Characterization of the early steps of human parvovirus B19 infection

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Abstract

The early steps of human parvovirus B19 (B19V) infection were investigated in UT7/Epo cells. B19V and its receptor globoside (Gb4Cer) associate with lipid rafts, predominantly of the non-caveolar type. Pharmacological disruption of the lipid rafts inhibited infection when added prior to virus attachment but not after virus uptake. B19V internalizes by clathrin-dependent endocytosis and spreads rapidly throughout the entire endocytic pathway reaching the lysosomal compartment within minutes, where a substantial proportion is degraded. B19V did not permeabilize the endocytic vesicles, indicating a mechanism of endosomal escape without apparent membrane damage. Bafilomycin A1 (BafA1) and NH4Cl, which raise endosomal pH, blocked the infection by preventing endosomal escape resulting in a massive accumulation of capsids in the lysosomes. In contrast, in the presence of chloroquine (CQ), transfer of incoming viruses from late endosomes to lysosomes was prevented, the viral DNA was not degraded and the infection was boosted. In contrast to untreated or BafA1-treated cells, the viral DNA was progressively associated with the nucleus in CQ-treated cells, reaching a plateau by 3 h post-internalization and coinciding with the initiation of viral transcription. At this time more than half of the total intracellular viral DNA was associated with the nucleus; however, the capsids remained extranuclear. Our studies provide the first insight into the early steps of the infection by B19V and reveal mechanisms involved in virus uptake, endocytic trafficking and nuclear penetration.
Introduction

Human parvovirus B19 (B19 virus; B19V) was discovered in 1975 (16) and has been classified within the Erythrovirus genus of the Paroviridae family. Transmitted mainly via the respiratory route, B19V is generally associated with a mild, self-limiting childhood disease named erythema infectiosum or fifth disease. However, during pregnancy or in individuals with underlying immune or hematologic disorders, B19V can cause more severe syndromes such as acute and chronic arthropathies, severe cytopenias, hydrops fetalis and fetal death (49). 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% are VP1 (83 kDa) (17). Two other open-reading frames coding for small proteins of unclear functions have also been identified (51, 64).

Paroviruses have a similar strategy for the delivery of the viral genome into the nucleus for replication; however, there are significant differences depending on the specific virus and cell type (18, 25, 42). While some paroviruses are internalized following interaction with a single receptor, others require complex interactions with receptors and co-receptors before they can be internalized. In addition to clathrin-mediated internalization, caveolae-dependent internalization and macropinocytosis have also been described (2, 5). A common feature of the endocytic trafficking of paroviruses is the requirement of endosomal acidification. However, the timing of virus trafficking through the endosomal pathway, the cellular elements involved and the sites of escape into the cytosol vary considerably among virus species and cells. However, the mechanisms by which paroviruses enter the nucleus remain unclear. Having a small size (22-25 nm), they can theoretically pass through the nuclear pore complex (NPC) without disassembly. However, an alternative mechanism based on local disruption of the outer nuclear envelope has
been proposed (14, 15). For most parvoviruses, the majority of capsids accumulate in a perinuclear location from which the viral DNA is imported into the nucleus either as an intact viral particle (24, 50, 61) or after capsid disassembly (33). Three cell receptors/co-receptors have been identified for B19V, the glycosphingolipid globoside (globotetraosylceramide; Gb4Cer) (12), α5β1 integrin (59) and Ku80 autoantigen (40). Gb4Cer is mainly expressed in the human erythroid progenitor cells in the bone marrow, which are also the main target cells for the virus, and it seems to be the primary attachment receptor. Ku80 might also function for virus attachment in certain cells (40), while the α5β1 integrin is thought to act as a co-receptor required for internalization (58, 59).

The mechanisms of B19V uptake and intracellular trafficking have remained elusive (48). These studies are limited because a well-established cell line system for B19V infection is lacking; thus, it is not possible to propagate the virus to high titers. Therefore, highly viremic plasma from naturally infected individuals without virus-specific antibodies remains the main source of infectious native virus. UT7/Epo cells are commonly used to study B19V infection (30). Although entry and intracellular trafficking of B19V is not restricted in UT7/Epo cells, the infection is limited to a small number of cells due to intracellular factors induced by erythroid differentiation (23). An improved B19V infection has been described in ex vivo expanded primary human erythroid progenitor cells (EPCs) (21, 60), where B19V infects a larger number of cells. The improvement operates at the level of replication/transcription and not at the level of entry and trafficking (13); however, the production and egress of an infectious progeny is still restricted. Considering that the susceptibility of EPCs to B19V infection is limited to a narrow time frame due to large variations in virus receptor expression (60), UT7/Epo cells provide more stable conditions to study entry/trafficking events.
Using complementary approaches, we have studied the early events of B19V infection in UT7/Epo cells. Although B19V and its receptor globoside associate with lipid rafts, the internalization mechanism occurs through clathrin-mediated endocytosis. Viral particles are routed to the lysosome for degradation. Escape into the cytosol depends on the low endosomal pH and occurs without apparent membrane damage. Nuclear entry is highly inefficient but can be boosted by chloroquine, which prevents the degradation of incoming viruses by blocking their transfer to lysosomes. The viral DNA is then efficiently imported into the nucleus while the capsids remain extranuclear.
**Materials and Methods**

**Cells and viruses.** UT7/Epo cells were cultured in RPMI with 10% FCS and complemented with 2 U/ml of recombinant human erythropoietin (Epo; Janssen-Cilag, Midrand, South Africa) at 37°C and 7.5% CO₂. A B19V-infected plasma sample (Genotype 1; CSL Behring AG, Charlotte, NC), without detectable levels of B19V-specific IgM or IgG antibodies, was used as a source of native infectious virus. The virus was pelleted by ultracentrifugation through 20% (w/v) sucrose and the concentration of virions was determined using quantitative PCR.

**Antibodies and chemicals.** For the detection of B19V by immunoprecipitation and immunofluorescence, a monoclonal antibody against B19V (mAb 860-55D) was kindly provided by S. Modrow (Regensburg, Germany). The mAb 860-55D targets a conformational epitope in VP2 and recognizes exclusively intact capsids (22). A rabbit polyclonal antibody against VP1u (aa 142-163; referred as α-VP1u) was obtained as previously described (7). Early endosomes were detected using immunofluorescence with a rabbit polyclonal antibody against EEA1 (Abcam, Cambridge, MA). A mouse mAb against the mannose-6-phosphate receptor and a mouse-mAb against Lamp-1 (Abcam), were used to detect late endosomes and lysosomes, respectively. Flotillin-1 was obtained from BD Biosciences (San Jose, CA), and caveolin-1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-labeled cholera toxin was purchased from Sigma, and FITC-labeled human holo transferrin was purchased from Exbio (Vestec, Czech Republic). Rhodamine-conjugated lysine-fixable dextran (Mr, 3,000) was obtained from Invitrogen (Carlsbad, CA). All drugs were purchased from Sigma (St. Louis, MO). Ammonium chloride (NH₄Cl) was dissolved in water, nystatin and bafilomycin were dissolved in dimethyl sulfoxide (DMSO), and filipin was dissolved in ethanol.
Control of interfering drugs. To test the drugs for cytotoxicity and specificity, B19V replication was quantified in the presence of increasing doses of the drugs added a few hours after internalization. Only doses that showed no effect on B19V replication were used. In addition to ensuring that none of the doses used have an effect on the cellular S-phase, which is required for parvovirus replication, the method allows to rule out possible pleiotropic effects on later stages of the infection not related to virus binding/internalization and trafficking.

Isolation of lipid rafts. The insolubility of lipid rafts in cold nonionic detergents is the most common method for lipid raft isolation. However, the presence of detergents can produce artifacts or interfere with sphingolipid distribution. Therefore, we used a novel detergent-free method in which rafts are purified by shearing cells in an isotonic buffer with cations, followed by separation along an OptiPrep™ gradient (34). A total of 5x10^7 UT7/Epo cells were infected or not at 4°C for 1 h and intensively washed to remove unbound virus. Cells were lysed in 500 μl of detergent-free lysis buffer (1× TBS; pH 8), in the presence of 1 mM CaCl₂ and 1 mM MgCl₂ to stabilize the rafts and supplemented with protease inhibitor cocktail (Complete Mini; Roche). The homogenate was sheared through a 22 g x 3” needle and centrifuged at 1,000 g for 10 min at 4°C. The resulting post-nuclear supernatant was collected and maintained on ice. The procedure was repeated on the pellet, and the obtained supernatant was combined with the first. The pooled supernatant (225 μl) was mixed with an equal volume of 50% OptiPrep™ (Sigma) in SW60 ultracentrifuge tubes. The mixture was carefully overlaid with 3 ml of 20% Optiprep™ and 675 μl of 10% Optiprep™. The tubes were centrifuged at 38,500 rpm (200,000 g) for 18 h at 4°C. Acceleration and deceleration rates were set to zero. Fractions of 520 μl each were collected from the top of the tube. An aliquot (20 μl) from each fraction was analyzed by Western blotting with an antibody against the lipid raft component flotillin-1.
**Immunofluorescence.** UT7/Epo cells were infected with B19V at $2 \times 10^4$ viral particles per cell in RPMI for 1-2 h at 4°C to allow binding, followed by several washing steps to remove unbound virus. After different incubation times at 37°C, the cells were fixed with acetone-methanol (1/1 [v/v]) for 5 min at -20°C. Following staining with specific antibodies, the cells were washed and mounted with Mowiol (Calbiochem, La Jolla, CA) containing 30 mg/ml of Dabco (Sigma) as an antifade agent. Confocal laser scanning microscopy was performed using a LSM 510 Meta with an inverted Zeiss microscope (Axiovert 200M, Carl Zeiss A.G., Feldbach, Switzerland).

**Electron microscopy.** UT7/Epo cells were infected with B19V at $3 \times 10^4$ viral particles per cell in RPMI without serum for 1.5 h at 4°C. Following a washing step to remove unbound virus, the cells were incubated at 37°C. After 0, 1, 5, 10 or 30 min, the cells were fixed in 2.5% glutaraldehyde. Cells were washed in PBS and embedded in 3% agarose. The cubes were fixed for 1 h in osmium tetroxide (1% in PBS) on ice, washed and further incubated for 1 h in tannin solution (0.05 M HEPES + 0.01 g Tannin) at RT. The samples were washed with sodium sulfate solution (1% in 0.05 M HepesHEPES) and water. Filtrated 2% uranyl solution was added to the samples for 1 h at RT, washed with water and dehydrated in increasing ethanol concentrations. Samples were incubated in Epon, embedded in gelatine capsules and polymerized for 48 h at 60°C. Capsules were dissolved at 70°C, and 70 nm ultrathin sections were cut, stained with 1% lead citrate and 2% uranyl acetate and finally analyzed in a Zeiss EM 902 electron microscope, equipped with a TRS digital camera.

**Infectivity assay.** Cells ($3 \times 10^5$) in RPMI containing 10% fetal calf serum and 2 U/ml of Epo were seeded into 12-well plates and pre-incubated with the drugs for 30 min at 37°C before infection. In some cases, cells were pulse-treated with drugs for specific times, as indicated. As
controls, drugs were added 3-4 h post-infection. Cells were infected with B19V at 5x10³ genome equivalents per cell and further incubated at 37°C for 24 h. The cells were transferred to RNase-free tubes (Eppendorf Biopur®, Hamburg, Germany) and pelleted. The pellets were washed twice with PBS and stored at -20°C until use. Total poly(A) mRNA was isolated using the Dynabeads mRNA direct kit (Invitrogen) following manufacturer’s instructions. The isolated viral NS1 mRNA was reversed transcribed and cDNA was quantified. Cells collected at 30 min post-infection served as input controls to define the background signal.

**Quantitative PCR.** Amplification of B19V DNA or cDNA and real-time detection of PCR products were performed using a LightCycler system (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR green (Roche). PCR was performed using a FastStart DNA SYBR green kit (Roche) following the manufacturer’s instructions. Plasmids containing the genome of B19V were used at 10-fold dilutions as external standards. The number of cells used for each experiment was determined with the quantification of the cell β-actin gene, as previously described (44).

**Immunoprecipitation.** UT7/Epo cells were infected with B19V, as described above. Cells were lysed in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, and 5 mM EDTA), supplemented with protease inhibitor cocktail (Complete Mini; Roche). Viral particles were immunoprecipitated with a human mAb against intact capsids (mAb 860-55D). After overnight incubation with 20 μl protein G agarose beads at 4°C, the beads were washed four times (three times with PBS-1% bovine serum albumin and once with PBS) and resuspended in protein loading buffer to analyze the immunoprecipitated capsids by Western blotting or in PBS to quantify the viral DNA by PCR. Total DNA was extracted using a the DNeasy tissue kit (Qiagen) and quantified as specified above.
Analysis of endosomal membrane permeabilization. To examine whether B19V can induce a detectable endosome membrane permeabilization or damage during the escape process, rhodamine-labeled dextrans (Mr, 3,000) were co-endocytosed with B19V for 2 h at 37°C. The cells were washed and further incubated at 37°C in the presence of B19V to maintain a constant flow of incoming viruses in endocytic vesicles. Uninfected cells were used as controls. The capacity of the endosomal vesicles to retain the endocytosed dextrans was monitored with fluorescence microscopy at increasing incubation times.

Quantification of B19V DNA nuclear import. UT7/Epo cells were infected as specified above. At increasing times post-infection, the cells were washed twice with ice-cold PBS and the pellets resuspended in 100 µl EZ buffer before adding an additional 900 µl EZ buffer (Sigma). The samples were vortexed and kept on ice for 5 min, then pelleted at 2,400 rpm for 5 min at 4°C. This step was repeated. Pellets were then resuspended in 500 µl EZ buffer containing 0.25 M sucrose and layered on top of 500 µl EZ buffer with 0.5 M sucrose. The purified nuclei were collected by centrifugation at 5,000 rpm for 10 min at 4°C. The purity and integrity of the isolated nuclei was assessed via light microscopy after trypan blue staining. Nuclear pellets were resuspended in nuclei lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1 % Triton X-100, and 1 mM EDTA [pH 7.4]), supplemented with protease inhibitor cocktail (Complete Mini; Roche) and maintained on ice for 10 min. The homogenate was sheared through a 26 g x 1” needle and centrifuged at 8,000 g for 10 min at 4°C. The supernatant was used to quantify the viral DNA or to immunoprecipitate capsids, as specified above.
Results

**B19V and the globoside receptor associate with lipid rafts.** Lipid rafts are specialized plasma membrane microdomains that are enriched in cholesterol and glycosphingolipids and play roles in membrane receptor dynamics, signal transduction, intracellular trafficking, cell polarization and cell migration (31). The glycosphingolipid globoside (Gb4Cer) is the receptor of B19V (12). The presence of Gb4Cer in lipid rafts was examined using immunofluorescence staining with FITC-labeled cholera toxin B (CTxB), which binds to the ganglioside GM1, a common component of lipid rafts (62). Expression of GM1 in UT7 cells was irregular, as some cells showed little or no detectable expression. Gb4Cer colocalized partially with CTxB in cells expressing GM1 abundantly (Fig. 1A). Similar to Gb4Cer, B19V partially colocalized with the GM1-CTxB complex (Fig. 1A). Additionally, the distribution of the lipid raft marker flotillin-1 (Flot-1) along OptiPrep density gradients was examined. As expected, in uninfected cells Flot-1 was found in the light buoyant membrane fractions, predominantly in fractions 1 and 2. In contrast, when lipid rafts were isolated from infected cells, Flot-1 significantly shifted to denser fractions, suggesting a virus-mediated modification in the structure and/or lipid composition of the rafts (Fig. 1B).

Depending on the presence of the membrane protein caveolin-1 (Cav-1), lipid rafts can be divided into caveolar and non-caveolar rafts (38, 47), which are enriched in caveolin and flotillin proteins, respectively. Gb4Cer had a variable colocalization with Cav-1, from extensive colocalization in some cells, to modest or no colocalization in others (Fig. 1C). However, B19V rarely colocalized with Cav-1 (Fig. 1C), indicating that the large majority of B19V associates preferentially with non-caveolar lipid rafts.
Disruption of lipid rafts inhibits B19V infection. The infectivity of B19V was analyzed in the presence of nystatin, an antifungal reagent that disrupts cholesterol-enriched microdomains (6, 46). When added after virus binding/internalization, none of the doses tested caused significant inhibition. However, when added before virus infection, nystatin caused a dose-dependent inhibition of B19V infectivity (Fig. 1D). B19V infectivity was also sensitive to filipin, a sterol-binding agent that binds to cholesterol and disrupts lipid raft formation (1) (Fig. 1D). Similar to nystatin, filipin had no effect on the infection when added after virus binding/internalization, suggesting that the plasma membrane microdomains play a critical role during the process of B19V entry.

B19V internalizes through clathrin-mediated endocytosis. Electron micrographs from B19V-infected cells during the first minutes of incubation at 37°C showed the uptake of B19V capsids into plasma membrane invaginations and vesicles with the characteristic clathrin electrodense coating (Fig. 2A). One single virus particle was sufficient to accomplish the internalization process into clathrin-coated vesicles. Virus internalization in the typical small flask-shape caveolae invaginations or in other structures was not observed at any time. Furthermore, B19V did not colocalize with the ER, which is typically involved in caveolar-dependent internalization (29) (data not shown).

The uptake mechanism of B19V was followed using FITC-labeled human holo transferrin, which typically internalizes through clathrin (26). B19V and transferrin did not colocalize during the binding step at 4°C. However, when the temperature was shifted to 37°C, B19V and transferrin colocalized intensively, indicating a common internalization pathway (Fig. 2B). These results together indicate that clathrin-mediated endocytosis is the primary internalization mechanism used by B19V.
Incoming B19V capsids spread rapidly through the endocytic pathway and are routed to the lysosomes. The progression of intracellular capsids was examined using immunofluorescence confocal microscopy at increasing time points after internalization. Cells were washed, fixed and stained with an antibody against intact capsids (mAb 860-55D) and antibodies against early endosomes (EE; EEA1), late endosomes (LE; mannose-6-phosphate receptor), lysosomes (Lys; Lamp1), recycling vesicles (RV; Rab11), Golgi (58K Golgi protein) and endoplasmic reticulum (ER; GRP78 BIP). By 5 min post-internalization, B19V capsids were detectable in early endosomes, and by 30 min, capsids were observed throughout the entire endocytic pathway (Fig. 3A). Quantification of the colocalization signal from an average of 40 cells was performed with the BioImage XD software (27). Maximal colocalization with LE occurred by 10 min post-internalization and by 30 min with lysosomes (Fig. 3B). At later times post-infection, colocalization with both LE and Lys decreased progressively. By 1 h, capsids were detected in lysosomes but only few were detected in the LE. By 2-3 h post-internalization, capsids were clearly less abundant and appeared scattered in the cytoplasm, and with the exception of a few capsids detectable in lysosomes, colocalization with EE or LE was no longer observed. Significant colocalization of incoming particles with recycling vesicles, Golgi and ER was not observed at any time point (data not shown). The lack of colocalization with any relevant organelle marker suggests that following endocytic trafficking, a proportion of incoming viruses escapes into the cytosol.

B19V requires acidification for endosomal escape. Acidification inside the endosomes is required by certain viruses as part of their infectious entry route. All parvoviruses analyzed to date require endosomal acidification for the infection process (18, 25, 42). To investigate the requirement of B19V for low endosomal pH, cells were treated with either bafilomycin A1
(BafA1), a selective inhibitor of vacuolar ATPases, or the lysosomotropic weak base ammonium chloride (NH4Cl). These compounds raise the pH of intracellular compartments, which can be restored upon removal of the inhibitory substances (11, 36). UT7/Epo cells were pulse-treated for different times with BafA1 or NH4Cl. Although their mechanisms of action are different, BafA1 and NH4Cl treatment resulted in a remarkable similar reduction in B19V infectivity when applied at the beginning of the infection (Fig. 4A). However, BafA1 and NH4Cl had no significant effect on infectivity when added 1 h post-internalization, suggesting that most of the infectious particles had escaped from the endocytic vesicles.

The effect of BafA1 was further investigated using immunofluorescence. By 5 h p.i. in untreated cells, intact capsids did not colocalize with endocytic markers (Fig. 4B). Furthermore, no colocalization was observed with markers of recycling endosomes (rab 11 or transferrin), caveolin-containing vesicles, Golgi or ER (data not shown), indicating that these capsids may be in the cytosol. However, in the presence of BafA1, the capsids were retained inside late endocytic elements, notably in the lysosomes (Fig. 4B). These observations suggest that endosomal acidification is not essential for the progression of the virus along the endocytic pathway and in particular the transfer of the incoming viruses from late endosomes to lysosomes but is required by B19V to escape into the cytosol.

**A proportion of incoming capsids is degraded.** During the process of entry, B19V is routed to the lysosomes, reaching maximal colocalization by 30 min to 1 h after internalization (Fig. 3). To verify whether particles become degraded in this compartment, the integrity of the incoming viral DNA was examined using quantitative PCR. As shown in Figure 4C, from 1 to 5 h post-infection, significant degradation of the viral DNA was observed. The reduction was not due to virus detachment from the receptor or recycling, as no increase in viral DNA was
observed in the supernatant of the infected cells from 1 to 5 h post-infection (Fig. 4D). These observations indicate that during the process of viral entry, a proportion of incoming capsids are routed to the lysosomes for degradation.

Endosomal escape occurs without detectable membrane damage. At later times post-infection, intracellular capsids did not colocalize with markers of the endocytic pathway, including recycling endosomes (rab 11 or transferrin) and significant colocalization with caveolin-containing vesicles, Golgi or endoplasmic reticulum was not observed at any time (data not shown). This observation would suggest that the capsids have escaped into the cytosol. The mechanism by which these capsids reach the cytosol is unknown. Increasing evidence indicates that the PLA₂ activity of VP1u plays a role by disrupting the endosomal membrane (63). We have examined whether B19V can induce a detectable permeabilization or damage in the endocytic membranes through monitoring the capacity of the endosomal vesicles to retain co-endocytosed dextran of small size (Mr, 3,000). Dextrans were retained in the endocytic vesicles, without detectable cytosolic or nuclear stain (Fig. 5A and B). The lack of a detectable dextran leakage would suggest that endosomal escape occurs without membrane permeabilization/disruption through a yet unidentified mechanism or it occurs from only few vesicles resulting in an undetectable leakage.

B19V nuclear entry is highly inefficient. Parvoviruses deliver their genome into the nucleus for replication. In general, this step has been found to be inefficient in all parvoviruses analyzed (25). We have previously showed that incoming MVM accumulates and persists in the lysosomal compartment without noticeable cytosolic capsids and that incoming nuclear viral DNA or proteins remain undetectable even after the onset of viral DNA replication and RNA transcription in the nucleus (35). To examine the presence of incoming B19V DNA in the
nucleus, total DNA was extracted from purified nuclei at increasing post-internalization times in untreated as well as in BafA1-treated cells. A sample taken 10 min post-internalization was used to define the background signal. As shown in figure 6A, no increase over the background was observed in untreated and BafA1-treated cells, even after the onset of viral transcription in the nucleus (Fig. 6B).

The presence of viral particles in the nucleus was examined by confocal microscopy. Viral capsids appeared scattered throughout the cytoplasm, and although some particles were in close contact with the nuclear envelope and nuclear invaginations, they were not observed inside the nucleus (Fig. 6C). Immunoprecipitation of capsids from total cells or from isolated nuclei further confirmed that viral capsids did not enter the nucleus in detectable quantities (Fig. 6D).

**Chloroquine boosts nuclear import.** In previous studies, we have shown that chloroquine (CQ) enhances B19V infection in UT7, HepG2 and primary bone marrow mononuclear cells (8). To understand the mechanism underlying the boosting effect of CQ, UT7/Epo cells were pulse-treated for different times with CQ. As shown in figure 7A, when added at the time of the infection for 1 h, CQ had a modest inhibitory effect on the infection. However, when added 1 h after the infection, CQ was beneficial for the infection, and this effect was more significant with the increasing time in the presence of the drug. This observation would suggest that immediately after internalization, the endosomal acidification is beneficial for the infection and that the effect of CQ can be attributed to a subsequent process.

We next analyzed the efficiency of B19V nuclear entry in the presence of CQ. In sharp contrast to untreated or BafA1-treated cells, CQ boosted nuclear import. The viral DNA accumulated in the nuclei and reached a plateau by 3 h post-infection (Fig. 7B). Comparison of the amount of viral DNA from complete cells and from isolated nuclei at 3 h post-infection
revealed that the viral DNA associated to the nuclei represents a significant fraction of the total intracellular viral DNA (Fig. 7C). Although more than half of the incoming viral DNA was imported into the nuclei, viral capsids were not detectable inside the nucleus (Fig. 7D). Immunoprecipitation of viral capsids from total cells or from isolated nuclei confirmed that viral capsids remained extranuclear (Fig. 7E and F). These results would suggest that the viral DNA is imported into the nucleus from capsids that are immediately uncoated or that uncoating takes place prior to import of the DNA into the nucleus.

**Chloroquine modifies the endocytic trafficking of B19V.** The increase of virus nuclear import by CQ could be explained through the particular effects of CQ on endosomal vesicles. Although CQ, BafA1 and NH4Cl have similar effects on the endosomal pH, CQ has the ability to destabilize endocytic membranes leading to vesicle swelling. The intense lysosomal dysfunction caused by CQ results in a deficient transfer of cargo to the degradative lysosomes, promoting escape from a pre-lysosomal vesicle and avoiding the degradation in lysosomes (28, 37). This ability has been routinely used to improve the efficiency in transfection experiments (32). Using confocal microscopy, we confirmed that in CQ-treated cells but not in untreated or BafA1-treated cells, lysosomes appeared severely enlarged (Fig. 8A). The extensive vacuolization of endocytic vesicles did not induce a detectable leakage of endocytosed dextrans (Fig. 8B). Confocal microscopy pictures taken 5 h p.i. confirmed the swelling of the endocytic vesicles, the accumulation of intact capsids in late endosomes, but not in lysosomes, and the lack of viral DNA degradation (Fig. 8C and D). Although the viral DNA was not degraded, the amount of intact capsids decreased significantly during the entry process (Fig. 8E). Therefore, this result cannot be attributed to degradation as is the case for untreated cells, but the more likely cause is the uncoating of the incoming virus.
Lipid rafts are small, heterogeneous, cholesterol and glycosphingolipids-enriched domains that play important roles in cellular processes such as membrane signaling and trafficking (31). They are also involved in multiple stages of the virus life cycle, such as attachment, internalization, uncoating, protein transport, assembly and budding (53, 55). Several pieces of evidence indicate that B19V exploits lipid rafts during the process of entry; (i) colocalization of B19V and its receptor globoside (Gb4Cer) with the lipid raft marker GM1 (Fig. 1A), (ii) shift of flotillin migration toward non-raft fractions in infected cells (Fig. 1B), and (iii) inhibition of B19V infection following lipid raft disruption (Fig. 1D). The fact that lipid raft disruption had no effect a few hours after infection indicates that plasma membrane rafts are important for the infectious entry/trafficking of B19V and not for later steps. The exact mechanism regarding how these membrane microdomains contribute to virus entry is not known. Preliminary results indicate that lipid raft disruption does not inhibit virus attachment (data not shown). Gb4Cer is required but is not sufficient for B19V infection. Other receptors have been shown to be important in the process of viral entry (12, 40, 58, 59). Thus, lipid rafts may act as platforms for the concentration of virus receptors/coreceptors required for B19V infection, as has been shown for other viruses (55).

Clathrin-dependent endocytosis is the default uptake mechanism for paroviruses (3, 5, 20, 41, 54). To date, only AAV-5 and porcine parvovirus use additional routes based on caveolae and macropinocytosis, respectively (2, 5). As caveolae are integral parts of some lipid rafts and B19V associates with rafts, a possibility of caveolae-mediated internalization of B19V into UT7/Epo could be envisioned. Although Gb4Cer was associated with both caveolar and non-caveolar rafts, B19V associated preferentially to non-caveolar rafts (Fig. 1C). By means of
electron microscopy, B19V was recurrently observed in clathrin-coated invaginations and
vesicles (Fig. 2A); however, viruses in flask-shape invaginations characteristic of caveolae were
not observed. While at the attachment step B19V did not colocalize with transferrin, which is
internalized through clathrin-mediated endocytosis (26), extensive colocalization was observed
during the internalization step (Fig. 2B). In contrast to the slow internalization by caveolae,
cargo internalized by clathrin-mediated endocytosis is quickly delivered to early endosomes (39).
Consistent with a rapid internalization mechanism, immunofluorescence pictures taken 5 min p.i.
confirmed the presence B19V capsids in early endosomes (Fig. 3A). Therefore, although B19V
interacts with plasma membrane rafts, internalization occurs by clathrin-mediated endocytosis
and do not involve caveolae. This mechanism of internalization based on lipid raft and clathrin
have been observed in other viruses (19).

We have found that BafA1 and NH₄Cl inhibited viral infection by blocking viral escape,
resulting in the accumulation of viral particles in the degradative lysosomes. However, CQ,
which also alkalinizes the endosomes, enhances the infection. We have previously shown that
CQ enhances B19V infection in UT7, HepG2 and primary bone marrow mononuclear cells (8).
The case of HepG2 cells is particularly striking. These cells are considered non-permissive for
B19V infection (10); however, in the presence of CQ, HepG2 cells support B19V infection (8).
The reason for this enhancement can be explained, at least in part, by the particular effects of CQ
on endosomal vesicles. CQ causes vesicle swelling and hampers fusion of endosomes and
lysosomes, preventing the transfer of endocytosed material to the degradative lysosomal
compartment (28, 37). Because of these particular properties, CQ is frequently used in
transfection experiments to increase transduction efficiency (32). We confirmed that CQ, but not
BafA1, induced vacuolization of endocytic vesicles (Fig. 8A) and prevented the transfer of the
incoming capsids to the degradative lysosomes (Fig. 8C). We could also confirm that CQ, but not BafA1, prevented the degradation of incoming B19V DNA (Fig. 8D). The capsids retained in vacuolated prelyosomal vesicles would profit to progressively escape into the cytosol.

Although the effect of CQ on endosomal vesicles is independent of the cell type, only B19V and no other parvovirus can benefit (4, 20, 44, 50). All paroviruses studied to date exploit the endosomal acidification for capsid modifications required for subsequent steps, primarily the externalization of the N-VP1 and its constitutive PLA₂ domain, which is thought to be required for endosomal escape (35, 50, 56). In addition, nuclear localization signal (NLS) sequences have been identified in the N-VP1 from some paroviruses, which might assist in the transport of capsids toward the nucleus (57). We have recently shown that B19V is unique among paroviruses in that N-VP1 becomes already externalized upon receptor binding (7, 9).

Therefore, in contrast to other paroviruses, B19V would not depend on the low endosomal pH for this critical conformational rearrangement. However, B19V would require acidification to facilitate the process of endosomal escape, for example, by promoting interactions between the PLA₂ domain of N-VP1 and the endocytic membranes.

At increasing post-internalization times, incoming capsids appeared dispersed throughout the cytoplasm and did not colocalize with endocytic markers, ER, caveolin-containing vesicles, Golgi or recycling vesicles, suggesting that they have escaped into the cytosol. The PLA₂ activity of N-VP1 is thought to play a role in endosomal escape, however, the mechanism is not known. Adenovirus is able to release coendocytosed dextran of different sizes, implying a mechanism of escape based on endosomal disruption. Human rhinovirus 2 is able to release only small dextrans, suggesting a less invasive mechanism of escape based on pore formation (43). The escape mechanism of CPV has been studied by cointernalization of alpha-sarcin, where no
disruption of endosomes was found (41). In other studies, however, CPV was able to release rhodamine-labeled dextrans of 3 kDa but not of 10 kDa (52). However, the effect was only evident 20 h post-infection, while endosomal escape of CPV occurs earlier. In addition, a parvovirus capsid would not be able to escape through a pore that selectively allows the escape of dextrans of 3 kDa but not of 10 kDa. In the case of B19V, there was no detectable leakage of dextrans of 3 kDa at any time or in the presence of CQ (Fig. 5 and 8B). Two possibilities can be envisaged, either capsids permeabilize only a minor amount of endocytic vesicles, or capsids escape through a yet unidentified mechanism, which do not involve membrane damage.

Viral capsids (Fig. 6C and D) or viral DNA (Fig. 6A) were not detected inside the nucleus, even after the onset of viral transcription (Fig. 6B). Thus, B19V is inefficiently imported into the nucleus, which makes it difficult to study this step of the infection. In clear contrast, in CQ-treated cells, B19V DNA was efficiently imported into the nucleus, reaching a plateau by 3 h post-infection (Fig. 7B). At this time, more than half of the total viral DNA was found associated to the nuclear fraction (Fig. 7C). Examination of the incoming nuclear DNA confirmed that the viral DNA was not associated to capsids, which remained extranuclear (Fig. 7D, E and F). In CQ-treated cells, the amount of intact capsids decreased significantly (Fig. 8E). As the viral DNA remained stable (Fig. 8D) and a large proportion was found in the nucleus (7B and C), the decrease in the amount of intact capsids cannot be attributed to degradation, as was the case for untreated cells. These results would suggest that the viral DNA is imported into the nucleus from intact capsids that are immediately uncoated or that uncoating takes place prior to import of the DNA into the nucleus. This second possibility seems more plausible, as capsids were not found in the nucleus at any time point.
Our studies provide the first insight into the early steps of the infection of B19V and reveal mechanisms involved in virus uptake, endocytic trafficking and nuclear import. This study also outlines novel questions that warrant further investigation, such as the precise role of lipid rafts in the process of virus entry, the mechanism by which B19V escapes from endosomes without detectable permeabilization/damage or the pathway involved in the nuclear import of the viral DNA.

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Figure legends

**Figure 1. Lipid rafts and B19V infection.** (A) Colocalization of globoside (Gb4Cer) and B19V with the lipid raft marker GM1. UT7/Epo cells were incubated with FITC-labeled cholera toxin and infected with B19V at 4°C to allow virus binding. Cells were fixed and stained with an antibody against Gb4Cer or against capsids. (B) B19V causes a shift of flotillin to high density fractions. UT7/Epo cells were infected with B19V at 4°C for 1 h. Following intensive washings to remove unbound viruses, the cells were lysed, and lipid rafts were prepared as specified in the Materials and methods. An aliquot from each fraction was analyzed by SDS-PAGE followed by Western blot for the detection of flotillin-1. (C) Colocalization of Gb4Cer and B19V with Cav-1. Cell showing extensive (i) or poor (ii) colocalization of Gb4Cer with Cav-1. (iii) Representative cell showing a modest colocalization of B19V with Cav-1. (D) Effect of nystatin (15-40 µM) and filipin (0.5-2 µM) in B19V infection. UT7/Epo cells were pre-incubated with the drugs for 30 min at 37°C before infection. As a control, the drugs were added 3-4 h post-infection. Data are the mean ±SD of duplicate samples from three independent experiments.

**Figure 2. Clathrin-dependent B19V uptake.** (A) Electron micrographs showing internalization of B19V in UT7/Epo cells by clathrin-mediated endocytosis. Scale bar; 100 nm. a) Early coated pit, b) coated pit, c) newly formed coated vesicle, d) coated vesicle in cytoplasm. (B) Colocalization of B19V with transferrin at time 0 min (binding) and 10 min post-internalization. UT7/Epo cells were incubated with FITC-labeled human holo transferrin and infected with B19V at 4°C to allow virus binding. Following washes to remove unbound virus, the cells were incubated at 37°C to initiate virus internalization.
**Figure 3. Endocytic trafficking of B19V.** (A) Confocal immunofluorescence images showing colocalization of B19V capsids with markers of the endocytic pathway. EE; early endosomes (EEA1), LE; late endosomes (Mannose 6-phosphate receptor), Lys; lysosomes (Lamp1). (B) Kinetics of the colocalization of B19V capsids with LE and Lys. Quantification analysis was performed by the BioImage XD software with the Pearson’s correlation coefficient. The values represent the mean ±SD of two measurements from an average of 40 cells.

**Fig. 4. Low pH-dependent entry of B19V.** (A) Effect of bafilomycin A1 (BafA1) or ammonium chloride (NH₄Cl) in B19V infection. UT7/Epo cells were pulse-treated with BafA1 (20 nM) or NH₄Cl (25 mM) for the indicated periods of time. (B) Effect of BafA1 treatment in B19V endosomal trafficking. Infected cells were washed and fixed 5 h post-infection in the absence or presence of BafA1 (20 nM). Cells were stained with mAb 860-55D against intact capsids and antibodies against late endosomes (M6PR) and lysosomes (Lamp1). (C) Analysis of B19V DNA degradation at increasing times post-internalization in untreated cells or in the presence of BafA1 (100 nM). The drug was added 30 min before the infection and removed 3 h post-infection. (D) Analysis of B19V release into the extracellular milieu from 1 to 5 h post-infection. Data shown are the mean ±SD of duplicate samples from three independent experiments.

**Fig. 5. Endosomal escape occurs without apparent membrane damage.** (A) Effect of incoming virus on endosomal membrane integrity. UT7/Epo cells were incubated in the presence of rhodamine-conjugated lysine-fixable dextran (Mr, 3000; 3 mg/ml) and B19V for 2 h. The cells were subsequently washed to remove unbound virus and extracellular dextran and further incubated at 37°C. At the indicated times, the cells were fixed with 4% paraformaldehyde. Uninfected cells were used as controls. (B) Cells from the same experiment were fixed with
methanol/acetone 7 h post-infection and stained with mAb 860-55D. A representative cell is shown.

**Fig. 6. Analysis of nuclear entry of B19V DNA and capsids.** (A) Nuclei from infected cells (untreated or BafA1-treated) were isolated at increasing times post-infection. B19V DNA was extracted and quantified from the nuclear samples. Samples taken 10 minutes post-infection served as background controls. Data shown are the mean ±SD of duplicate samples from two independent experiments. (B) Kinetics of NS1 mRNA synthesis in infected cells. At increasing post-infection times, total mRNA was isolated and NS1 mRNA quantified. Samples taken 10 minutes post-infection served as background controls. Data are the mean ±SD of two independent experiments. (C) Confocal images of cells 3 h post-infection. B19V capsids were stained with mAb 860-55D (green), and nuclei were counterstained with DAPI (blue). Rendering in 1D made from the Z stack and orthogonal sections are shown. (D) Immunoprecipitation of capsids with mAb 860-55D from total cells (0 h and 3 h post-infection) and isolated nuclei (3 h post-infection). A double amount of cells and nuclei were used for the 3 h post-infection.

**Fig. 7. Chloroquine boosts B19V nuclear import and the infection.** (A) Effect of chloroquine (CQ) on B19V infection. UT7/Epo cells were pulse-treated with CQ (25 μM) for the indicated periods of time. (B) Nuclei from CQ-treated cells (25 μM) were isolated at increasing post-infection times. B19V DNA was extracted and quantified from the nuclear samples. Samples taken 10 minutes post-infection served as background controls. (C) Quantification of total incoming B19V DNA from the whole cell and from isolated nuclei at 3 h post-infection. The amount of cells and nuclei was normalized by quantification of the human β-actin gene. (D) Confocal images of cells 3 h post-infection. B19V capsids were stained with mAb 860-55D (green) and nuclei were counterstained with DAPI (blue). Rendering in 1D made from the Z stack.
stack and orthogonal sections are shown. (E) Immunoprecipitation of capsids with mAb 860-55D from total cells (0 and 3 h post-infection) and isolated nuclei (3 h post-infection). Twice the amount of cells and nuclei were used for the 3 h post-infection. (F) Quantification of the total amount of B19V DNA from the nuclear fraction (grey bars) or following immunoprecipitation with mAb 860-55D (dark bars) at 3 h post-infection. As a control, isolated nuclei from uninfected cells were spiked with B19V. Data shown are the mean ± SD of duplicate or triplicate samples from two independent experiments.

Fig. 8. Chloroquine prevents transfer of B19V to lysosomes and degradation of the incoming viral DNA. (A) CQ induces lysosomal vacuolization. UT7/Epo cells were treated with BafA1 (20 nM), CQ (25 μM) or left untreated. After 3 h, cells were fixed and stained with an antibody against lysosomes (Lamp1). Nuclei were stained with DAPI. (B) CQ does not permeabilize endocytic vesicles. UT7/Epo cells were treated with BafA1 (20 nM), CQ (25 μM) or left untreated. Cells were incubated in the presence of rhodamine-conjugated lysine-fixable dextran (Mr, 3000; 3 mg/ml) for 2 h. The cells were subsequently washed to remove extracellular dextran and further incubated at 37°C. Cells were fixed after 2 h with 4% paraformaldehyde. (C) CQ prevents transfer of B19V to lysosomes. CQ-treated cells (25 μM) were fixed 5 h post-infection. Cells were stained with mAb 860-55D against capsids and antibodies against late endosomes (LE; Mannose 6-phosphate receptor) and lysosomes (Lys; Lamp1). (D) CQ was added 30 min before the infection and removed 3 h post-infection. B19V DNA was quantified at increasing post-infection times. Data are the mean ± SD of duplicate samples from two independent experiments. (E) CQ-treated cells were fixed 1 and 5 h post-infection and stained with antibody 860-55D against intact capsids.
Fig. 1

A Time= 0 min

Gb4Cer   GM1   Merge

B19V

Gb4Cer GM1 Merge

B raft fractions

Mock

B19V

% Optiprep™

C Time= 0 min

Gb4Cer Cav-1

Gb4Cer Cav-1

B19V Cav-1

D

Nystatin (μM)

Fillipin (μM)

NS1 mRNA
Fig. 2

A

Time = 5 - 30 min

(b)

(c)

(d)

A

B

Time = 0 min

Time = 10 min

B19V

Trf

Trf

B19V
Fig. 3

A

Time = 5 min
EEA1  
M6PR  
Lamp1 

Time = 30 min
EEA1  
M6PR  
Lamp1 

Time = 3h
EEA1  
M6PR  
Lamp1

B

Colocalization (% capsids) vs. Post-infection time (min)

Colocalization (% capsids)

0  10  20  30  40  50

0  50  100  150  200  250  300

Post-infection time (min)

M6PR

Lamp1
Fig. 4

A

BafA1

NH4Cl

NS1 mRNA (% of untreated)

B

Time= 5 h

Untreated

B19V

M6PR

Merge

B19V

Lamp1

Merge

B19V

M6PR

Merge

BafA1

B19V

Lamp1

Merge

C

B19V DNA (x10³)

Time post-internalization (h)

D

Virus released (%)

Time post-infection

BafA1

removed

Untreated

BafA1

0 
20 
40 
60 
80 
100 
input 
0-1h 
0-2h 
0-3h 
0-4h 
1-2h 
1-3h 
1-4h 
NS1 mRNA (% of untreated) ... 
C 
0 
40 
80 
120 
1h 2h 3h 
Virus released (%) 
Time post-infection 
D 
BafA1 
removed 
Untreated 
BafA1
Fig. 5

A

Dextran (3 kDa)

Mock B19V

Time= 2h

Time= 7h

Time= 20h

B

Time= 7h

Mock B19V

Dextran

B19V

Dextran
Fig. 7

A. NS1 mRNA expression over time post-internalization.

B. Nuclear import of B19V DNA over time post-internalization.

C. Percentage of total and nuclear viral DNA.

D. 3D rendering from Z-stack showing orthogonal sections.

E. Western blot analysis of VP1 and VP2 expression with Chloroquine treatment.

F. Genome copies comparison between total virions and virions from IP capsids.
**Fig. 8**

A. Untreated, Baf A1, CQ

B. Dextran (3 kDa), Untreated, Baf A1, CQ

C. B19V, M6PR, Merge, Chloroquine

D. B19V DNA (x10³) vs. Time post-internalization (h)

E. Chloroquine removed: Time = 1h, Time = 5h