Characterization of Parvovirus B19 Genotype 2 in KU812Ep6 Cells

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An infectious parvovirus B19 (B19V) genotype 2 variant was identified as a high-titer contaminant in a human plasma donation. Genome analysis revealed a 138-bp insertion within the p6 promoter. The inserted sequence was represented by an additional 30 bp from the end of the inverted terminal repeat adjacent to a 108-bp element found also, in inverted orientation, at the extreme right end of the unique sequence of the genome. However, despite the profound variations in the promoter region, the pattern of gene expression and DNA replication did not differ between genotype 1 and genotype 2 in permissive erythroid KU812Ep6 cells. Capsid proteins of both genotypes differ in their amino acid sequences. However, equivalent kinetics of virus inactivation at 56°C or pH 4 indicated a comparable physicochemical stability of virus capsids. Sera from six individuals infected by B19V genotype 1 were investigated on cross-neutralization of B19V genotype 2 in vitro. Similar neutralization of both B19V genotypes was observed in sera from three individuals, while the sera from three other individuals showed weaker cross-neutralization for genotype 2. In conclusion, the in vitro replication characteristics and physical stability of B19V capsids are very similar between human parvovirus B19 genotypes 1 and 2, and cross-neutralization indicates a close antigenic relation of genotypes 1 and 2.

B19 virus (B19V or parvovirus B19) is a human virus belonging to the genus Erythrovirus within the family Paroviridae. Paroviruses are single-stranded DNA viruses with small nonenveloped capsids known to be highly resistant to physicochemical inactivation. The clinical manifestation of infection depends on the immunologic and hematological state of the host (for a recent review see reference 36). In otherwise healthy immunocompetent individuals, B19V infections are mostly asymptomatic or cause a mild disease with a rash, called erythema infectiosum. Since B19V replication is restricted to erythroid progenitor cells, infected patients with underlying hematopoietic disease may develop a severe transient aplastic crisis, and immunocompromised patients can suffer from a persistent infection manifesting as chronic pure red-cell anemia. Pregnant woman are at special risk because of B19V-induced hydrops fetalis and fetal loss.

Parovirus B19 can contaminate human blood or plasma donations. The frequency of B19V DNA-contaminated blood donations has been found to range from 1 to 800 and 1 in 1,590 (3, 33). Recently, low-level viremia from persistent infections has been reported at a frequency of approximately 1 in 100 (9). Viremia may be up to 1012 genomes per ml, albeit at a lower frequency of 1 in 8,000 (33), with some seasonal variation. Since viral contamination may be at high titer and because paroviruses are resistant to many inactivation procedures, there is a risk that plasma-derived products contain infectious B19V (4). Transmission cases with plasma-derived products have been investigated in detail (6).

Replication of paroviral DNA is carried out by eukaryotic host cell DNA polymerases which have a low error rate at DNA synthesis. This and presumably some selective pressure on the small virus genome results in a very low genetic variability of conventional B19V DNA sequences (up to 1%). However, in recent years, several virus strains have been identified showing more than 10% divergent nucleotide sequences (12). The first described variant of B19V, called V9, was obtained in 1995 from a child presenting with aplastic anemia and suffering from glucose-6-phosphate-dehydrogenase deficiency (21, 22). The V9 genome showed 11% variability from the already known B19V sequences. Two virus samples, termed LAli and HaAM, representing a novel genotype variant, were identified from skin samples of healthy individuals (15), and another virus strain (A6) very similar to LAli and HaAM was identified in a sample from a human immunodeficiency virus-infected individual presenting with chronic anemia in 1991 (23). The A6 variant was divergent from conventional B19V isolates (87.8% similarity) and the V9 isolate (92.0% similarity). In a large study encompassing more than 1,000 serum or plasma samples, several V9-like and A6-like sequences were found, and it was suggested to group the human erythrovirus sequences into three distinct genotypes (29). Genotype 1 represents the most frequent “conventional” B19V isolates, genotype 2 is represented by the LAli or A6-like isolates, and genotype 3 encompasses the V9 or D91.1 related viruses (29).

The discovery of distinct genetic B19V variants posed important questions regarding their biological properties, pathogenetic potential, and epidemiological relevance. Until now, data on the biological activity of variant erythroviruses were limited. In this report, we describe the identification and characterization of a genotype 2 B19V virus which was present at high titer in a plasma donation. The virus productively infected erythroid KU182Ep6 cells. Despite the significant variation in the whole nucleotide sequence encompassing the promoter region, the NS-1 gene, and viral capsid genes, no differences in the replication process were observed between the genotype 1 and genotype 2 viruses. Furthermore, similar inactivation kinetics during heat treatment and...
at low pH indicated that the physicochemical stability of virus particles does not differ between these genotypes. Cell culture studies indicated cross-neutralization of genotype 2 by sera raised against genotype 1 infection.

**MATERIALS AND METHODS**

**Viruses and cells.** Highly viremic plasma donations were used as the source of infectious B19V. The donations were identified with Baxter’s routine screening program for highly contaminated donations and are termed S-1, IM-71, IM-72, IM-73, IM-80, and IM-81. The IM-81 sample was from a donation in Germany. A high-titer plasma donation containing B19V genotype 1 (termed S-9) was kindly provided by N. Ikeda (Fujirebio Inc., Tokyo, kindly provided by W. K. Roth (DRK-Hessen). Viruses S-1 and S-9 have been infectious B19V. The donations were identified with Baxter’s routine screening study indicated cross-neutralization of genotype 2 by sera raised against genotype 1 infection.

**DNA sequencing.** Overlapping PCR products spanning the B19V genome were generated for direct sequencing analysis. PCR products were purified by agarose gel electrophoresis using the QiAquick Gel Extraction kit (QiAGEN, Hilden, Germany). Approximately 50 ng PCR product was added to cycle sequencing reactions (10 μl final volume) containing 10 pmol sequencing primers. In addition to sequencing of PCR products, viral DNA extracted from human serum was also directly subjected to sequencing reactions using a single primer. The primers for direct sequencing of genomic DNA were JS 27 (TATGATTAGGGGTTTTCGCT, nt 2122 to 2102), 200 nM probe (6-carboxyfluorescein/FAM)-labeled 20-mer, and JS 38 (TAAAGCATCAAACATTGCGGTTGCCCGAG), starting with their 5’ end at nucleotides (nt) 553 and 255, respectively, of the sequence of reference B19V strain Au. Note that the primers do not exactly match the Au sequence, as they were specifically adapted to the IM-81 sequence. An AmpliTag FS BigDye Terminator kit (Applied Biosystems, Weiterstadt, Germany) was used for sequencing reactions. Twenty-five cycles with 10 s of denaturation at 96°C, 5 s of annealing, and 2 min of elongation were performed. Sequencing products were purified by ethanolation, and 2 min of elongation were performed. Sequencing primers were the same as those used for generation of PCR products, and the annealing temperature was set accordingly. Sequencing products were purified by ethanol precipitation, and products were run on polyacrylamide gels using an ABI sequencing apparatus (Applied Biosystems, Weiterstadt, Germany). Sequencing data were analyzed using the BioEdit software (14).

**DNA quantification by TaqMan PCR.** DNA was isolated using the QIAamp Blood Mini kit (QiAGEN, Hilden, Germany). DNA was finally eluted in 100 μl H2O. Ten microliters of DNA extract was added to 40 μl master mix containing final concentrations of 0.15% gelatin, 0.01% Tween 80, 5 mM MgCl2, 2.5 mM deoxynucleotides (dATP, dGTP, dCTP), 5 mM dUTP, 300 nM primer TP1 (5’GGCGCGCTGAAACGACTGGAAC, nt 2030 to 2088) and primer TP7 (5’CTTCCCGAGAAAATGCTGCC, nt 2122 to 2120), 200 nM probe (6-carboxyfluorescein/FAM)-labeled 20-mer, and 2.5 U TaqMan DNA polymerase (Applied Biosystems, Weiterstadt, Germany). Amplification was carried out for 30 min at 50°C and stopped by incubation at 95°C for 15 min. Thereafter, 5 PCR cycles (15 s at 94°C, 15 s at 60°C, 15 s at 72°C) were performed. The reaction mixture was transferred to the ABI Prism 7700 system (Applied Biosystems, Weiterstadt, Germany). After 2 min at 50°C and 10 min at 95°C, 45 cycles (15 s at 95°C and 30 s at 60°C) were performed. In each run, the infectivity (mRNA) from a standard sample was determined by the endpoint dilution method (2 wells per dilution) and was calculated as the 50% mRNA-inducing dose (mRNA50) per ml using the Spearman-Karber method (16, 31), resulting in an absolute quantification of the mRNA/mL titer with a variation of ±0.8 log10. Furthermore, this sample was used to generate a standard curve (2 to 3 wells per dilution) for relative quantification of viral mRNA. One well per dilution was analyzed for mRNA, and diluted samples with cycle threshold values falling into the linear range of the standard curve were used for calculation of the titer using the TaqMan software.

The 95% detection limit of the B19V infectivity assay was calculated according to the formula:

\[ c \sim -\log_{10}{P}v \]

where \( c \) is the volume of the analyzed sample and \( P \) is the concentration which would be detected with 95% probability (\( P = 0.05 \)). Considering that a sample volume of 0.03 ml went into the mRNA assay, this resulted in a detection limit of 2.0 log10 mRNA/ml.

**Kinetics of mRNA expression.** Approximately 3 × 10⁵ cells were infected with 30 μl serum containing B19V genotype 1 (S-9) or 100 μl serum containing genotype 2 (IM-81), samples at 3 different dilutions. At 24 hpi, cells were collected, mRNA was isolated using the mRNA Capture kit (Roche, Mannheim, Germany). RNA was quantified by TaqMan PCR as described above. Primers for detection of spliced VP mRNA were the same as described in the section above, while for detection of unspliced NS-1 mRNA (both genotypes), primers JS42 (5’GACC CGC GTA AAA TGG CTT T, nt 1066 to 1588) and JS43 (5’GGCG CGC CAA GTA CAG GAA AA, nt 1418 to 1437) and probe NS-1Zb (5’CTG CTT GTG GTT TGT GGA TGA AGT CAT TAA GTC TA-TAMRA, nt 1533 to 1570) were used. Amounts of mRNA were expressed in relative units according to the standard curve, which was created from the mRNA obtained 72 h postinfection.

**Protein extraction and Western blotting.** Proteins were made from approximately 10⁶ plaque-forming units (PFU)-washed cells using 100 μl 1× lysis buffer (125 mM Tris–Cl, pH 6.8, 1% [vol/vol] sodium dodecyl sulfate [SDS], 25% [vol/vol] glycerin, 0.01% [wt/vol] bromphenol blue, 2.5% [vol/vol] 2-mercaptoethanol). Samples were heated for 8 min at 95°C and stored at −80°C. Aliquots (12 μl) were subjected to SDS-gel electrophoresis in 8% Tris–glycine gels and blotted onto nitrocellulose membranes using a wet-blot apparatus. Membranes were blocked for 1 h in PBS containing 3% dry milk and 0.1% Tween 20. The primary monoclonal antibody H1/10 (Chemicon International, Temecula, Calif.) was incubated in a 1:500 dilution in blocking buffer overnight, and membranes were washed three times for 15 min in PBST (PBS containing 0.1% Tween 20). Anti-mouse immunoglobulin G (IgG) peroxidase conjugate was added for 1 h in a 1:10,000-fold dilution in blocking buffer, and thereafter membranes were washed three times (15 min) in PBST. Bound antibodies were detected using the ECL plus Western Blotting Detection System (Amersham, Freiburg, Germany).

**DNA extraction and Southern blotting.** Approximately 10⁶ cells were washed with PBS, pelleted by low-speed centrifugation, and resuspended in 120 μl Ca- and Mg-free PBS. Cells were mixed with 120 μl 1% low-melting-point agarose (Bio-Rad, Munich, Germany) and casted into a plug mold. Cell lysis was done by incubation of the agarose blocks for 48 h in ES (0.5 mM EDTA [pH 9.5], 1% sodium lauryl sarcosine, 1 mg/ml proteinase K). Blocks were washed three times in 15 ml TE (10 mM Tris–Cl pH 8, 1 mM EDTA) and stored at 4°C until use. Agarose blocks were loaded onto 0.7% agarose gel, and DNA was separated by gel electrophoresis.

DNA was transferred by neutral capillary Southern transfer onto nylon membranes (Roche, Mannheim, Germany) with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as transfer buffer. A digoxigenin (DIG)-labeled probe was generated from 500 ng of a B19V genome-containing plasmid (20), and 250 ng of IM-81 viral DNA was extracted from serum using the DIG High Prime kit (Roche, Mannheim, Germany). Hybridization was carried out in DIG Easy Hyb buffer (Roche, Mannheim, Germany) at 42°C. The membrane was washed three times (15 min at room temperature) in 2× SSC, 0.1% SDS, followed by two washes (30 min at 65°C) in 0.2× SSC, 0.1% SDS. Digoxigenin-labeled DNA was detected using Fab fragments of alkaline phosphate-labeled
anti-DIG antibody (Roche, Mannheim, Germany) and CDP-Star (Roche, Mannheim, Germany) as chemiluminescence substrate.

Pasteurization. Pasteurization experiments were performed as described previously (5). A commercial human serum albumin product was preheated to 56°C in an Eppendorf 5436 Thermoblock (Hamburg, Germany). The temperature was monitored throughout the experiment by reading from a thermometer in an unspiked albumin sample, which was incubated in parallel. One milliliter preheated albumin was spiked with 100 µl virus-containing plasma, and 100 µl samples were taken after 1 min, 5 min, 10 min, and 30 min of heat treatment. Samples were placed on ice and immediately titrated on KU812Ep6 cells.

Cytotoxicity controls. Cells were seeded into 24-well plates. One-hundred-microliter samples of a 10-fold dilution series of albumin were inoculated into each well, and after 7 days cells were visually examined for cytotoxic effects using a light microscope.

Interference controls. A 10-fold dilution series of B19V in undiluted albumin was prepared, and 100 µl from each dilution was inoculated into cell cultures and examined for virus. Additionally, a 10-fold dilution series of B19V in 1:10-diluted albumin was prepared, and 100 µl from each dilution was inoculated into cell cultures and examined for virus.

Virus control. In each virus experiment, the titer of the virus stock was verified by titration on KU812Ep6 cells (B19V).

Bench controls. In parallel to each pasteurization experiment, albumin was spiked at room temperature. This material was held at room temperature and monitored throughout the experiment by reading from a thermometer in an Eppendorf 5436 Thermoblock (Hamburg, Germany). The temperature was monitored throughout the experiment by reading from a thermometer in an unspiked albumin sample, which was incubated in parallel. One milliliter preheated albumin was spiked with 100 µl virus-containing plasma, and 100 µl samples were taken after 1 min, 5 min, 10 min, and 30 min of heat treatment. Samples were placed on ice and immediately titrated on KU812Ep6 cells.

Inactivation at low pH. Experiments for inactivation at low pH were performed as described previously (7). Virus was spiked (1/1, vol/vol) into 2% human serum albumin solution. The solution was adjusted to pH 4 with 1 M HCl. Incubation was carried out at 37°C for the indicated period of time. Samples were withdrawn and immediately neutralized with 1 M NaOH, buffered with phosphate buffer (100 mM, pH 7.4), and subjected to virus titration. Control experiments were analogous to those described above.

Neutralization. Fifty microliters of virus at a concentration of approximately 9.6 log_{10} genomes per ml was mixed with 50 µl test serum from a twofold dilution series in PBS. The test serum had been subjected to inactivation of complement at 56°C for 30 min before use in neutralization experiments. The 100-µl mixture of virus and serum was incubated for 2 h at 37°C for neutralization. Thereafter, the whole sample was subjected to KU812Ep6 cells for titration of infectious virus as described above.

Antibody detection. Detection of anti-B19 IgG and IgM was performed using the enzyme-linked immunosorbent assay (ELISA) based on recombinant virus capsid protein 2 (VP2) expressed in insect cells (Biotrin, Dublin, Ireland). Except for the calibration curve generated with the International Standard for IgG Antibodies to Parvovirus B19 NIBSC 93/724 (11), the assay was carried out according to the instructions of the manufacturer.

Characterization of B19V from various plasma samples. DNA obtained from five B19V-containing plasma samples was digested with XmnI and subjected to Southern analysis using a mixture of digoxigenin (DIG)-labeled DNA from a plasmid containing the B19V genome 1 genotype and DIG-labeled IM-81 viral DNA. (A) DNA sizes are given in kilobase pairs (kb). Samples IM-71, IM-72, IM-73, and IM-80 contained genotype 1 B19V, while sample IM-81 contained the variant genotype 2. (B) The theoretical pattern and length of the restriction fragments is sketched. P6 indicates the location of the p6 promoter. Additionally, genome concentrations were determined by quantitative PCR (1), and the infectivity was quantified by titration on Ku812Ep6 cells (C).

FIG. 1. Characterization of B19V from various plasma samples. DNA obtained from five B19V-containing plasma samples was digested with XmnI and subjected to Southern analysis using a mixture of digoxigenin (DIG)-labeled DNA from a plasmid containing the B19V genome 1 genotype and DIG-labeled IM-81 viral DNA. (A) DNA sizes are given in kilobase pairs (kb). Samples IM-71, IM-72, IM-73, and IM-80 contained genotype 1 B19V, while sample IM-81 contained the variant genotype 2. (B) The theoretical pattern and length of the restriction fragments is sketched. P6 indicates the location of the p6 promoter. Additionally, genome concentrations were determined by quantitative PCR (1), and the infectivity was quantified by titration on Ku812Ep6 cells (C).
Similarity to genotype 3 sequences V9 and D91.1 was 90.8%, and similarity to “conventional” genotype 1 sequences (strains Au, Wi, HV, Rm, N8, and Mi) ranged from 88.9 to 90.8%. The nucleotide sequence of the p6 promoter region is shown in Fig. 2. The IM-81 promoter was very similar to the other described genotype 2 promoters (A6, LaLi). However, a 138-bp insertion was located between nt 173 and 174 of the B19 Au sequence. This insertion corresponded well to the Southern analysis depicted in Fig. 1A, where the fragment spanning the p6 promoter region was 100 to 150 bp longer than conventional sequences. In order to verify the insertion, we performed additional sequencing reactions using viral DNA extracted from human serum as template and single primers. The binding sites of the two sequencing primers used were located downstream of the insertion and were directed towards the left inverted terminal repeat (ITR). Single-read sequences confirming the arrangement of the IM-81 promoter as shown in Fig. 2 were obtained with both primers. The insertion is not contained in the genotype 2 LaLi sequence and in the genotype 3 V9 and D91.1 sequences. It is not known if such an insertion is contained in the A6 isolate, as the published sequence starts downstream of the potential location. The inserted sequence was represented by an additional 30 bp from the beginning of the ITR adjacent to a 108-bp element found also, in inverted orientation, at the extreme right end of the unique sequence of the genome. Sequence elements similar to the 108-bp element were also found at the corresponding positions in the far right-hand end of the unique region from the A6, V9, and Au sequences.

**Kinetics of mRNA expression.** B19 virus-specific mRNAs are expressed from a single promoter, the p6 promoter, which is located next to the left-end inverted terminal repeat (ITR) region of the genome. Different mRNAs are produced by alternative splicing. The NS-1 protein is translated from a non-spliced mRNA, while the two viral capsid proteins VP1 and VP2 are produced from single- and double-spliced mRNAs, respectively. The striking differences in the promoter sequences from genotypes 1 and 2 (Fig. 2) lead to the question of whether mRNAs are differently expressed in infected cells. The erythroid cell line KU812Ep6 can be permissively infected with B19V (19). Therefore, we investigated accumulation of unspliced (NS-1 specific) or spliced (VP) mRNAs after infection with a genotype 1 or genotype 2 virus, respectively (Fig. 3). Unspliced transcripts from both genotypes accumulated very early after infection (4 h) with comparable kinetics and remained at high levels throughout the replicative cycle (Fig. 3).
This indicated that NS-1 expression does not differ between the two genotypes in KU812Ep6 cells. It is noted that a background signal was detected immediately after adsorption of virus to the cells (0 h postinfection). This signal was presumably caused by the high level of input DNA genomes (10^10 genomes/ml), as the quantitative RT-PCR assay does not discriminate between unspliced reverse-transcribed RNA or DNA. Controls using RNase showed that it was difficult to separate mRNA completely from the high input of viral genomic DNA (more than 10 log genomes) used for infection. However, the more than 2 log increase of the signal 4 h after infection is clearly not caused by an increase of viral DNA, as DNA replication does not start until 12 to 24 h after infection (see Fig. 5).

Spliced transcripts encompassing the VP1 and VP2 open reading frames were detected at low levels soon after infection (4 to 12 h postinfection [hpi]), and a 2 to 3 log increase was observed between 12 hpi and 24 hpi (Fig. 3B). Again, the pattern of expression did not differ between genotypes 1 and 2. VP-specific transcripts could be detected without any background signal from input DNA, as the distance between the primers on DNA was too long for amplification and detection by TaqMan PCR in contrast to the distance on the spliced reverse-transcribed mRNA.

**Kinetics of capsid protein expression.** Expression of viral capsid proteins was investigated by Western blotting using a VP2-specific antibody (Fig. 4). The VP2 protein (58 kDa) accumulated between 12 and 24 h postinfection. Again, there was no difference in the pattern of VP2 protein expression between genotypes 1 and 2. The accumulation of VP2 proteins at 24 h postinfection corresponded well to the increase of VP-mRNA expression from 12 to 24 h postinfection. No signal corresponding to the VP1 capsid protein (84 kDa) was observed. However, it has been shown that B19V-infected KU812Ep6 cells express VP1 proteins (19). It seems, therefore, that the monoclonal antibody used for Western blotting was not suitable for VP1 detection.

**DNA replication.** The pattern of viral DNA replication was investigated by Southern analysis and quantitative DNA determination (Fig. 5). For Southern analysis, DNA was prepared in agarose plugs by lysis of agarose-embedded cells. Southern analysis revealed two bands of B19V DNA. The band at 5.6 kb corresponds to the monomeric replicative intermediates and to progeny genomes which anneal to double-stranded forms after DNA extraction. The bands at 11 kb correspond to dimeric DNA replication intermediates. A strong signal from viral DNA which did not enter the gels was observed in the loading wells. The nature of this signal remains to be clarified. DNA from both B19V genotypes was...
replicated with the same kinetics. Quantitative DNA analysis by TaqMan PCR showed a 100-fold increase of genomes from ca. 12 hpi until 72 hpi (Fig. 5). Southern analysis of XmnI-cleaved DNA collected late in infection (48 h) showed that the 138-bp insert from genotype 2 strain IM-81 is not lost during DNA replication (data not shown).

In summary, no difference in DNA replication was observed between both genotypes.

Physicochemical stability of virus capsids. We have shown that B19V is more readily inactivated at high temperatures or low pH than the animal paroviruses porcine parovirus and mice minute virus (5, 7). Inactivation occurs by disintegration of viral capsids. This was indicated by DNase I sensitivity of viral DNA in virus-containing plasma samples after heat or low-pH treatment. The B19V capsid consists of two proteins, VP1 and VP2. VP2 represents the major capsid protein (95% of the capsid), while VP1 differs from VP2 only by the N-terminal “unique region” composed of 227 additional amino acids. The VP1 unique domain is mostly located outside the virion and, therefore, is accessible to antibody binding (2, 24).

Capsid proteins from genotype 1 and genotype 2 differ remarkably in their amino acid sequence. The DNA sequences from genotype 1 strain S-1 and genotype 2 strain IM-81 were determined, and the differences in the amino acid sequences of viral capsid proteins VP1 and VP2 are shown in Fig. 6C. There are 28 positions with different amino acids in the VP1/VP2 open reading frame. Eighteen of the 28 differences are located in the VP1 unique region (amino acid positions 1 to 227), while the other 10 affect both capsid proteins VP1 and VP2.

Despite the variations in amino acid composition of viral capsid proteins, the sensitivity of infectious virus particles to heat treatment in 5% albumin at 56°C (Fig. 6A) or sensitivity to pH 4 at 37°C (Fig. 6B) did not differ between the two
genotypes. Both viruses were inactivated with the same kinetics. It is concluded that the physicochemical stability of virus particles (viral capsids) from both genotypes does not differ significantly.

**Cross-neutralization.** Most of the amino acid variations between genotypes 1 and 2 are located in the VP1 unique region, and it has been indicated that the neutralizing immune response against B19V is mainly elicited by this region (25).
Additionally, several antigenic target regions have been described for VP2 (8, 27, 32, 35). We therefore asked whether antibodies from a genotype 1 infection would be effective against genotype 2. We tested sera from six individuals who had recovered from an infection with B19V genotype 1 for their effectiveness to neutralize in vitro B19V genotype 1 (isolate S-1) or genotype 2 (isolate IM-81). Infection of the six individuals with B19V genotype 1 was confirmed by partial nucleic acid sequencing of viral DNA obtained from the acute phase of infection. Convalescent-phase sera obtained 11 months, 6 months, 16 months, 10 months, 9 months, and 18 months after infection were used for in vitro neutralization (Fig. 7A to F, respectively). In three cases (Fig. 7A to C), complete neutralization of both genotypes was observed. Infectivity was reduced by more than 4 log10. In two cases, only genotype 1 virus was neutralized completely, while virus inactivation by neutralization of genotype 2 was limited to ca. 2 log10 of virus (Fig. 7D and E). One serum (Fig. 7F) did not neutralize genotype 2 virus, and neutralization of genotype 1 was limited. This serum showed the lowest antibody titer measured with a VP2-specific ELISA and calibrated to the international standard serum for parvovirus B19 antibody. There was no clear correlation between the neutralizing capacity and the VP2 antibody titer of sera or the time after infection. In summary, these experiments indicate that human sera raised against genotype 1 cross-neutralize genotype 2 in most instances completely or partially and that viral capsids of both genotypes have related antigens.

DISCUSSION

The discovery of variant human erythrovirus (B19V) genotypes posed important questions about their biological properties, their pathogenetic potential, and the epidemiological relevance. The pathogenic properties of the B19V genotypes seem similar. The V9 strain (genotype 3) was detected in blood from a child with transient aplastic crisis (21), and the A6 virus was from a human immunodeficiency virus-infected patient with chronic anemia. Furthermore, a case of recurrent genotype 2 viremia in a transplant recipient has been recently described (18). The exact epidemiology of variant B19V genotypes remains to be clarified. Genotype 2 was detected in Finland (15) and in a sample from Italy (23). Strain IM-81 characterized in this report was from a blood donor in Germany. Genotype 3 was initially detected mainly in French samples (21, 29). In a recent study (9), genotype 3 was found to be prevalent in samples from Ghana but not from Malawi, South Africa, or the United Kingdom. However, until now, experimental data on the biological activity of variant human erythroviruses is limited.

In this report, we describe the identification and characterization of a genotype 2 B19 virus which was detected at high titer in a plasma donation. The nucleotide sequence of this genotype 2 isolate, termed IM-81, was very similar to published genotype 2 sequences, such as those of LaLi and A6. One unexpected feature was, however, that the viral DNA was ca. 130 bp longer than that from genotype 1 isolates. We have not yet detected a further isolate with such an elongation of the genome by screening of available samples. It remains, therefore, unclear if this insertion is an extraordinary feature of IM-81 or if such an insertion can be found more commonly in genotype 2 isolates. The LaLi sequence did not contain this insertion, and the A6 sequence spanning the analogous site of potential insertion has not been described. The rearrangement within the promoter region might have been generated by duplication of the first 30 bp from the end of the ITR and an inverted insertion of the 108-bp element which is also found at the extreme right end of the unique genome sequence. An alternative explanation would be that the rearrangement represents the inverted insertion of a 138-bp sequence from the right end of the genome that includes the extreme 108 bp from the right-end unique region and the first 30 bp from the right-end ITR. In this context, it is interesting that the 30-bp element encompasses the nick site as established for the genotype 1 genome. The complete ITR of the IM-81 or any other genotype 2 isolate has not yet been determined. There are two reports characterizing the complete ITRs of B19V genotype 1 after molecular cloning that managed several technical difficulties, as there is a risk of excision of sequence elements flanked by direct repeats in the ITR (10, 37). However, in the latter report, functionality of the cloned sequences was confirmed by creating a plasmid enabling the production of infectious virus from transfected cells.

The p6 promoter region of genotype 2 differs significantly from that of genotype 1. There are several potential transcription factor binding sites (E4BP4 and SP1/MZF1) containing nucleotide sequence variations or lacking the genotype 2 promoters. This leads to the speculation that the promoters are differentially expressed. However, we did not observe different kinetics of both unspliced early mRNA and spliced late mRNA expression in the permissive erythroid cell line KU812Ep6. This is in accordance with the results from transient transfection experiments investigating a luciferase expression reporter plasmid in erythroid UT7/Epo-S1 cells (15). It is concluded that, despite the striking structural differences, the p6 promoter of both genotypes is efficiently expressed in erythroid cells. Additionally, no differences in the kinetics of the capsid protein expression and DNA replication in KU812Ep6 cells were observed, indicating similar virus replication of B19V genotypes 1 and 2 in erythroid cells. Efficient virus replication of genotype 2 in erythroid cells is further supported by the detection of the isolate IM-81 at very high titers in human plasma (11.3 log10 genomes per ml). It remains to be clarified if the different promoter structures affect viral gene expression and replication in other tissues than erythroid progenitor cells. B9V genotype 2 has been found in human skin (15) and liver samples (34 and A. M. Eis-Hüninger, unpublished observations).

Since B19V genotype 2 can be detected in human blood at high titers, it should be considered with respect to the viral safety of blood and medicinal products (e.g., clotting factor concentrates, immunoglobulins, albumin), which are produced from pooled human plasma. The exact prevalence of genotype 2 in the population has not yet been characterized. However, genotype 2 DNA has been detected recently in 2.5% of plasma-derived clotting factors, all of which were cocontaminated with genotype 1 DNA (28). This indicates that genotype 2 has a significant prevalence in the donor population.

In order to obtain virus safety, production processes of plasma-derived medicinal products contain steps for virus inacti-
viation or virus removal. B19V genotype 1 has been shown to be susceptible to pasteurization (10 h, 60°C) of albumin (5) and pH 4 treatment (7), which is occasionally used during manufacture of immunoglobulins. We previously showed that virus inactivation by these processes occurs by disintegration of viral capsids (5, 7). Since significant differences between the amino acid compositions of both genotypes exist, we investigated whether there are differences in the physicochemical stability of virus capsids from both genotypes. However, the identical inactivation kinetics at pasteurization or pH 4 treatment indicates identical stability of viral capsids. This is important for assessment of the safety of plasma-derived medicinal products, as the results indicate that the viral inactivation techniques will work with both genotypes equally. There is an inherent risk that the large plasma pool used for production might be contaminated with genotype 2. Genotype 2 DNA has already been detected in various products (28). The efficient inactivation of B19V genotype 2 at 56°C observed in this study may also explain the failure to infect erythroid cells with the A6 strain, as the A6-containing sample had been heat treated (23). It was already speculated in this report that heat treatment of the A6 sample could have led to virus inactivation. The exact structure of B19 virus-like capsids consisting of VP2 has been determined recently at high resolution by X-ray crystallography (17). Relating these data to both virus isolates used in this study, one could assume that some variations were located in the external loops of VP2 protruding to the outer surface while others were located in internal regions. However, it should be noted that most of the amino acid variations clustered to the small VP1 unique region encompassing 227 amino acids. The three-dimensional structure of VP1 has not been determined so far; however, there are indications that VP1 is an important antigenic site for immune neutralization.

Cross-neutralization is a common in vitro test to characterize the antigenic relation of different viruses. We tested several human sera collected from individuals after infection with genotype 1 for their capacity to neutralize in vitro genotype 1 and genotype 2 isolates. While three sera did completely neutralize both genotypes, neutralization of genotype 2 was more limited in three other cases. Two sera (sera C and F) were from children with asymptomatic infection, while the other sera were from patients with common symptoms of B19V infection (rash, cough, myalgia, arthralgia). Serum B was still weakly positive on B19V-IgM, while all others were B19V-IgM negative. There was no correlation between the neutralizing capacity and the VP2 antibody titer or the time after infection. As VP1 is known as an important immunodeterminant protein, the lack of correlation of neutralization with the VP2 antibody titer as determined by the ELISA is not unexpected. It may be speculated that the neutralizing capacity of the different sera depends on the specific constellation of the amino acid sequences of capsid proteins of the infecting genotype 1 virus eliciting the antibodies and of both challenge viruses used in the in vitro neutralization assay. Although the reasons for the variability in neutralizing capacities of sera remain unclear, it is indicated from these experiments that both genotypes have related antigens resulting mostly in partial or complete cross-neutralization. Further studies are needed to clarify whether a partial in vitro cross-neutralization mediates protection of individuals from genotype 2 infection. However, it should be noted that very high challenges of virus (9.6 log10 genomes per ml) were applied in the in vitro assay in order to achieve highly infectious titers, which may not reflect a natural virus challenge situation. The observed in vitro cross-neutralization might also indicate some protection against reinfection with B19V genotype 2 in vivo. Determining the immune status against B19V would be especially important for women, as infection of pregnant women may lead to severe damage of the fetus if such an infection is not recognized and adequately treated.

In summary, the data provided here support the concept that B19V genotypes 1 and 2 are very similar and should be grouped as one virus species. The similar kinetics of both mRNA and protein expression as well as in DNA replication in KU812Ep6 cells indicate similar virus replication kinetics of genotypes 1 and 2 in erythroid progenitor cells. Furthermore, virus particles were shown to have similar physicochemical stability, and in vitro cross-neutralization experiments indicated that both genotypes express common viral antigens. These data support grouping of B19V genotypes 1 and 2 into a common virus species.

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