are rare, possibly also in part because they are difficult to identify – i.e. to be distinguished from dormant persistence and from possible re-infections among immunocompromized hosts (Muetherig *et al.*, 2007; Soderlund *et al.*, 1997a). However, B19 type 2, being rarely seen in primary infection but commonly detected in the tissues, (III; Heegaard *et al.*, 2001; Norja *et al.*, 2006), has been encountered in the blood of HBV/HCV co-infected patients or in immunodeficient subjects conspicuously often (Table 9).

The persistence of B19 genomes of virus type 2 can not be attributed to reinfections, since that virus type is absent from current circulation (III; Heegaard *et al.*, 2001; Norja *et al.*, 2006). One hypothetical mechanism for the life-long DNA persistence could be equilibrium between viral replication and efficiency of host immunity. However, an immunological difference is not likely to explain the absence of B19 type 2 DNA in tissues of the young because of the similarity in B-cell immunity between the three virus types (IV; Blumel *et al.*, 2005; Heegaard *et al.*, 2002b; Parsyan *et al.*, 2006) and the paucity of viremic type 2 infections during the recent decades. To investigate the role of the immune system in controlling replication of the persisting viral DNA molecules, Manning *et al.* recently examined the levels of B19 DNA in bone marrow, lymphoid tissue and brain collected from HIV infected subjects at pre-AIDS or in terminal AIDS, and from HIV uninfected individuals. No positive correlation was observed between median viral loads and immunosuppression. On the contrary, the loads in the HIV uninfected individuals were the highest (Manning *et al.*, 2007).

Table 9: Reported B19 type 2 and 3 cases

Type 2 Strain	Sample	Year	Clinical signs	Reference
E99.3	Serum	1999	Rash	Servant <i>et al.</i> 2002
E99.4	Serum	1999	Anemia, immunocompromised	Servant <i>et al.</i> 2002
A6	Serum	1991	Chronic anemia, HIV	Nguyen <i>et al.</i> 2002
IM-81	Plasma	?	?, Plasma donation	Blumel <i>et al.</i> 2005
	Serum	2001	Anemia, renal transplant recipient	Liefeldt <i>et al.</i> 2005
04BR0081	Bone marrow	2004	Renal transplantation, HHV6, pancytopenia	Sanabani <i>et al.</i> 2006
	Serum (2)	2000-2002	HBV	Toan <i>et al.</i> 2006
B19-SJ	Serum/plasma	2000	Renal transplant, anemia	Cohen <i>et al.</i> 2006
GESTgt1	Serum	1996-2002	Pregnant woman with rythema infectiosum	Enders <i>et al.</i> 2006
	Factor III concentrates	<1980		Schneider <i>et al.</i> 2004
	Factor III concentrate	<2004		Schneider <i>et al.</i> 2004
	Livers (5)	?	HBV or HCV	Wong <i>et al.</i> 2003
	skin	birth year \leq 1972	B19 non-related	II
	skin, synovia, tonsils, liver	birth year \leq 1972	B19 non-related	Norja <i>et al.</i> 2006

Type 3 Strain	Sample	Year	Clinical signs	Reference
A95.1	Serum	1995	Chronic anemia, HIV	Servant <i>et al.</i> 2002
D91.1	Serum	1991	Aplastic crisis, G6PD defect, minor thalassemia	Servant <i>et al.</i> 2002
E99.2	Bone marrow	1999	Pancytopenia, HCV	Servant <i>et al.</i> 2002
E00.2	Serum	2000	Pregnant, fever	Servant <i>et al.</i> 2002
E01.1	Serum	2001	Aplastic crisis, immunocompetent	Servant <i>et al.</i> 2002
03BR0440	Bone marrow	2003	Endometriosis, febrile pancytopenia, hemophagocytic syndrome	Sanabani <i>et al.</i> 2006
03BR0570	Bone marrow	2003	Hepatic transplantation, colitis, pancytopenia	Sanabani <i>et al.</i> 2006
03BR0057	Bone marrow	2004	Hepatic transplantation, anemia	Sanabani <i>et al.</i> 2006
04BR0290	Bone marrow	2004	Hepatic transplantation, diarrhea, pancytopenia	Sanabani <i>et al.</i> 2006
04BR0445	Bone marrow	2004	T-cell leukemia, anemia, neutropenia	Sanabani <i>et al.</i> 2006
04BR0448	Bone marrow	2004	Diabetes, pancytopenia, hemophagocytic syndrome	Sanabani <i>et al.</i> 2006
V9	Serum	1995	microcytic anemia, G6PD defect,	Nguyen <i>et al.</i> 1999
R1	Serum	1998	chronic renal insufficiency, macrocytic anemia	Nguyen <i>et al.</i> 1999
E99.1*	Blood	1999	Chronic anemia, HIV	Servant <i>et al.</i> 2002
E00.1*	Bone marrow	2000	Aplastic crisis, Waldenstrom disease	Servant <i>et al.</i> 2002
E00.3*	Bone marrow	2000	Chronic anemia, HIV	Servant <i>et al.</i> 2002
E00.4*	Serum	2000	Anemia, immunocompromised	Servant <i>et al.</i> 2002
B19-AQ	Serum	2005	Chronic renal failure, anemia, red cell aplasia	Cohen <i>et al.</i> 2006
	Plasma	2001-2003	blood-donors, Ghana (1.3%)	Candotti <i>et al.</i> 2004
	Plasma		pregnant women, Ghana (1.8%)	Candotti <i>et al.</i> 2006
	Plasma	2003-2005	blood-donors, Ghana (1.8 %)	Parsyan <i>et al.</i> 2006
Liver	Liver	?	HBV or HCV infection	Wong <i>et al.</i> 2003
LiverH41	Liver	?	Fulminant hepatitis, HCV not tested	Wong <i>et al.</i> 2003

Concluding remarks

This thesis describes the discovery and characteristics of a new B19 variant, LaLi, a representative of B19 genotype 2. As is known for the B19 prototype, DNA of this new virus type was shown to persist in solid tissues of nonsymptomatic humans with serological evidence of pre-existing immunity. Comparison of the viral genomes from synovial tissues of subjects with acute-phase infection and long-term immunity suggested that the viral DNAs persist apparently as intact molecules without persistence-specific mutations. Interestingly, type 2 viremias were not found among blood-donors, strongly suggesting that (in Europe) B19 type 2 is absent from wide circulation.

Even though type 2 and 3 viremias are rare, and in most studies only their DNA has been detected, our work concerning the biological and immunological relations of the new B19 types show that they indeed are biologically active viruses, with the capacity for mRNA production and expression of both non-structural and structural proteins leading to formation of infectious virions. In concordance with recent studies showing similar kinetics for mRNA expression of types 1 and 2 in cell culture (Blumel *et al.*, 2005), our results showed similar transcription efficiency for the p6 promoters of all three virus types. Furthermore, the comparison of p6 activity in various cell types suggested similar tropism. However, their striking difference in temporal occurrence and geographical distribution (Norja *et al.*, 2006) still awaits explanation. The cessation of B19 type 2 from circulation might be a fitness-related phenomenon resembling the replacement of CPV2 by the more effective lineage CPV2a (Parrish *et al.*, 1988; Shackelton *et al.*, 2005). Additional investigations, including measurement of the phospholipase A2 activity (Dorsch *et al.*, 2002; Lu *et al.*, 2006; Zadori *et al.*, 2001), definition of the additional functions of NS1, and elucidation of the impact of the 7.5 and 11 kDa proteins (Zhi *et al.*, 2006) and the polyadenylation regulation sites (Yoto *et al.*, 2006), may be needed to identify the molecular background of the epidemiological difference between the three B19 virus types. However, our studies on their molecular biology show that the three B19 genotypes are, despite their sequence and epidemiological differences, highly similar variants of the same species. Furthermore, immunological studies by us and others suggest that the three B19 types would constitute a single serotype.

Life-long persistence of B19 genomic DNA in human solid tissues is the normal outcome of B19 infection, complicating the interpretation of positive B19 DNA findings in human tissue. Thus far, the persistence in healthy individuals has been shown for B19 types 1 and 2. Further studies are needed to investigate the molecular and cellular mechanisms, as well as the biological activity and possible pathogenetic potential of the B19 genome persistence.

The tissue / cell types harbouring the persistent B19 DNA, the molecular and cellular mechanisms by which the persistence is maintained, as well as the biological activity and possible pathogenetic potential of the persistent B19 genomes needs to be elucidated. Additional studies are also needed to determine whether the persistence mechanism is shared among all the known B19 types (including type 3), and among the newly found species of human parvoviruses, human bocavirus (HBoV) (Allander *et al.*, 2005) and PARV4 (Fryer *et al.*, 2006; Jones *et al.*, 2005). Such information will help in development of new methods (e.g. based on quantification of viral loads, mRNA or antigen detection, and serology) for the diagnosis of acute and chronic B19 infections.

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