Interaction of parvovirus B19 with human erythrocytes alters virus structure and cell membrane integrity

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Abstract

The unique region of the capsid protein VP1 (VP1u) of B19 virus (B19V) elicits a dominant immune response and has a phospholipase A\textsubscript{2} (PLA\textsubscript{2}) activity required for the infection. Despite these properties, we have observed that the VP1u-PLA\textsubscript{2} occupies an internal position in the capsid. However, brief exposure to increasing temperatures induced a progressive accessibility of the PLA\textsubscript{2} motif as well as a proportional increase of the PLA\textsubscript{2} activity. Similarly, upon binding on human red blood cells (RBCs), a proportion of the capsids externalized the VP1u-PLA\textsubscript{2} motif. Incubation of B19V with RBCs from 17 healthy donors resulted in an extensive virus attachment ranging between 3,000 and 30,000 virions per cell. B19V empty capsids represent an important fraction of the viral particles circulating in the blood (30-40\%) and bind to RBCs similarly to full capsids. The extensive B19V binding to RBCs did not cause direct hemolysis but an increased osmotic fragility of the cells by a mechanism involving the PLA\textsubscript{2} activity of the exposed VP1u. Analysis of a blood sample from an individual with a recent B19V infection revealed, that at this particular moment of the infection, the virions circulating in the blood were mostly associated to the RBC fraction. However, the RBC-bound B19V was not able to infect susceptible cells. These observations indicate that RBCs play a significant role during B19V infection by triggering the exposure of the immunodominant VP1u including its PLA\textsubscript{2} constituent. On the other hand, the early exposure of VP1u might facilitate viral internalization and/or uncoating in target cells.
Introduction

Human parvovirus B19 (B19 virus; B19V) was discovered in 1975 (7) and it has been classified within the *Erythrovirus* genus of the *Parvoviridae* family. B19V is generally associated with a mild and frequent childhood disease named erythema infectiosum or fifth disease (1). However, in certain situations or in individuals with altered immunologic or hematologic conditions, B19V can cause other more severe syndromes such as acute and chronic arthropathies (28), hemolytic disorders (32), hydrops fetalis and fetal death (5, 10).

The single-stranded DNA genome of B19V is packaged into a small nonenveloped, icosahedral capsid consisting of 60 structural subunits, of which approximately 95% are VP2 (58 kDa) and 5% VP1 (83 kDa). VP1 and VP2 originate from overlapping reading frames and are identical except for a stretch of 227 additional amino acids at the VP1 N-terminal region, the so-called VP1 "unique region" (VP1u) (9, 26). Despite of being the minor component of the capsid, the dominant immune response against B19V is elicited by the VP1u region, which harbors strong neutralizing epitopes (2, 31, 45). A poor immune response against VP1u has been linked to persistent infections (21). Apart of its immunodominant role, VP1u harbors a phospholipase A₂ (PLA₂) motif (13), which is required for the infection (14, 18, 44). Growing evidence indicate that VP1u also plays a central role in the induction of autoimmune reactions and inflammatory processes (22, 36, 37, 41) by mechanisms still not well understood. Despite all these properties, we have recently shown that the most N-terminal part of VP1u harbouring strong neutralizing epitopes (2) is not external to the capsid. However, brief exposure
to mild temperatures or low pH rendered this region accessible and triggered the VP1u-PLA$_2$ activity of the virus (30). B19V would therefore be similar to other parvoviruses in which the internal VP1u can become exposed \textit{in vitro} by mild heat or low pH treatments (3, 8, 14, 20, 34, 39) and \textit{in vivo} during the intracellular trafficking of the virus (24, 29, 33, 39). Given to the fact that VP1u of B19V is the immunodominant constituent of the capsid, it is reasonable to expect that these regions should become exposed, not during the intracellular trafficking of the virus, but already in the extracellular milieu. In this sense, it is tempting to speculate that binding of B19V to receptors on the cell surface would act as the trigger to render VP1u accessible. It has been previously shown for several viruses that binding to the cellular receptor can trigger changes in capsid conformation (27). Upon attachment to susceptible cells, polioviruses, rhinoviruses, and most related enteroviruses, undergo conformational transitions which alter the accessibility of several regions (11, 15). Such changes have not yet been observed in parvoviruses.

The cellular receptor of B19V is globoside (4), which is necessary but not sufficient for the infection (42). Other required co-receptors have been identified such as α5β1 integrin and Ku80 (25, 40). The co-receptor α5β1 integrin is thought to be required for internalization (40). In order to be fully immunogenic, VP1u would have to be stably exposed on the cell surface without the subsequent virus internalization. B19V binding without internalization might occur in different cell types lacking the required conditions. Mature human red blood cells (RBCs) are one of these types of cells. They are very abundant, express large quantities of the B19V receptor globoside (16) but are unable to internalize B19V (40). Moreover, B19V does
not bind membrane-associated globoside in vitro (19), indicating that B19V probably binds globoside in concert with other molecular structures present on cell membranes. Despite the fact that the RBCs are major viral targets, particularly during the viremic phase of the infection when large amounts of infectious viral particles circulate in the blood, the interaction of B19V with the RBCs has not yet been investigated.
Materials and methods

Cells and viruses. UT7/Epo cells were cultured in RPMI, 10% FCS and 2 U/ml of recombinant human erythropoietin (Epo; Janssen-Cilag, Midrand, South Africa) at 37°C and 7.5% CO$_2$. Heparinized blood samples were obtained from healthy donors. The red blood cells (RBCs) were washed four times with PBS before use. A blood sample was obtained from an individual with a recent B19V infection (IgM and IgG positive). A B19V-infected plasma sample was obtained from our donation center (Genotype 1; CSL Behring AG, Charlotte, NC). The viremic serum sample did not contain B19V-specific IgM or IgG antibodies. B19V was concentrated from the infected serum by ultracentrifugation through 20% sucrose. The viral pellets were washed and resuspended in PBS. B19V titers were determined by quantitative PCR as DNA-containing particles per microliter. Gradient-purified B19V full capsids (FC) and empty capsids (EC) were prepared from the viremic serum sample as previously described (23). The amount of FC and EC was examined by dot-blot hybridization with a mouse antibody against B19V viral proteins (1:500; US biologicals, Swampscott, MA), followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution). Additionally, the amount of FC as well as the purity of EC was examined by quantitative PCR.

Antibodies. A synthetic peptide derived from the PLA$_2$ motif of B19V (aa 142-163) was used to immunize rabbits (Fig. 1A). This peptide was chosen because it was previously shown to induce neutralizing antibodies (2). The peptide was synthesized as a multiple antigenic peptide, built on a tetra-branched lysine core with a C-terminally amidated cysteine residue for
coupling purposes. Peptide specific antibodies were affinity purified. Peptide synthesis, immunization and affinity purification were performed by ImmunoGlobe (Himmelstadt, Germany). Two monoclonal antibodies (mAbs), one specific for the most N-terminal region of VP1u (mAb 1418-1), and the other recognizing only intact capsids (mAb 860-55D), were kindly provided by S. Modrow (Regensburg, Germany). The two mAbs were produced from peripheral blood mononuclear cells from normal, healthy individuals with high titers of serum antibodies against B19 virus proteins (17). A globoside-specific IgM monoclonal antibody (AME-2; 40) was kindly provided by J. de Jong (the Netherlands Red Cross, Amsterdam, Netherlands). A mouse non-specific IgM isotype control was purchased from Sigma (St. Louis, Miss).

**Quantitative PCR.** Amplification of B19V DNA and real-time detection of PCR products were performed by using the LightCycler system (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR Green (Roche). PCR was carried out using the FastStart DNA SYBR Green kit (Roche) following the manufacturer’s instructions. Primers used for B19V-DNA amplification were; B19V-forward (5’-GGGCAGCCATTTTAAGTGTTT-3’) and B19V-reverse (5’-GCACCACCAGTTATCGTTAGC-3’). As external standards, plasmids containing the genome of B19V were used in ten-fold dilutions.

**Analysis of B19V binding to human RBCs.** Binding of B19V to RBCs was examined by quantitative PCR. RBCs from a total of 17 individuals were washed with PBS and resuspended at a concentration of 2 x 10^7 RBCs per ml. A volume of 10 µl of the RBC suspension was mixed with B19V (2 x 10^5 virions per cell). After 2 h at 4°C the cell/virus suspension was washed 6 times with PBS to remove unbound virus. The cell pellet was resuspended in 200 µl
of PBS and a fraction was used to count the cells. Total DNA was extracted from the cell suspension by using the DNeasy tissue kit (Qiagen, Valencia, CA). RBC-associated viral DNA was quantified by using real-time PCR as specified above. The results were compared with values obtained from corresponding amounts of RBCs spiked with increasing amounts of B19V.

Additionally, the RBCs were incubated with a mouse IgM antibody specific for the cellular receptor globoside (AME-2; 40), with a similar concentration of a non-specific mouse IgM isotype control antibody (Sigma) or with empty capsids (5 x 10^4 particles per cell). After 1h at 4°C, the cells were washed and B19V was added (2.5 x 10^4 virions per cell) and further incubated at 4°C for 2h.

Binding of B19V empty capsids to RBCs was examined by immunofluorescence. RBCs were incubated with full or empty capsids as specified above and the cells were fixed with acetone/MeOH (1/1 v/v) for 5 min at -20°C. After fixation, the cells were air dried and washed with PBSA (PBS, BSA 1%). Subsequently, a human mAb recognizing only intact capsids (mAb 860-55D) was applied (1:50 diluted in PBSA) for 1 h at RT. The cells were washed 3 times with PBSA for 5 min and as secondary antibody, a goat anti-human IgG-FITC was added (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at RT. After final washings with PBSA, the cells were mounted with mowiol (Calbiochem, La Jolla, CA) containing 30 mg/ml of Dabco (Sigma) as an antifading agent and examined by fluorescence microscopy.

**Infectivity assay.** B19V particles were bound to RBCs as specified above. Following binding, the cells were washed to remove unbound particles.
The amount of virus bound per cell was quantified as previously described. Subsequently, the RBCs were added as intact or lysed cells to UT7/Epo cells (10^5 cells) at 1,000 B19V DNA-containing particles per cell in RPMI without serum. As controls, similar amounts of free B19V without RBCs or in the presence of RBCs that were pre-blocked with EC, were used. After 1h at 4°C, the cells were washed 3 times in PBS and transferred to 12-well plates and further incubated at 37°C in RPMI containing 10% FCS and erythropoietin. For viral RNA analysis, the cells were transferred after 24h to RNase-free tubes (Safe-Lock Tubes 1.5 ml, Eppendorf Biopur®) and pelleted. The pellet was washed twice with PBS and stored at -20°C until use. Total poly-A-mRNA was isolated with an mRNA isolation kit (Roche). Following reverse transcription, cDNA was quantified by using the LightCycler® 2.0 system (Roche) and the LightCycler® FastStart DNA Master SYBR Green I kit (Roche). Primers were chosen to amplify a 133 nt-long NS1 cDNA fragment: NS1 forward (5’-GGG GCAGCATGTGTTAAAG-3’ (nucleotide 1017-1035) and NS1 reverse (5’-CCATGCCATATACT GGAACACT-3’ (nucleotide 1129-1150). For viral DNA analysis, the cells were collected 84h post-infection. Total DNA was extracted and viral DNA was quantified as specified above.

Immunoprecipitation. B19V capsids were immunoprecipitated with a human mAb against intact capsids (850-55D) or with a rabbit polyclonal against the PLA2 region (VP1u 142-163). After overnight incubation at 4°C in the presence of 20 µl of protein G agarose beads, the supernatant was discarded. The beads were washed four times with PBSA. Immunoprecipitated viral capsids were resolved by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE). After transfer to a PVDF
membrane, the blot was probed with a mouse antibody against B19V proteins (1:500; US biologicals, Swampscott, MA), followed by a horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution). The viral structural proteins were visualized with a chemiluminescence system (Pierce, Rockford, IL). Immunoprecipitation of B19V bound to RBCs was performed after cell lysis in NP40 buffer (50 mM Tris-HCl; 150 mM NaCl; 5 mM EDTA; 1% NP40; pH 7.2). Following centrifugation at 10,000 x g for 10 min, the supernatant was used for immunoprecipitation. Quantification of the immunoprecipitated virions was performed by real-time PCR as specified above.

**Phospholipase A<sub>2</sub> activity assay.** Native B19V capsids were assayed for PLA<sub>2</sub> activity by using a colorimetric assay (sPLA<sub>2</sub> assay kit; Cayman Chemical, Ann Arbor, MI), following the manufacturer’s instructions. The absorbance at 414 nm was determined every minute. The PLA<sub>2</sub> activity was expressed as micromoles of substrate hydrolyzed per minute per milliliter.

**Osmotic fragility test.** The osmotic fragility (OF) test measures RBC resistance to hemolysis when exposed to hypotonic saline solutions. An increased OF indicates a damage or an alteration of the RBC. RBCs (2 x 10<sup>6</sup>) were incubated with increasing amounts of B19V per cell in a final volume of 10 µl of PBS. Following virus attachment for 1h at 4°C, the cells were incubated in a buffer containing calcium (20 mM Hepes; 150 mM NaCl; 10 mM CaCl<sub>2</sub>; 0.5 µg/ml BSA) for 3h at 37°C. After centrifugation, the amount of hemoglobin of each supernatant was measured in a spectrophotometer at 545 nm. The results were expressed as per cent of hemolysis compared with the absorbance obtained after total cell lysis (corresponding amount of RBCs in
water) and were referred as direct hemolysis. Subsequently, the RBC pellets were resuspended in a hypotonic NaCl buffer (0.6g/dl) and incubated ON at 37°C. The percentage of hemoglobin release was measured in the same way and was referred as osmotic fragility.
Results

The PLA\textsubscript{2} motif of native B19V capsids is not accessible to antibody binding but can become exposed and active upon mild heat treatment. We have previously found that the PLA\textsubscript{2} activity is barely detectable in the native B19V capsids but can be triggered upon mild heat or low pH treatment (30). The question remained whether the lack of activity was due to an internal position within the capsid. In the present studies, we have verified the accessibility of this region by using an antibody (VP1u 142-163) targeting the PLA\textsubscript{2} enzymatic region (Fig. 1A). The results showed that the PLA\textsubscript{2} region of B19V is internal and not accessible to antibodies (Fig. 1B). Treatment of the native capsids to increasing temperatures induced a progressive accessibility of the PLA\textsubscript{2} motif (Fig. 1C) as well as a proportional increase of the PLA\textsubscript{2} activity (Fig. 1D). Therefore, in order to be active, the PLA\textsubscript{2} region of B19V has to become fully exposed in a conformation which is accessible to antibody binding.

B19V empty capsids represent an important fraction of the viral particles circulating in the blood and bind RBCs similarly to full capsids. For some animal parvoviruses it has been shown that during the infection process in cell culture DNA-containing capsids (full capsids; FC) and empty capsids (EC), are generated. We have examined the proportion of FC and EC from a naturally infected individual. Gradient-purified FC and EC were prepared from a viremic serum sample. A dot-blot hybridization performed with the CsCl fractions showed the separation of EC and FC (Fig. 2A). The same experiment performed with pool fractions showed that the EC represent an important proportion (estimated 30-40\%) of the B19V particles circulating
in the blood (Fig. 2B). The purity of the EC as well as the concentration of FC was verified by quantitative PCR (Fig. 2C).

Given the important proportion of B19 EC in the viremic serum, it was of interest to verify the attachment of EC to RBCs. The immunofluorescence experiments showed that EC also bind to RBCs (Fig. 2D). Pre-incubation of RBCs with pure B19V-EC resulted in a total binding inhibition of FC (Fig. 2E), indicating that both FC and EC bind to RBCs through the same receptor structures.

**Quantification of B19V binding to RBCs from different donors.** Incubation of B19V with RBCs from 17 healthy donors resulted in an extensive virus attachment ranging between 3,000 and 30,000 virions per cell. Most of the samples showed a binding rate of approximately 4,000 to 12,000 virions per cell. Two of the 17 samples had a higher binding capacity reaching 20,000 to 30,000 virions per cell (Fig. 3A).

**B19V binding to RBCs can be disturbed by an antibody specific for globoside.** It has been previously shown that binding of B19V to susceptible cells involves other receptors apart from globoside; namely, Ku80 and α5β1 integrin (25, 43). Human RBCs have globoside but lack integrin (43). We have investigated the blocking effect of a mAb specific for globoside (AME-2; 40) on the binding efficiency of B19V to RBCs. Pre-incubation of RBCs with AME-2 disturbed binding of B19V to RBCs (Fig. 3B). This result indicates that globoside is involved in the binding of B19V to RBCs. However, since the blocking effect is not complete, other molecular structures might also be involved in the binding.
Binding of native B19V to RBCs triggers the exposure of the VP1u-PLA$_2$ motif. We have previously shown that the most N-terminal part of VP1u, containing neutralizing epitopes, is not exposed on the surface of native B19V particles (30). In the present studies we also show that the PLA$_2$ region, distant from the N-terminal part, is also not accessible to antibodies. Given the fact that VP1u contains critical neutralizing epitopes, it should become accessible to antibodies in the extracellular milieu. In view of that, the possibility that binding of B19V to human RBCs would trigger the exposure of VP1u was investigated.

Following binding to RBCs at 4°C, the viral capsids present in the supernatant (unbound virus) and cellular fractions (bound virus) were immunoprecipitated with a mAb against assembled capsids (860-55D), and a polyclonal antibody against the VP1u-PLA$_2$ motif (VP1u 142-163). The amount of virus present in the immunoprecipitated material was examined by Western blot. As shown in Fig. 4A, viral capsids with exposed VP1u-PLA$_2$ were only detected in the cell fraction. From the immunoblot results it was evident that not all capsids bound to RBCs had VP1u exposed. In order to quantify the proportion of virions with exposed VP1u-PLA$_2$, the viral DNA present in the immunoprecipitated material was quantified by real-time PCR. Following binding to RBCs, the amount of virions that have changed in conformation exposing VP1u-PLA$_2$ was approximately 1/3 of the total particles (Fig. 4B). Therefore, there are capsids bound to RBCs that did not exposed VP1u. This result might be due to the observation that binding of B19V to RBCs is complex and might involve various molecular structures, which would have a different effect on the conformation of VP1u.
B19V is not directly hemolytic but increases the osmotic fragility of RBCs. Binding of B19V to human RBCs results in the exposure of the PLA₂ motif present in the VP1u. Treatment of human red cells with PLA₂ from bee venom does not cause direct hemolysis but increases the osmotic fragility in parallel with the cleavage of the accessible phospholipids (38). Since B19V binds extensively to human RBCs without internalization, it was of interest to examine whether the exposed PLA₂ enzyme affects the integrity of the RBC membranes. Similar to the PLA₂ from bee venom, no direct hemolysis was observed after 3h incubation at 37°C with B19V capsids, however the osmotic fragility of the RBCs increased progressively with the number of virus per cell (Fig. 5), indicating a damage or an alteration in the cell membranes.

Inhibition of the PLA₂ activity of B19V capsids. The PLA₂ activity of B19V capsids was assessed in the presence of antibodies against VP1u. The antibody 1418-1 which targets a region of VP1u distant from the PLA₂ motif; aa 30 to 42 (12) had no effect on the enzyme activity. The antibody against the PLA₂ region only moderately inhibited the activity (Fig. 6A). This result indicates that the antibody does not bind any critical amino acid required for the enzymatic activity with a soluble substrate.

The VP1u-associated PLA₂ activity is responsible for the increase in the osmotic fragility of RBCs. In order to investigate whether the B19V-PLA₂ activity is responsible for the increase in the osmotic fragility (OF) of the RBCs, the OF test was performed in the absence of calcium, which is required for the PLA₂ activity (6) or in the presence of antibodies against VP1u (1 µg). The mAb against the most N-terminal part of VP1u (aa 30-42; mAb 1418), which is distant from the PLA₂ motif, had no influence in the
increase of OF caused by B19V. However, under calcium depletion or in the presence of the PLA\textsubscript{2} antibody, B19V was not able to modify the OF of the RBCs (Fig. 6B). Heat-treated capsids (60°C / 3 min), which were previously shown to expose the PLA\textsubscript{2} motif (Fig. 1C and D) did not cause any detectable increase in OF, indicating that the PLA\textsubscript{2}-phospholipid interaction depends on a specific conformation of the capsid-receptor complex, which might be different in the heat-treated virus. None of the conditions tested; calcium depletion, VP1\textsubscript{u} antibodies or mild heat treatment, disturbed B19V binding to RBCs and did not modified the OF of the RBCs in the absence of the virus (data not shown).

**Quantification of B19V from plasma and RBC fractions from an individual with a recent infection.** Despite the fact that RBCs have large amounts of the globoside receptor, B19V viremia is always examined in the plasma fraction, while the RBC fraction is ignored. The amount of B19V in the plasma and RBC fractions of a blood sample from an individual with a recent B19V infection (IgG and IgM positive) was examined by quantitative PCR. In plasma and RBC fractions, the amount of B19V-DNA was $1.7 \times 10^7$ and $2.7 \times 10^8$ virions/ml, respectively. This result indicates that in this particular individual and at this particular moment of the infection, more than 90% of the virions circulating in the blood were associated to RBCs (Fig. 7A).

**B19V bound to RBCs cannot infect UT7/Epo cells.** Given the fact that B19V particles circulating in the blood can be associated to RBCs, it was of interest to investigate whether such particles are still able to infect a susceptible cell line. Following binding of B19V to RBCs, the cells were extensively washed to remove unbound virus. The RBCs were added as
intact or lysed cells to UT7/Epo cells. B19V infectivity was measured by NS1-mRNA and viral DNA quantification, as specified in materials and methods. The results showed that viruses attached to RBCs are not able to initiate an active infection in UT7/Epo cells, most probably due to the firm attachment of the virus to the RBCs (Fig. 7B and C).
Discussion

Human red blood cells (RBCs) are the most abundant cell type in the blood. Despite the fact that RBCs have large quantities of the B19V receptor globoside (16) and that B19V viremia is typically high, the interaction between B19V and RBCs have not yet been studied. Our observations indicate that B19V interacts extensively with human RBCs. This important interaction results in changes in virus structure and membrane integrity.

The VP1u region is immunodominant and has a PLA₂ activity (13) that is required for the infection (14, 18, 44). VP1u is also suspected to contribute to autoimmune reactions and inflammatory processes by mechanisms still not well understood. VP1u has been shown to generate the production of antiphospholipid antibodies and antiphospholipid syndrome-like autoimmunity via molecular mimicry (36, 37). The PLA₂ activity of VP1u is also suspected to contribute to inflammatory processes induced by the production of potent eicosanoid lipid mediators (22). All these immunological features contrast with our recent findings indicating that the most N-terminal part of VP1u is internal and that the capsids are enzymatically inactive (30). In the present studies we have analyzed the accessibility of the PLA₂ region of VP1u, which is close to the VP1-VP2 junction and found that this region of VP1u is also internal in native capsids. It is well known for some viruses that binding to their cellular receptors leads to capsid structural rearrangements required for entry into susceptible cells. When poliovirus attaches to its receptor, the particle changes in conformation and internal components, including VP4 and the N-terminal of VP1, are externalized (27, 35). In the case of parvovirus, changes occurring during virus binding to cellular receptors have not been observed so
far. In the present studies we have observed changes in capsid conformation following B19V binding to RBCs leading to the externalization of VP1\textsubscript{u} sequences. It remains unknown the function of the exposed VP1\textsubscript{u} sequences at the cell surface. It is tempting to speculate that the early exposure of VP1\textsubscript{u} on the membrane of target cells is followed by interaction with a co-receptor, which would facilitate virus entry. In line with this hypothesis is the fact that globoside is necessary but not sufficient for B19V infection (42). Other co-receptors have been identified such as Ku80 and α5β1 integrin (25, 43). The role of these co-receptors in B19V infection is still not clear, but it has been postulated that α5β1 integrin is required for viral entry. Therefore, the externalization of VP1\textsubscript{u} sequences at the cell surface would facilitate entry and/or uncoating of B19V. The exposure of VP1\textsubscript{u} sequences at the cell surface would also have implications in the immunology of B19V infection. Antibodies derived from the exposed VP1\textsubscript{u} sequences would not target and neutralize free infectious particles circulating in the blood, whose VP1\textsubscript{u} epitopes are not accessible, but receptor-attached virus. This mechanism of neutralization at the cell surface has already been observed for mAb 1418-1, which target the most N-terminal part of VP1\textsubscript{u}. This antibody is highly neutralizing (17), but is not able to bind B19V in a cell-free system (30).

The exposure of VP1\textsubscript{u} has an impact on the red cell membrane. Similar to the effect observed during treatment of red cells with bee venom PLA\textsubscript{2} (38), binding of B19V to RBCs did not cause direct hemolysis but an increased osmotic fragility of the cells. The increased fragility was not observed in the absence of calcium, which is required for the PLA\textsubscript{2} activity (6) or in the presence of the PLA\textsubscript{2}-specific antibody. It is interesting that although
the PLA₂ antibody was able to fully protect the RBCs, the antibody could only moderately inhibit the PLA₂ activity in an *in vitro* assay with a soluble PLA₂ substrate. This result likely indicates that the antibody protects the membrane by interpolating between the enzyme and the phospholipids of the cell membrane. The PLA₂ inhibitor manoalide was able to inhibit the B19V-PLA₂ activity. However, the dose required for the inhibition (above 75 µM) was cytotoxic and caused the direct hemolysis of the RBCs (data not shown), therefore it was not possible to use it in the OF test. Further studies would be required to verify whether the B19V-induced membrane damage also occurs during the natural infection.

B19V receptor globoside is the most abundant neutral glycolipid in the RBC membrane with more than $10^7$ antigens per cell (16). In our studies, the amount of capsids bound per cell is largely bellow this amount. A possible explanation is that B19V binding to RBCs is complex and involve globoside in concert with other molecular structures. In agreement with this is the fact that B19V does not bind to membrane-associated globoside *in vitro* (19) and that an antibody specific for globoside was not able to totally block B19V binding to RBCs (Fig. 3B). Moreover, in our studies the amount of virus bound per cell ranged between 3,000 and 30,000. This significant variation exceed that of globoside, whose concentration is rather similar between different individuals, regardless whether they are P₁ or P₂ phenotype (16).

B19V viremia is always evaluated in the plasma fraction of the blood, while the RBC fraction is systematically neglected. Cell-associated viremia is therefore unknown. The analysis of a blood sample from an individual with a recent virus infection revealed that at this particular moment of the infection
B19V was mostly associated to the RBC fraction. However, since B19V IgM and IgG-specific antibodies were present, the result could reflect a faster elimination of B19V from plasma compared to RBC-associated B19V. Therefore, in order to characterize cell-associated viremia and determine the extent of B19V-binding to RBCs in a natural infection, more samples should be analysed, particularly from early viremic blood samples without detectable B19V specific antibodies. These results together highlight the importance of the human red blood cells in the context of B19V infection.

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Legends to figures

Fig. 1. Accessibility of the PLA$_2$ region of B19V-VP1u (A) Schematic representation of the VP1/VP2 proteins of B19V. Circular dots indicate the calcium binding domain and squared dots indicate the PLA$_2$ enzymatic core. The sequence of the peptide used for the immunization of rabbits is indicated. (B) Accessibility of VP1u-PLA$_2$ motif in native B19V capsids. B19V-DNA was quantified following immunoprecipitation with an antibody against capsids ($\alpha$-capsids; mAb 860-55D) or an antibody against the PLA$_2$ region ($\alpha$-PLA$_2$; VP1u 142-163). (C) Accessibility of VP1u-PLA$_2$ region after heat treatment. B19V capsids were treated at increasing temperatures for 3 min, cooled on ice and immunoprecipitated with mAb 860-55D ($\alpha$-caps) or Ab VP1u 142-163 ($\alpha$-PLA$_2$). The 75-kDa band corresponds to a LC–HC dimer resulting from partial denaturation of the antibody (band present without virus). (D) Activation of B19V-PLA$_2$ enzyme by heat-treatment. PLA$_2$ activity of B19V capsids was measured following exposure to increasing temperatures for 3 min. Error bars represent the deviation from three independent experiments.

Fig. 2. Proportion of full and empty capsids from an infected plasma sample. (A) Dot-blot hybridization from CsCl fractions and (B) from dilutions of the pooled fractions. (C) Quantification of B19V-DNA from FC and EC pool fractions. (D) Detection of FC and EC bound to RBCs by immunofluorescence. (E) RBCs were incubated empty capsids ($5 \times 10^4$ particles per cell). After 1h at 4°C, the cells were washed and B19V was added and further incubated at 4°C for 2h. Following intensive washing to remove unbound virus the amount of
B19V associated to RBCs was quantified by real-time PCR. Deviations from three independent experiments are indicated.

**Fig. 3.** (A) Quantification of B19V binding to RBCs from different donors. B19V was incubated with RBCs from 17 healthy donors. Following washings to remove unbound virus, the amount of virus bound per cell was quantified by real-time PCR (B) Effect of pretreatment of RBCs with an antibody specific for globoside on B19V binding. RBCs where either untreated (PBS), treated with anti-globoside IgM (AME-2) or a similar concentration of a non-specific mouse IgM isotype control before the addition of B19V (2.5 x 10⁴ virions per cell). Deviation from three independent experiments are indicated.

**Fig. 4.** VP1u-PLA₂ is exposed on the capsid surface following attachment to P antigen on human RBCs. (A) Following binding to RBCs at 4°C, the supernatant (unbound virus) and cellular fractions (bound virus) were incubated in lysis buffer and centrifuged at 10,000 x g for 10 min. The viral capsids present in the supernatants were immunoprecipitated with a mAb against assembled capsids (α-Caps; 860-55D), and a polyclonal antibody against the VP1u-PLA₂ motif (α-PLA₂; VP1u 142-163). The amount of virus present in the immunoprecipitated material was examined by Western blot. (B) The amount of virions immunoprecipitated with the antibody against capsids, with the antibody against the PLA₂ motif or with the pre-immune serum from the rabbits used to produce the PLA₂ antibody, were quantified by real-time PCR. Error bars represent the deviation from four independent experiments.
Fig. 5. B19V increases the osmotic fragility of human RBCs. RBCs (2 x 10^6) were incubated with increasing amounts of B19V per cell (virus concentrated from serum by ultracentrifugation through 20% sucrose and resuspended in PBS). Following virus attachment for 1h at 4°C, the cell suspension was incubated in an isotonic buffer containing calcium for 3h at 37°C. The percentage of hemoglobin release was measured in the supernatant and referred as direct hemolysis. Subsequently, the RBC pellet was resuspended in a hypotonic NaCl buffer (0.6g/dl) and incubated ON at 37°C. The percentage of hemoglobin release was measured in the same way and was referred as osmotic fragility. Results are mean values from three independent experiments.

Fig. 6. The VP1u-associated PLA2 activity is responsible for the increased osmotic fragility of human RBCs. (A) B19V-PLA2 activity in the presence of antibodies against VP1u. The PLA2 activity from heat-treated (60°C/3 min) B19V (1.2 µg) was measured after incubation with antibody 1418-1 (α-N-VP1u; against the most N-terminal part of VP1u) or with antibody VP1u 142-163 (α-PLA2; targeting the PLA2 region). (B) Osmotic fragility of RBCs incubated with B19V under different conditions. RBCs (2 x 10^6) were incubated with 3 x 10^4 virions per cell. Following virus attachment for 1h at 4°C, the cells were incubated in an isotonic buffer with or without calcium or in the presence of antibodies against VP1u, for 3h at 37°C. Following centrifugation, the RBC pellets were resuspended in a hypotonic NaCl buffer (0.6g/dl) and incubated ON at 37°C. The percentage of hemoglobin release was measured. The upper and lower dotted lines indicate the average OF
value of RBCs incubated with B19V (upper line) or without B19V (lower line). B19\(^{60}\): B19V exposed to 60°C for 3 min. Error bars from three independent experiments are indicated.

**Fig. 7.** (A) Quantification of B19V in the plasma and RBC fractions from an individual with a recent B19V infection (IgG and IgM positive). The heparinized fresh blood sample was centrifuged to separate plasma and RBCs. The RBCs were extensively washed in PBS. B19V DNA was quantified from a volume of 100 µl of plasma or washed RBCs, as specified in materials and methods. (B) Infectivity of B19V bound to human RBCs based on NS1-RNA or (C) viral DNA quantification. RBCs were pre-incubated with B19V. Subsequently, the RBCs were washed intensively with PBS to remove unbound virus. The RBCs were added as intact (in PBS) or lysed cells (in water) to UT7/Epo cells. As controls, similar amounts of B19V alone or in the presence of RBCs that were pre-blocked with EC, were used. NS1 RNA and viral DNA were quantified after 24h and 84h, respectively. Error bars from three independent experiments are indicated.
Fig. 1

A

1 227 780

VP1 unique region VP1-VP2 common region

130 195

α-PLA2

B

Virions immunoprecipitated

α-caps α-PLA2 PI

C

α-caps α-PLA2 α-caps α-PLA2 α-caps α-PLA2

37°C 55°C 60°C

VP1 HC-LC dimer VP2

D

PLA activity (nmol/min/ml)

37°C 55°C 60°C 65°C
Fig. 3
Fig. 4

A

B

Values with accessible PLA2 modified

[Fig. 4 diagram and legend]
Fig. 5

The graph shows the comparison between osmotic fragility and direct hemolysis. As the number of virus particles added per RBC increases, the hemolysis percentage also increases. The black square represents osmotic fragility, and the grey square represents direct hemolysis.
Fig. 6

A

PLA2 activity (μmol/min/ml)

B19
B19-50°C
B19-60°C
B19-80°C

No antibody
α-N-VP1u
α-PLA2

B

Hemolysis (%)

B19
B19-50°C
B19-60°C
B19-80°C
B19-α-PLA2

PBS
Fig. 7

A

Vironce
(percentage virions in whole blood)

Plasma
RBCs

B

B19 NS1 mRNA copies (x 10^4)

B19 bound to RBC
B19 bound to RBC-RBC
B19 + V-blocked RBC-RBC
B19 alone
Mock

C

B19 genome copies (x 10^5)

B19 bound to RBC
B19 + V-blocked RBC-RBC
B19 alone
Mock