minipools that yielded a B19 DNA concentration higher than 10^5 IU per mL were resolved by creating subpools from archive plates. Next the identified B19 DNA–positive samples were discarded and all negative or weakly positive B19 DNA samples included in the minipool were released for transfusion.

NAT. Real-time quantitative amplification of B19 DNA was performed with a CE labeled B19 PCR kit (DRK Baden-Württemberg-Hessen, Frankfurt, Germany) according to the manufacturers' instructions with a thermocycler (ABI PRISM 7000, 7700, 7300, or 7900HT; Applied Biosystems, Foster City, CA). Five microliters of extract was analyzed in a total volume of 25 µL. The assay contains reagents and enzymes for the specific amplification of the VP1-capsid protein gene of B19. Thermal cycling was as follows: 50°C for 2 minutes, 95°C for 15 minutes, 10 cycles of 95°C for 10 seconds and 62°C for 30 seconds, 40 cycles of 93°C for 10 seconds, and 56°C for 40 seconds.

Data analysis was performed with the computer software (sequence detection software, Version 1.6.3, Applied Biosystems). A positive real-time PCR result is reflected by an increase in the fluorescence intensity of a reporter dye. After PCR, the number of PCR cycles necessary to reach a defined fluorescence threshold in each sample was defined as the cycle threshold (C_t). The C_t value is related to the amount of PCR product and therefore to the original amount of target present in the PCR procedure. Low C_t values indicate a high initial target amount and high C_t values indicate the opposite.

Sensitivity and specificity of the DRK B19 PCR kit. Sensitivity was analyzed in accordance with the directive of European Commission 98/79/EC. Probit analysis was done on at least 24 replicates of each dilution from a dilution series containing at least six steps. The calculation was performed on nonlog converted data. Specificity was tested with 200 negative plasma samples. Additionally, the amplification efficiency of different B19 genotypes (Genotype 1, Genotype 2 [Subtype A6], and Genotype 3 [Subtype V9]) was evaluated. Genotype 3 was obtained from a Ghanaian blood donor service.[20-22]

Precautions to prevent B19 DNA cross-contamination. All steps of NAT (pooling, enrichment by centrifugation and extraction, master mix preparation, and amplification) were performed in separate rooms. All rooms were equipped with ultraviolet light and were decontaminated once per week. The daily decontamination procedure included decontamination of all workbenches, pipettes, and centrifuges with a disinfectant (Bacillol Plus, Bode, Hamburg, Germany) and sodium hypochlorite (Roth, Karlsruhe, Germany). All PCR procedures were monitored by the addition of at least six negative controls. PCR procedures were only valid if all negative controls gave a negative test result. All personnel performing nucleic acid extraction and resolution of highly B19 DNA–positive pools have been thoroughly trained to be competent in performing these procedures without cross-contamination.

Screening for B19 antibodies. Samples were screened with two assays for IgM and IgG antibody detection. A parvovirus IgG and IgM assay (recomLine, Mikrogen, Neuried, Germany) was used to analyze antibodies against VP-2, VP-N, VP-1S, VP-2r, VP-C, and NS-1 epitopes. Band intensities were compared with a control band and were scored as –, +/–, 1+, 2+, 3+, or 4+. Additionally all samples were screened with the microtiter plate–based B19 enzyme immunoassay (EIA; Biotrin, Dublin, Ireland) for IgG and IgM antibodies. All antibody assays were performed according to the manufacturers' instructions.

Antibody adsorption. Eight samples with a B19 DNA concentration of more than 10^5 IU per mL and eight samples with a B19 DNA load below 10^5 IU per mL were analyzed for B19 IgG antibodies by use of the recomLine assay. In these samples, virus load was determined by real-time NAT before and after treatment with a protein G column (MAb Trap kit, Amersham, Uppsala, Sweden). One-hundred microliters of each sample was filtered through a protein G column and washed with 5 mL of binding buffer. The flowthrough of the binding step was centrifuged at 58.000 × *g* for 1 hour at 4°C followed by a standard minipool extraction protocol.

B19 sequence analysis. Sequence analysis was performed as described in detail by Hokynar and colleagues.[23] Overlapping amplicons of 1000 bp that spanned the entire protein coding region of the genome were used. Primers (NSofwd and NSirev, NSsfwd and NSorev, p6 and p3, p9 and rtsrev, and rt1 and VP2orev) were used for sequencing plus and minus strands. Amplification products were sequenced directly with a cycle sequencing ready reaction kit (BigDye Terminator, Applied Biosystems, Darmstadt, Germany) and a DNA sequencer (ABI PRISM 310, Applied Biosystems).

Statistical analysis

The sensitivity, standard deviation (SD), and coefficient of variation (CV) of the real-time PCR test were calculated with computer software (Excel 2000, Microsoft Corp., Redmond, WA). For the Probit analysis, another computer program (SPSS 12.0, SPSS, Chicago, IL) was used. Comparison between the case and control groups was calculated with Fisher's exact test or the t test. Statistical significance was assumed if p values were less than 0.05.

RESULTS

Incidence of B19 in different areas

B19 incidence between 2003 and 2006 was demonstrated for six different areas (Fig. 1). There was a high incidence

period of B19 from May 2004 to January 2006 in all screened regions. The highest incidence was found in Areas 4 and 5, although the incidence of B19 DNA–positive donors with a high virus load (B19 DNA concentration $\geq 10^5$ IU/mL) as well as with low B19 DNA virus loads (B19 DNA concentration $<10^5$ IU/mL) did not differ significantly between the areas (Table 2).

Sequence analysis of the 50 B19 NAT–positive blood donors included in the substudy identified only Genotype 1 strains. Sequence analyses of all B19 DNA–positive samples are currently being processed to better understand the genotype distribution in our donor population.

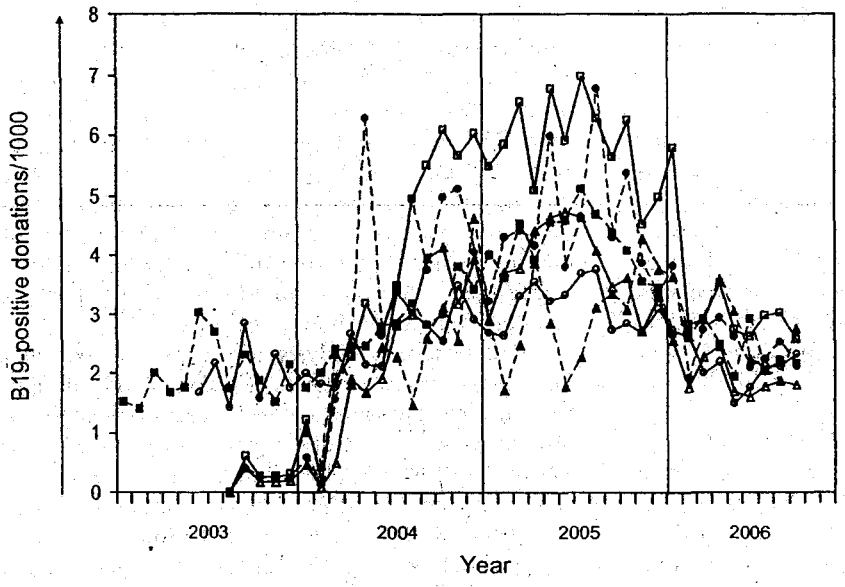

Fig. 1. Incidence of B19 virus infections between 2003 and 2006. Donations were screened for B19 by real-time minipool NAT. Incidence was increased between May 2004 and January 2006, especially in Areas 4 and 5. (■) Area 1 = GRC Institute Frankfurt; (▲) Area 2 = German Armed Forces; (△) Area 3 = Medical University of Graz; (□) Area 4 = Austrian Red Cross Institute Klagenfurt; (●) Area 5 = Austrian Red Cross Institute Feldkirch; and (○) Area 6 = Austrian Red Cross Institute Vienna.

Monitoring of B19 DNA–positive blood donors

In the substudy, two additional donations were taken from 50 B19 DNA–positive multiple-time donors (high-virus-load group) randomly selected from all B19 DNA–positive samples in Test Area 1 to determine B19 DNA concentration and the course of antibody development to B19.

All donors included in the substudy were B19 DNA–positive with a virus load of more than 10^5 IU per mL at the index donation (Time Point T0). The virus load was significantly reduced within 12 weeks from a median of 4.85×10^7 IU per mL (T0; SD) to 4.6×10^2 IU per mL (SD; T1; Fig. 2) and either remained at this level or declined further at Time Point T2. Additional follow-up in a subset of these donors beyond Time Point T2 revealed that B19 DNA concentration was stable around the NAT detection level for up to 1 year (range, 100 and 1500 IU/mL; data not shown). All samples from donors of the case group were below the release level of 10^5 IU per mL at Time Point T1.

B19 antibody levels were investigated with an enzyme-linked immunosorbent assay and a line probe assay. Both commercially available B19 antibody assays gave comparable results for B19 IgM (Table 3) and IgG (Table 4) antibodies. At each time point, IgM antibodies were detected more frequently ($p < 0.05$) in the case group compared with the control group, and antibody titers generally showed an increase from Time Point T0 to Time Point T1 followed by a decrease at Time Point T2. IgM antibodies against the nonstructural protein (NS-1) were not detected at any time point. In contrast, neutralizing IgG antibodies against VP-2 were detected in all samples of the high-virus-load group

TABLE 2. Incidence of B19 virus infections in different areas per 100,000 donations*

	B19 DNA virus load (IU/mL)													
	Area 1		Area 2		Area 3		Area 4		Area 5		Area 6		All	
Year	$>10^5$	$<10^5$	$>10^5$	$<10^5$	$>10^5$	$<10^5$	$>10^5$	$<10^5$	$>10^5$	$<10^5$	$>10^5$	$<10^5$	$>10^5$	$<10^5$
2003	9.3	185.2	1.0	200.3	0.0	19.6	0.0	29.9	0.0	23.9	ND†	ND	6.3	172.0
2004	17.9	254.3	13.6	239.4	9.3	217.0	25.1	340.3	50.0	279.1	11.5	212.9	16.3	247.7
2005	25.0	395.1	12.6	300.6	36.0	345.8	3.9	580.9	6.2	434.7	2.7	295.7	19.4	362.8
2006	5.7	237.1	3.7	209.9	6.0	170.8	0.0	317.1	0.0	255.4	3.6	268.9	4.5	227.6
All	15.0	269.8	9.1	245.0	15.7	221.3	9.3	366.1	17.6	289.7	6.1	259.1	12.7	261.5

* Donations were tested from six different areas in Germany and Austria. Incidence was demonstrated in two groups: 1) donations with high B19 DNA virus load over 10^5 IU/mL and 2) donations with low B19 DNA virus load below 10^5 IU per mL. Incidence increases were observed in all areas in 2004 and 2005 for both groups.
† ND = not done.

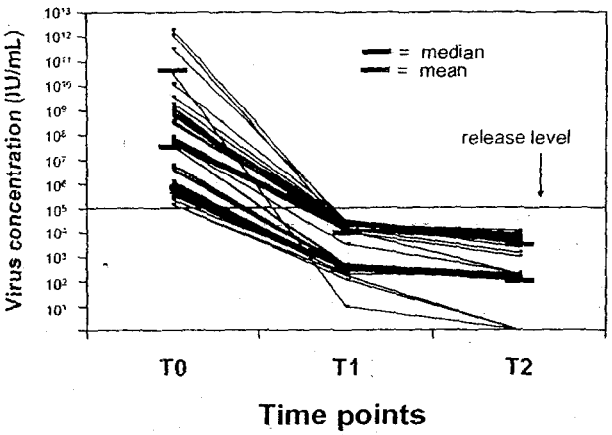

Fig. 2. Virus load during the 6-month study period. All donors of the case group were B19 DNA–positive at the index donation with a value of more than 10^5 IU per mL (highest concentration was 2.1×10^{12} IU/mL). The black bar represents the median virus concentration and the gray bar indicates the mean virus concentration of all donations for each time point. Virus load was significantly reduced from Time Point T0 to Time Point T1. The SDs were 3.5×10^{11}, 1.0×10^4, and 3.5×10^3 for T0, T1, and T2, respectively.

(case group) at Time Point T1 and T2 without any exception. Both antibody assays and titers were significantly higher in the case group compared to the control group. Likewise, antibodies against nonstructural antigens of NS-1 increased up to 92.3 percent from Time Point T0 to Time Point T2.

In one experiment, plasma from donors with B19 DNA concentrations of more than 10^5 IU per mL and with B19 DNA concentrations of less than 10^5 IU per mL was filtered through protein G columns. The viral load was determined before and after IgG absorption. Reduction of the B19 virus concentration was significantly higher in samples with low virus load and high IgG antibodies titers as shown in Table 5. In two of eight samples (viral load, $<10^5$ IU/mL), no virus was detectable after column filtration. In the other six samples, low virus concentrations were detected (mean C_t value, 30.6; virus concentration, <100 IU/mL).

All donors included in the case-control substudy were matched by age and sex and were interviewed with a standard B19 questionnaire about clinical symptoms (Table 1). Typical clinical symptoms for B19 infections such as tiredness, joint pain, or complications between pregnancies did not significantly differ between groups.

TABLE 3. IgM antibodies in the case group and the control group*

	Biotrin EIA	Mikrogen immunoblot					
Group	VP2 (%)	VP-2p (%)	VP-N (%)	VP-1S (%)	VP-2r (%)	VP-C (%)	NS-1 (%)
Case							
T0†	42.9	42.9	42.9	57.1	42.9	35.7	0.0
T1	71.4	71.4	85.7	85.7	50.0	35.7	0.0
T2	23.1	46.2	69.2	69.2	38.5	30.8	0.0
Control							
T0	1.8	9.2	4.6	6.1	1.5	1.5	0.0

* Fifty B19 DNA–positive donors were screened for IgM antibodies by two different assays to detect antibodies against structural (neutralizing) and nonstructural antigens at three time points, and the data were compared to results from 100 B19 DNA–negative blood donors (control group).
† T0 = index donation.

TABLE 4. IgG antibodies in the case and control groups*

	Biotrin EIA	Mikrogen immunoblot					
Group	VP2 (%)	VP-2p (%)	VP-N (%)	VP-1S (%)	VP-2r (%)	VP-C (%)	NS-1 (%)
Case							
T0†	35.7	35.7	28.6	28.6	28.6	21.4	0.0
T1	100	100	100	100	100	85.7	57.1
T2	100	100	100	100	100	76.9	92.3
Control							
T0	74.8	73.3	71.0	68.7	46.6	16.0	14.5

* Fifty B19 DNA–positive donors were screened for IgG antibodies by two different assays to detect antibodies against structural (neutralizing) and nonstructural antigens at three time points, and the data were compared to results from 100 B19 DNA–negative blood donors (control group).
† T0 = index donation.

TABLE 5. IgG adsorption by protein G columns*

Sample B19 virus load (IU/mL)	Total number	Mean C_t Before column absorption	Mean C_t After column absorption	Mikrogen immunoblot mean reactivity ΔC_t	VP-2p	VP-N	VP-1S	VP-2r	VP-C	NS-1
$>10^5$	8	7.5	8.0	0.5	0.4	0.5	0.5	0.8	0.4	0.0
$<10^5$	8	25	30.6	5.6†	2.0	2.9	2.9	3.1	1.8	2.4

* Samples with B19 virus load of more than 10^5 IU per mL and less than 10^6 IU per mL were filtered through protein G columns. The virus load was determined before and after filtering. Additionally, B19 IgG antibodies were analyzed by immunoblots. Band intensities were compared with a control band and were scored as –, +/–, 1+, 2+, 3+, or 4+. For each sample, the mean reactivity is given. Samples with high levels of neutralizing antibodies (higher mean reactivity values) showed significantly higher virus reduction after filtering (higher ΔC_t).
† $p < 0.01$.

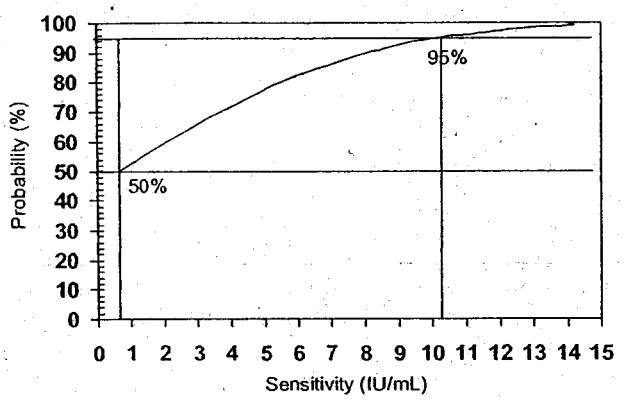

Fig. 3. Sensitivity of B19 NAT kit. The WHO standard (99/800) was diluted into six concentrations. Each standard concentration was tested in 24 replicates. Probit analysis was performed with SPSS Version 12.0 on nonlog converted data. Sensitivity of the minipool NAT was 10.2 IU per mL at 95 percent probability (CI, 7.5 and 18.8 IU/mL) and 0.65 IU per mL at 50 percent probability (CI, –4.1 to 2.47).

Sensitivity and specificity of the B19 PCR kit

As demonstrated in Fig. 3, the 95 percent detection probability of the NAT assay was 10.2 IU per mL (confidence interval [CI], 7.5-18.8 IU/mL) per processed volume. Sensitivity for an individual donation present in a minipool was 982 IU per mL (CI, 724-1811 IU/mL). Specificity was 100 percent as 200 of 200 negative samples gave a negative test result. The amplification efficiency of the DRK B19 PCR kit was comparable for all three genotypes (data not shown). Precision is defined as the degree of scattering within a series of analyses. It is expressed as the SD and the percent CV (%CV). SD and %CV were 0.6, 0.58, and 0.67 and 2.47, 2.37, and 4.20 for intraassay variability, interassay variability, and interbatch variability, respectively.

DISCUSSION

The frequency of B19 viremia in voluntary blood donors has been estimated to range from 1:260 to 1:50,000 and to depend on both the sensitivity of the screening method and the season.[24-26] Here we report results from screening blood donors over a period of more than 4 years with a sensitive real-time NAT method. The mean frequency of DNA-positive blood donors was 274 per 100,000 donations, which was within the range previously reported.[27,28]

Although the incidence of B19 DNA–positive blood products is high, transfusion-transmitted infections have rarely been reported when compared to other transfusion-relevant virus infections like human immunodeficiency virus-1, hepatitis C virus, or hepatitis B virus. This could be explained by the fact that most recipients already have B19 antibodies due to previous infections and that many B19 DNA–positive blood products were also positive for the presence of B19 VP-1 or VP-2 antibodies, resulting in neutralization of the virus. Another possible explanation is that B19 infections were underreported because most recipients get only mild or no clinical symptoms.[29] In the present study, we analyzed the development of anti-B19 and the decrease of B19 DNA in 50 blood donors and compared the data to a control group. In accordance with the literature,[30,31] VP-2 IgG antibodies already existed in the majority (75%) of B19-negative donors (control group). Without exception, all donors in the high-virus-load group (case group) were anti-VP-2 IgG–positive at Time Points T1 and T2. The increase in VP-2 antibodies correlated directly with a significant decrease in B19 virus load. The obvious explanation for this is that the antibodies neutralize the virus.[32]

Although antibodies persist for a long period of time, however, B19 DNA was detectable by real-time NAT for more than 1 year. The question is whether blood products with low levels of B19 DNA and B19 antibodies are infectious. This question is controversial in the literature and is currently being examined by a retrospective donor-recipient study.

Since B19 screening was initiated, the following release procedure was used in our blood donor service. Donations with high B19 DNA concentrations (equal or higher than 10^5 IU/mL) were discarded, but donors were permitted to make subsequent donations and were not informed about their infection. Blood products with B19 DNA concentrations less than 10^5 IU per mL are thought

to contain neutralizing antibodies. Therefore, minipools containing low B19 DNA–positive blood products were not dissolved, and all products contained in those minipools were transfused. The in vitro experiment with a protein G column indicates a significantly higher virus reduction in samples containing low viral loads and high levels of neutralizing antibodies compared to samples containing high viral loads and low B19 antibodies. This observation supports our release procedure, because it suggests that these samples are unlikely to be infectious (Table 5).

Nonetheless, for special-risk patients (immunocompromised patients, young children, or pregnant women), our blood donor service offers B19 DNA–negative blood products. The opportunity to obtain blood testing negative for the presence of B19 DNA has been available since summer 2003. Until now, however, less than 10 B19-negative blood products have been ordered, which demonstrates that physicians are relatively unaware of this infection.

In summary, all blood products have been screened for B19 by a real-time minipool PCR since 2000. A high-incidence period was observed between May 2004 and January 2006. Transfusion of blood products with a low virus concentration seems to be safe because of the coexistence of neutralizing antibodies, whereas blood products with high virus concentrations may pose a risk for transfusion recipients. Therefore, these donations were discarded at our blood donor service. Transfusion-transmitted B19 infections might be underreported and should be examined in donor-recipient studies.

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別紙様式第2-1

No. 11

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2008. 1. 21	該当なし	

一般的名称	人免疫グロブリン	研究報告の公表状況	Modrof J, Berting A, Tille B, Klotz A, Forstner C, Rieger S, Aberham C, Gessner M, Kreil TR. Transfusion. 2008 Jan;48(1):178-86. Epub 2007 Sep 27.	公表国 オーストリア
販売名(企業名)	人免疫グロブリン「日赤」(日本赤十字社)			

研究報告の概要

○血漿および静注用免疫グロブリン製剤によるヒトパルボウイルスB19の中和

背景:ヒトパルボウイルスB19(B19V)は広く蔓延する病原体であり、血漿由来製剤原料血漿プールは、B19V抗体(B19V免疫グロブリンG[IgG])を含有することが示されている。

試験デザインおよび方法:巨核芽球細胞株UT7/Epo-S1はB19V 遺伝子型1に感染し、また、本試験において、免疫組織学的方法、ウエスタンブロット法、B19V特異的mRNAのRT-PCR法を用いて示したとおり、最近発見された遺伝子型2にも感染する。感染UT7/Epo-S1細胞のB19V RT-PCR解析に基づき、感染実験を確立し、B19V中和検査を実施した。B19V IgG力価に関係したB19V中和抗体の役割を検討するため、製造血漿プール1000以上について酵素免疫測定法による検査を実施した。

結果:血漿プールは、B19V IgG力価:平均33±9IU/mL(最小値11IU/mL)を含有することが判明した。これらの11IU/mLのB19V IgGは、B19V遺伝子型1の感染性を4.6 log、遺伝子型2の感染性を3.9 log以上を中和した。このため、このようなプール由来の10%静注用免疫グロブリン製剤(IVIG)は、さらに高いB19V中和活性を含有することが分かった。

結論:分画用血漿プールにおけるB19遺伝子型1、2中和活性の高さは、当該プールのB19V IgG力価が一貫して高いために備わった特徴であることが示された。検討した10%IVIG製剤において、B19V IgGの中和活性を維持することが示された。

報告企業の意見	今後の対応
血漿由来製剤原料血漿プールにおけるヒトパルボウイルスB19中和活性の高さは、当該プールのB19V IgG力価が一貫して高いために備わった特徴であり、検討した10%IVIG製剤において、B19V IgGの中和活性を維持することが示されたとの報告である。	本製剤は現在製造・供給しておらず、当面特別な対応を必要としない。

使用上の注意記載状況・その他参考事項等

人免疫グロブリン「日赤」

ウイルス等の感染性

12

Neutralization of human parvovirus B19 by plasma and intravenous immunoglobulins

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BACKGROUND: Human parvovirus B19 (B19V) is a highly prevalent pathogen, and plasma pools for manufacturing of plasma-derived products have been shown to contain antibodies against B19V (B19V immunoglobulin G [IgG]).

STUDY DESIGN AND METHODS: The megakaryoblastic cell line UT7/Epo-S1 can be infected with B19V Genotype 1 and as demonstrated here by immunocytochemistry, Western blot, and reverse transcription–polymerase chain reaction (RT-PCR) of B19V-specific mRNA, also with the more recently discovered Genotype 2. Based on B19V RT-PCR analysis of infected UT7/Epo-S1 cells, an infectivity assay was established and implemented for a B19V neutralization assay. To investigate the role of B19V neutralization in relation to B19V IgG titers, more than 1000 manufacturing plasma pools were tested by enzyme-linked immunosorbent assay.

RESULTS: Plasma pools were found to contain a mean B19V IgG titer of 33 ± 9 IU per mL, with the lowest titer at 11 IU per mL. These 11 IU per mL B19V IgG neutralized 4.6 log B19V Genotype 1 and greater than 3.9 log Genotype 2 infectivity. Accordingly, a 10 percent intravenous immunoglobulin (IVIG) product prepared from such pools was found to contain an even higher B19V neutralization capacity.

CONCLUSION: A high capacity of B19V Genotypes 1 and 2 neutralization was demonstrated in plasma pools for fractionation, an inherent feature based on the constantly high titer of B19V IgG in these pools. The neutralizing activity of B19V IgG was shown to be maintained in the 10 percent IVIG product tested.

Human parvovirus B19 (B19V) belongs to the genus *Erythrovirus* (family *Parvoviridae*), which has recently been reclassified to contain three different B19V genotypes (1-3).[1] Although B19V Genotype 1 is by far the most prevalent, Genotype 2 has also been sporadically detected in Europe and was shown to occur in plasma pools for manufacturing into plasma derivatives.[2,3] B19V Genotype 3 appears to be mostly restricted to West Africa.[4]

Soon after its initial identification,[5] B19V was recognized to cause fifth disease in children (erythema infectiosum), whereas more serious clinical manifestations of B19V infection were only recently understood to include arthropathy, transient aplastic crises, persistent anemia, and hydrops fetalis.[6] In addition, an association of B19V with inflammatory heart disease in adults has lately been suggested.[7,8] The only treatment option available for B19V infection so far is intravenous immunoglobulin (IVIG), although based on anecdotal evidence rather than, for example, established dose–response correlations.[9]

Owing to the global prevalence of B19V Genotype 1, 30 to 60 percent of adults carry antibodies against B19V (B19V immunoglobulin G [IgG]), with a good correlation between antibody prevalence and age.[10] Consequently, the presence of B19V IgG was found in all of the few plasma pools for manufacturing so far investigated.[11-14] Mostly due to the lack of a widely available B19V

ABBREVIATIONS: B19V = parvovirus B19; MOI = multiplicity of infection; NC_{50} = 50 percent B19V neutralization capacity; $TCID_{50}$ = 50 percent tissue culture–infectious dose.

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infectivity assay, however, no information was available with respect to antibody function, that is, B19V neutralization, and whether this potentially clinically relevant variable would correlate with the presence of B19V antibodies detected by, for example, enzyme-linked immunosorbent assay (ELISA).

Although still to date a continuous cell culture system that would allow detection of B19V infectivity by determination of a classical cytopathic effect does not exist, B19V replication has been demonstrated in a few specialized cell lines mostly utilizing the detection of de novo transcribed B19V-specific mRNA after infection of and virus replication in these cells.[15-19] Particularly the erythroid progenitor cell line KU812Ep6 was used to detect B19V infectivity and was found to be sensitive to both B19V Genotypes 1 and 2.[18,20] These cells are, however, not widely available. Alternatively, use of the megakaryoblastic cell line UT7/Epo-S1 has also allowed accurate quantification of B19V Genotype 1 infectivity over several orders of magnitude.[15,16,21]

With an internally controlled reverse transcription–polymerase chain reaction (RT-PCR) system (TaqMan, Applied Biosystems, Foster City, CA), an infectivity assay for both B19V Genotypes 1 and 2 in UT7/Epo-S1 cells was established. The presence of B19V IgG antibodies in plasma pools for further manufacturing was confirmed, and the titers of B19V IgG were quantified. With our infectivity assay, a correlation between B19V antibody presence as determined by ELISA and function as determined by neutralization of B19V infectivity was established for manufacturing plasma pools. Given the clinical relevance of B19V antibodies in IVIG as the only treatment option for human B19V infection, particularly given the recent recognition of more severe disease associations[7,8] and emerging B19V variants,[22] the level of B19V IgG function was also determined for a commercially available IVIG preparation.

MATERIALS AND METHODS

Cells, B19V, and plasma

UT7/Epo-S1 cells were provided by K.E. Brown (Health Protection Agency, London, UK; with permission of K. Sugamura, Tohoku University, Sendai, Japan). Cells were maintained in Iscove's modified Dulbecco's medium, containing 10 percent fetal calf serum (JRH Biosciences, Lenexa, CA), 1 percent L-glutamine, 1 percent gentamicin sulfate, and 2 IU per mL erythropoietin (Janssen-Cilag, Neuss, Germany) at 37°C with 5 percent CO_2.

Plasma donations containing high titers of B19V as detected by the routine B19V PCR donor screening program of Baxter BioScience (Plasma Analytics Department, Vienna, Austria) were used as the source of infectious B19V. Plasma Donation 990237 contained B19V Genotype 1 (titer, 11.8 log IU/mL) and Donation IM 81 contained B19V Genotype 2 (titer, 11.4 log IU/mL).

Infection of UT7/Epo-S1 cells with B19V and isolation of mRNA

UT7/Epo-S1 cells (10^5 per six-well) were infected with B19V at multiplicity of infection (MOI) of 10^{-3} to 10^6 and incubated for 7 days or mock infected with buffer for negative controls. Seven days after infection, mRNA was extracted with a direct mRNA miniprep kit (GenElute, Sigma-Aldrich, Vienna, Austria) according to the manufacturer's protocol.

Immunocytochemical staining and Western blot of B19V capsid proteins

For immunocytochemical staining, infected cells were pelleted and fixed on glass slides coated with 70 to 150 kDa poly-L-lysine (0.01 mg/mL in phosphate-buffered saline [PBS]) with 4 percent paraformaldehyde. After permeabilization with 0.5 percent Triton X-100, cells were incubated for 1 hour with a monoclonal antibody (10 µg/mL) specific for B19V capsid proteins VP1 and VP2 (R92F6/MAB8293, against amino acids 328-344 of VP2; Chemicon, Chandlers Ford, UK).[23] After washing (PBS), an anti-mouse–horseradish peroxidase polymer conjugate (SuperPicTure polymer detection kit, Zymed, Vienna, Austria) was applied, and the signal was developed by 15-minute incubation with 3,3'-diaminobenzidine chromogen solution before mounting slides with aqueous mounting medium. To determine the intracellular localization of B19V structural proteins, cells were counterstained with methyl green (Vector, Burlingame, CA) according to the supplier's instructions.

For Western blotting, approximately $10^{5.6}$ cells were collected, 2 to 7 days after infection and washed in Tris-buffered saline (TBS). Proteins were then separated by Bis-Tris sodium dodecyl sulfate (SDS)–4 to 12 percent polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes with a "semidry" discontinuous protein transfer chamber (Immobilon P, Bio-Rad, Munich, Germany). After blocking, membranes were incubated with the primary antibody R92F6 diluted 1:2000. Membranes were washed in 0.5× TBS containing (vol/vol) 0.2 percent Triton X-100 and incubated for 1 hour with polyclonal rabbit α-mouse immune globulins–alkaline phosphatase (at 1:5000; Dako, Glostrup, Denmark). Bound antibodies were detected with a color reaction (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution, Sigma-Aldrich).

RT-PCR of spliced B19V transcripts

Real-time RT-PCR (TaqMan) was performed with a sequence detection system (ABI Prism 7900HT PE Applied