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研究報告の概要

ヒトパルボウイルス B19（B19V）は血液試料に多く認められるヒト病原体であり、主に呼吸器経路を介して伝播される。B19V は他のパルボウイルスよりも物理化学的な処理に対する感受性が高いが、その理由は未だ明らかではない。
血漿プールは数百の血液で構成されるため、PCR 法で検査すると大半の血漿プールから B19V DNA が検出され、第 VIII および第 IX 凝固因子、ヒト血清アルブミン、静注用免疫グロブリン、筋注用免疫グロブリン、プロトロンビン複合体濃縮製剤、アンチトロンビン III などで報告があるが、いずれも B19V DNA を検出したことを証明しているのであって、必ずしも感染性のあるウイルスの存在を証明しているわけではない。しかし、血漿分画製剤による伝播の報告もある。
パルボウイルスは最も安定なウイルス群に属し、物理化学的な処理の多くに抵抗性であるが、B19V は乾熱または湿熱のほか、低 pH または高 pH、UVC 照射、光化学反応により不活化できる。
本報告では B19V 不活化能について「60℃・10 分」および「pH4・2 時間」の 2 条件で評価したところ、ウイルスの感染性は低下し検出限界未満となり、既発表データと一致していた。
熱または低 pH による B19V 不活化機序は、DNA を包むカプシドの分解ではなく、カプシドからの DNA の遊離によるものであることがわかったが、熱安定化剤としてクエン酸を用いると B19V DNA はカプシドから遊離せず、感染性が維持される。

報告企業の意見	今後の対応
ヒトパルボウイルス B19 の不活化について「60℃・10 分」および「pH4・2 時間」の 2 条件で評価したところ、感染性は検出限界未満となり、既発表データと一致していたとの報告で、他のパルボウイルスに比べ不活化されやすいとも述べられている。 なお、弊社血漿分画製剤は最終製品において核酸増幅検査によりヒトパルボウイルス B19 DNA が陰性であることを確認している。	今後ともヒトパルボウイルス B19 に関する血漿分画製剤の安全性に関する情報に留意していく。

使用上の注意記載状況・その他参考事項等

慎重投与の項
・溶血性・失血性貧血の患者［ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。］
・免疫不全患者・免疫抑制状態の患者［ヒトパルボウイルス B19 の感染を起こす可能性を否定できない。感染した場合には、持続性の貧血を起こすことがある。］

重要な基本的注意の項
(1) 本剤の原材料となる‥［スクリーニング項目、不活化・除去工程］‥投与に際しては、次の点に十分注意すること。
1) 血漿分画製剤の現在の製造工程では、ヒトパルボウイルス B19 等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。

妊婦、産婦、授乳婦等への投与の項
妊婦又は妊娠している可能性のある婦人には治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。［妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルス B19 の感染の可能性を否定できない。感染した場合には胎児への障害（流産、胎児水腫、胎児死亡）が起こる可能性がある。］

Molecular mechanism underlying B19 virus inactivation and comparison to other parvoviruses

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BACKGROUND: B19 virus (B19V) is a human pathogen frequently present in blood specimens. Transmission of the virus occurs mainly via the respiratory route, but it has also been shown to occur through the administration of contaminated plasma-derived products. *Parvoviridae* are highly resistant to physicochemical treatments; however, B19V is more vulnerable than the rest of parvoviruses. The molecular mechanism governing the inactivation of B19V and the reason for its higher vulnerability remain unknown.

STUDY DESIGN AND METHODS: After inactivation of B19V by wet heat and low pH, the integrity of the viral capsid was examined by immunoprecipitation with two monoclonal antibodies directed to the N-terminal of VP1 and to a conformational epitope in VP2. The accessibility of the viral DNA was quantitatively analyzed by a hybridization-extension assay and by nuclease treatment.

RESULTS: The integrity of the viral particles was maintained during the inactivation procedure; however, the capsids became totally depleted of viral DNA. The DNA-depleted capsids, although not infectious, were able to attach to target cells. Comparison studies with other members of the *Parvoviridae* family revealed a remarkable instability of B19V DNA in its encapsidated state.

CONCLUSION: Inactivation of B19V by heat or low pH is not mediated by capsid disintegration but by the conversion of the infectious virions into DNA-depleted capsids. The high instability of the viral DNA in its encapsidated state is an exclusive feature of B19V, which explains its lower resistance to inactivation treatments.

B19 virus (B19V) is the only well documented human pathogen of the *Parvoviridae* family. The virus belongs to the genus *Erythrovirus*. In most cases, the infection is either asymptomatic or accompanied by mild nonspecific symptoms. The most common syndrome caused by B19V is an erythematous rash illness named erythema infectiosum affecting children. B19V is also the causative agent for transient aplastic crisis, which may have severe effects on patients suffering from sickle cell disease and other anemic illnesses. Chronic infections accompanied by pure red cell aplasia and anemia affect immunocompromised patients. Furthermore, B19V may cause fetal death, autoimmune diseases, and arthropathies.[1]

B19V is a widespread pathogen. The serologic evidence of a past infection is 40 to 60 percent for young adults and 80 to 100 percent for elder people.[1,2] Owing to its high prevalence, blood donations are frequently contaminated with B19V. The measured incidence of contamination depends on the sensitivity of the detection method and ranges from 0.003 percent (immunodiffusion) to 1.2 percent (polymerase chain reaction [PCR]) of blood donations examined.[3-7] Because plasma pools are constituted of hundreds of donations, B19V DNA is found in the majority of plasma pools as determined by PCR.[8-10] The contamination of plasma-derived products, such as coagulation factors VIII and IX, human serum albumin, intravenous immune globulin, intramuscularly injected

ABBREVIATIONS: B19V = B19 virus; MVM = minute virus of mice; PBSA = phosphate-buffered saline containing 1 percent bovine serum albumin; PLA_2 = phospholipase A2.

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immune globulin, prothrombin complex concentrate and antithrombin III has been reported.[8,9,11,12] Therefore, there is a risk of transmitting B19V through the administration of plasma-derived products. In these studies however, the contamination was demonstrated with the presence of B19V DNA with PCR, which does not necessarily prove the presence of infectious virus. Nevertheless, direct evidence of B19V transmission through the administration of plasma-derived products has also been shown in several case studies.[13-16] Moreover, patients that receive such medication on a regular basis show a higher prevalence of B19V-specific antibodies than control groups.[17] Altogether, the contamination of plasma-derived products indicates a potential risk of a B19V infection for the treated patient with potentially severe consequences for pregnant women and anemic and immunocompromised patients.

To achieve maximal safety for plasma-derived clinical products, pathogen safety guidelines have been established, as a result of which manufacturers must demonstrate the effective elimination of viral agents during the manufacturing process of their products. Virus elimination is demonstrated either with the relevant pathogen itself or with one or several closely related model viruses. To date, there is no convenient cell culture infectivity test for B19V. For this reason, animal parvoviruses such as bovine parvovirus, canine parvovirus, porcine parvovirus, or minute virus of mice (MVM) are often used for validation studies regarding the inactivation of B19V. Parvoviruses are among the most stable viruses and have been shown to resist many common physicochemical inactivation procedures. B19V inactivation can be achieved with dry or wet heat,[18-21] as well as with low or high pH,[22,23] UVC irradiation,[24,25] or photochemical reactions.[26] Interestingly, B19V has been found to be more readily inactivated than other parvoviruses. Whereas B19V is inactivated beyond the detection limit after 10 minutes at 60°C or after 2 hours at pH 4, canine parvovirus,[21] MVM,[27] and porcine parvovirus[18] can withstand 1 hour at 60°C without considerable inactivation. Similarly, the treatment of MVM at pH 4 for 6 hours only moderately reduces its infectivity.[23] The reason why B19V is more sensitive to inactivation than other parvoviruses is not known. Although different inactivation conditions for B19V have been described, the underlying mechanism of B19V inactivation has not yet been elucidated. It is generally assumed that the inactivation occurs through capsid disintegration because the viral genome becomes accessible to DNases.[18,27] We have shown in a recent study, however, that after mild heat treatments, the DNA from B19V and MVM can be rendered accessible without capsid disintegration.[28]

In this study we have analyzed the B19V capsid rearrangements occurring during the inactivation process. The results revealed a sequence of structural transitions preceding capsid disintegration. The critical transition, which resulted in full virus inactivation, was the dissociation of the viral DNA from the still intact capsid. Comparison studies revealed that the DNA release from intact capsids is a common feature among parvoviruses but occurs much more prematurely in B19V, explaining its lower resistance to inactivation procedures.

MATERIALS AND METHODS

Cells and viruses

Human UT7/EPO cells were propagated in RPMI 1640 supplemented with 5 percent fetal calf serum (FCS) and 2 U per mL recombinant human erythropoietin (EPO; Janssen-Cilag, Midrand, South Africa) at 37°C and 5 percent CO_2. UT7 cells were provided by A. Gröner (CSL Behring, Marburg, Germany). Two B19V-containing plasma samples (Genotype 1) were obtained from two infected individuals (S-1 and S-2) and did not contain B19V-specific immunoglobulin M or immunoglobulin G (IgG) antibodies. B19V was concentrated from infected serum by ultracentrifugation through 20 percent sucrose. The viral pellet was washed and resuspended in phosphate-buffered saline (PBS). All other parvoviruses were derived from cell culture supernatant. H-1 parvovirus was provided by C. Dinsart (German Cancer Research Center, Heidelberg, Germany). Porcine parvovirus was provided by T. Novak (CSL Behring, Marburg, Germany).

Exposure of viral particles to inactivation conditions

Viral suspensions in PBS were heat-treated in thin-wall tubes for 3 or 10 minutes in a preheated thermoblock. A probe was used to monitor the temperature of the suspension. After the temperature treatment, the samples were rapidly cooled on ice and immediately used for subsequent reactions. For pH treatments, the viral suspensions were acidified by adding MES-buffered saline until the desired pH was achieved and incubated for 2 hours at 37°C. After the treatment, the pH of the viral suspension was neutralized by dilution (1:100) into PBS or in PBS containing 1 percent BSA (PBSA). Additionally, the heat sensitivity of B19V in citrate buffer, which has been recently reported to confer heat resistance to B19V,[28] was examined. The viral suspension was diluted in citrate buffer (0.5 mol/L trisodium citrate, 0.1 mol/L NaCl, pH 7) or in PBS and exposed to heat as specified above.

Infectivity assay

Titration of B19V was performed by limited dilution in quadruplicate. UT7 cells were seeded on 96-well plates (3×10^4 per well) in RPMI, containing 2 U per mL recombinant human EPO and 5 percent FCS. Virus was diluted geometrically by the factor 10 in RPMI. An equal volume of

diluted virus was added to each well and incubated at 37°C in 5 percent CO_2. After 4 days, the cell culture volume was carefully removed and cells were fixed with a solution of ice-cold methanol:acetone (1:1, v/v) for 1 hour at 20°C. After fixation, the cells were air-dried, washed with PBSA, and incubated with a mouse antibody against B19V (1:40 diluted in PBSA, clone R92F6 IgG_1, Novocastra, Newcastle upon Tyne, UK) for 1 hour at room temperature. The cells were washed with PBSA, and as secondary antibody, a conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulins was added (1:50 dilution, DakoCytomation, Glostrup, Denmark) for 1 hour at room temperature. After final washings with PBSA, the cells were overlaid with 50 µL of glycerin:PBS solution (1:1) and examined under fluorescence microscope. The infectivity titer was calculated with the Spaerman-Kärber method.[30]

Assessment of B19V capsid integrity

After exposure to heat or low pH, the integrity of the viral capsid was examined by immunoprecipitation with two different antibodies. One antibody is directed to a VP2 conformational epitope (monoclonal antibody [MoAb] 860-55D), which exclusively recognizes capsids and not denatured proteins. Another antibody recognizes an epitope in the N-terminal of VP1 (MoAb 1418).[31] The immunoprecipitation was performed overnight at 4°C in the presence of 20 µL of protein G PLUS-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.5 to 1 µg antibody in a total volume of 120 µL PBSA. The supernatant was carefully removed, and the beads were washed three times with PBSA. Immunoprecipitated viral capsids were resolved by sodium dodecyl sulfate (SDS)-10 percent polyacrylamide gel electrophoresis (PAGE). After the transfer to a polyvinylidene fluoride membrane, the blot was probed with a mouse anti-B19 VPs (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).

Assessment of B19V DNA accessibility

Subsequent to the temperature or pH treatments, the presence of externalized viral DNA was examined by a hybridization-extension assay as previously described.[28] Briefly, a probe consisting of a virus-specific 3'-end and a virus-unrelated 5'-end was hybridized to the target viral DNA and subsequently extended with sequenase (3.25 U, USB, Cleveland, OH). The extended probe was purified with a PCR purification kit (QIAquick, Qiagen, Valencia, CA) and quantified by real-time PCR. Alternatively, the presence of externalized viral DNA was examined by the treatment of the viral suspensions with DNase I (10 U, Amersham Biosciences, Piscataway, NJ) overnight at room temperature in PBS containing 6 mmol per L $MgCl_2$. The viral DNA was purified and quantified as specified below.

Quantitative PCR

The viral DNA was quantified with a real-time PCR system (LightCycler, Roche Diagnostics, Rotkreuz, Switzerland). PCR was carried out with the FastStart DNA SYBR Green kit (Roche Diagnostics) following the manufacturer's instructions. For the detection and quantification of probe-extended DNA generated from the hybridization-extension reaction, a forward primer specific for the 5' virus-unrelated tail of the probe and a downstream virus-specific reverse primer were used. All probes and primers used are shown in Tables 1 through 3.

Assessment of the viral DNA-capsid association

To verify whether the exposed viral DNA is still associated to the capsid or otherwise dissociated, the B19V capsids were immunoprecipitated with MoAb 860-55D as indicated above. The amount of viral capsid protein and viral DNA present in the immunoprecipitated and supernatant fractions was analyzed by SDS-PAGE and quantitative PCR, respectively.

FACS analysis

The presence of B19V on the cell surface was quantitatively analyzed by flow cytometry. UT7/EPO cells were infected with either intact or heat-inactivated B19V (100 copies/cell) under conditions allowing the binding but not the internalization of the virus (4°C). The cells were washed three times and incubated with an anti-B19V capsid MoAb (5 µg/mL, 8293, Chemicon International, Temecula, CA) at 4°C for 1 hour in PBS containing 2 percent FCS, followed by an incubation with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG (5 µg/mL, A85-1, BD Biosciences, San Jose, CA) at 4°C for 1 hour. The cells were analyzed by flow cytometry with a flow cytometer (FACScan, Becton Dickinson, San Jose,

TABLE 1. Probes used for the hybridization-extension assay

Virus	5' virus-unrelated sequence	3' virus-specific sequence
B19V	CGATCCGACTCACACCTGGACC.....	.....CCGCCTTATGCAAATG
BPV	GGGCGAAGAACGGTGGATTAA.....	.....CGAGGACAGGTGGACC
CPV	GGGCGAAGAACGGTGGATTAA.....	.....GCGGTTTGTGTGTTTA
H-1	CCACAGAGGTCCAAGCACGCA.....	.....AGCGGTTCAGAGAGTT
MVM	GGGGATGCGGGGAGTGTACGGGC.....	.....GATAAGCGGTTCAGGG
PPV	AGGCGGTTCATGGGTGGATAG.....	.....GTTGCTTACTTCAGTT

TABLE 2. Primers used for PCR after the hybridization-extension assay

Virus	Forward primer	Reverse primer
B19V	CGATCCGACTCACACCTGGACC	CCCCGGTAAGGTCAAGCTTAGAAGC
BPV	GGGCGAAGAACGGTGGATTAA	CCCCCACATAGTTCATAGAAGCCT
CPV	GGGCGAAGAACGGTGGATTAA	TCCATTGCTGTTTGTGCTCCTGTA
H-1	CCACAGAGGTCCAAGCACGCA	CCGCCCCTCGTTGTAGAGACTTC
MVM	GGGGATGCGGGGAGTGTACGGGC	CCAACCATCTGATCCAGTAAACAT
PPV	AGGCGGTTCATGGGTGGATAG	CCGTTTTGTGAGGCTCTCGATT

TABLE 3. Primers used for B19V genome detection

Forward primer	Reverse primer
TGGGGCAGCATGTGTTAAA	CACAGGTACTCCAGGCACAG

TABLE 4. Effect of temperature and low-pH treatments on B19V infectivity

	S-1*	S-2
Stock	4.75†	6
pH 7.4‡	4.85	5.35
pH 4	≤2.48 ≥2.37§	≤2.48 ≥2.87
37°C	4.1	5.1
60°C	≤2.48 ≥1.62	≤2.48 ≥2.62

* S-1 and S-2 are serum samples of two infected individuals.
† Titers are given in log $TCID_{50}$ per mL.
‡ pH and temperature treatments for 2 hours and 10 minutes, respectively.
§ Reduction of infectivity.

CA). Data acquisition and analysis were conducted with software (CellQuest Pro, BD Biosciences). The percentage of cells having B19V on their surface is indicated in the upper right quadrant of each panel.

RESULTS

B19V inactivation by heat and low-pH treatments

Two different conditions, 60°C for 10 minutes and pH 4 for 2 hours, were evaluated for their capacity to inactivate B19V. After these treatments, an immunofluorescence infectivity assay was performed as described above. The applied heat or low-pH treatments resulted in the reduction of the virus infectivity beyond the detection limit (Table 4). These results are consistent with previous data on the inactivation of B19V.[16,21,23]

B19V inactivation by heat or low pH is not caused by capsid disintegration

Subsequent to the inactivation treatments by heat and low pH, the integrity of the viral capsid was examined. Viruses were immunoprecipitated with MoAb 860-55D against a VP2 conformational epitope, which recognizes only capsids.[31] The results showed that the inactivating heat treatments did not cause capsid disassembly (Fig. 1A). The capsid integrity was also examined with an antibody specific to N-VP1. As shown in Fig. 1A, after heat inactivation of B19V, VP2 could be immunoprecipitated with the antibody directed to N-VP1. Capsid disintegration was only observed increasing the incubation times at 60°C (Fig. 1B) or increasing the temperature above 60°C (Fig. 1C). As expected, treatments at 85°C resulted in the complete destruction of the viral capsids.

Similarly to the temperature treatment, inactivation of B19V by low-pH treatment was not caused by capsid disintegration. As shown in Fig. 1D, viral capsids remained assembled after exposure for 2 hours at pH 4. Moreover, exposure to more severe acidic conditions (pH 3) did not cause capsid disintegration.

B19V inactivation by heat or low pH is due to the release of the viral DNA

After the heat and low-pH inactivation treatments, the accessibility of the viral DNA was examined with a hybridization-extension assay, as described above. The results showed that while the viral capsid remained assembled, the viral genome, however, became fully accessible. The amount of accessible viral DNA was similar to that detected after complete disintegration of the viral capsids at 85°C (Figs. 2A, 2B).

To determine whether the DNA that had become accessible by the inactivation treatments was still associated with the virus capsid or otherwise dissociated, viruses were immunoprecipitated with the MoAb against capsids, and the DNA content in the supernatant and immunoprecipitated fractions was determined with quantitative PCR. As expected, in the untreated virus samples, all the viral DNA was immunoprecipitated and only a minor amount of DNA was detectable in the supernatant. Exposure of viruses to the temperature of 60°C or higher, however, resulted in total release of the viral DNA from the capsids (Fig. 2C). The same results were obtained after inactivation at pH 4 for 2 hours (Fig. 2D), indicating that the inactivation mechanism of B19V by heat or low-pH treatments was similarly caused by the conversion of the infectious DNA-containing virions into noninfectious empty capsids.

B19V DNA is not externalized and the infectivity is preserved when using citrate as thermostabilizer

It has been recently reported that in the presence of citrate, B19V becomes resistant to inactivation by pasteurization. Citrate is used as a protein stabilizer in the preparation of some plasma-derived products.[29] The mechanism by which the presence of citrate considerably

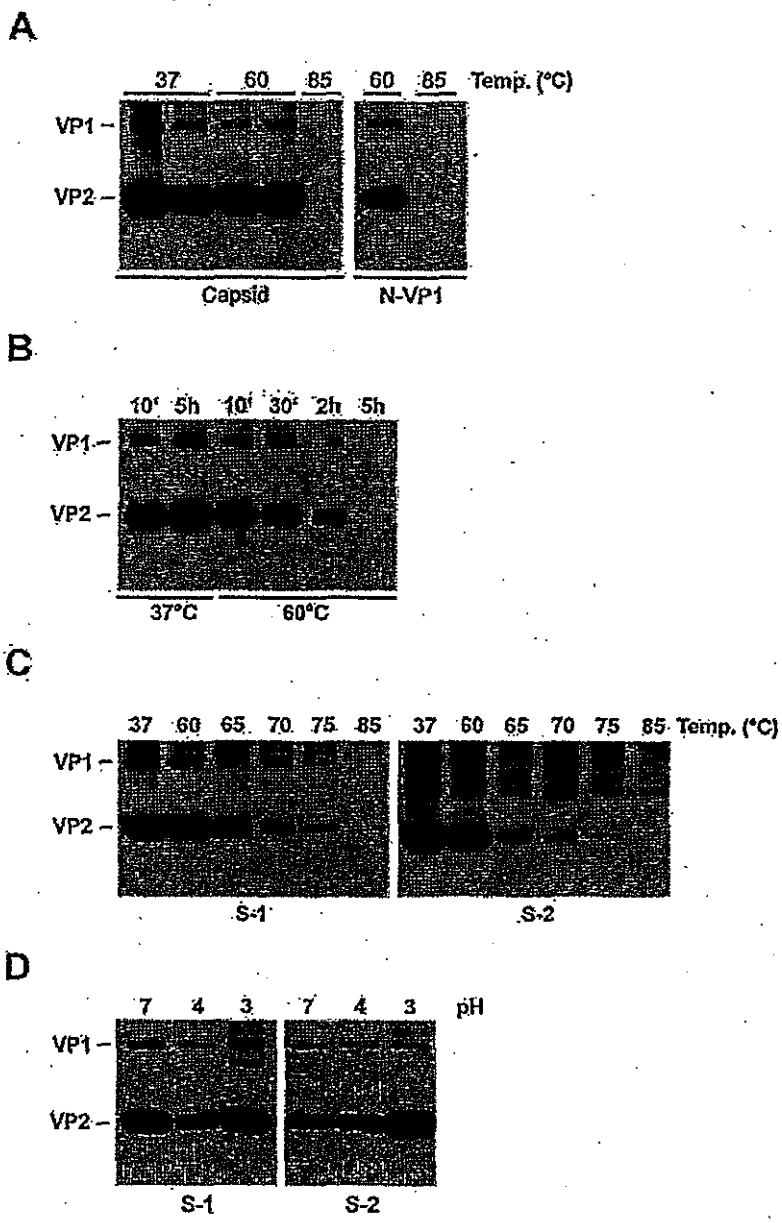

Fig. 1. Effect of inactivation by heat or low pH on B19V capsid integrity. After the exposure of B19V to different conditions, the intact capsids were immunoprecipitated and analyzed by Western blot. The immunoprecipitation was performed with an antibody directed to a VP2 conformational epitope (MoAb 860-55D), except for the right section in A, where an antibody recognizing an epitope in the N-terminal of VP1 (MoAb 1418) was used.[31] The immunoprecipitations were performed after exposure to (A) 60°C for 10 minutes, (B) increasing incubation times at 60°C, (C) increasing incubation temperatures, and (D) after exposure to low pH.

increases the heat resistance of B19V remains unknown. We have examined and compared the heat sensitivity of B19V in PBS and in a buffer containing citrate, as specified under Materials and Methods. The results confirmed that although the virus was fully inactivated in PBS, the presence of citrate conferred heat resistance and the virus could not be inactivated (data not shown). As expected, the viral DNA became fully accessible after the heat treatment of B19V in PBS but was not externalized in the presence of citrate (Fig. 3).

The inactivated DNA-depleted capsids preserve their capacity to bind cells

The capacity of the heat-inactivated B19V particles to bind the target cells was tested. The same amount of inactivated and infectious B19V was added to UT7 cells under conditions that allowed only viral binding and not internalization (4°C). Subsequently, flow cytometry analysis was performed with a B19V capsid proteins antibody as described above. The results revealed that the heat-inactivated and the infectious B19V bound to UT7 cells with a similar efficiency (Fig. 4).

B19V shows a unique DNA externalization pattern among parvoviruses

B19V is more readily inactivated than other parvoviruses. To understand the reason for this difference, the externalization of the B19V DNA was compared to that of other parvoviruses. B19V, bovine parvovirus, canine parvovirus, H1, MVM, and porcine parvovirus were exposed to increasing temperatures for 3 minutes, and the amount of accessible DNA was determined with the hybridization-extension assay. The rate of externalization was remarkably similar among all the examined viruses except for B19V (Fig. 5). At 50°C, approximately 40 percent of the B19V virions externalized their DNA, whereas barely any externalized DNA could be detected in the case of the other par-

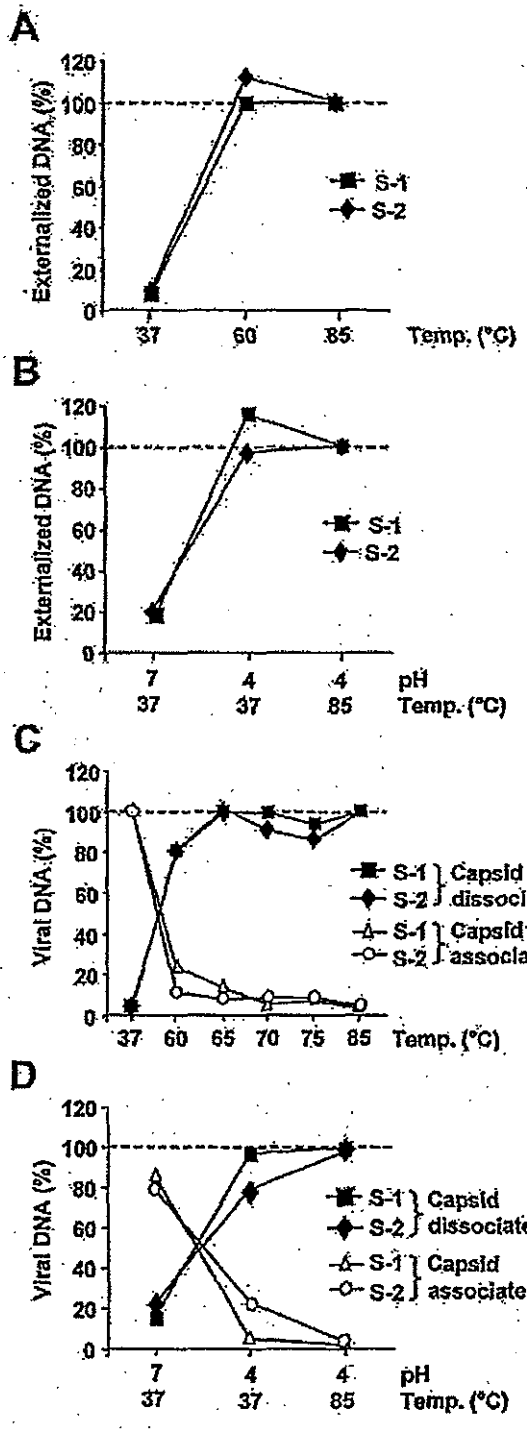

Fig. 2. Effect of inactivation by heat or low pH on B19V DNA accessibility and release. (A, B) Effect of inactivation on B19V DNA accessibility. The externalized DNA (%) refers to the amount detected at 85°C. (C, D) Effect of inactivation on B19V DNA release (dissociation from the capsid). Viral DNA (%) in relation to the input is shown.

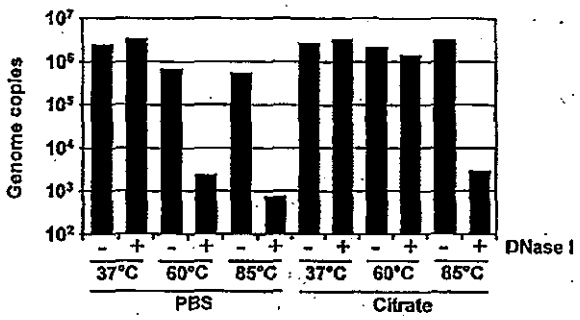

Fig. 3. Sensitivity of B19V DNA to DNase I after heat treatment in PBS or in citrate buffer.

voviruses. Although 60°C treatment leads to the externalization of nearly all the B19V genomes, the externalization in the rest of the tested viruses was at approximately 20 percent and in the range of 40 to 80 percent at 70°C. These results imply that the reason for the faster inactivation of B19V is due to the higher instability of its DNA in the encapsidated state.

DISCUSSION

To date, the lack of an appropriate cell culture to propagate B19V has complicated the experimental work with this virus. In contrast, optimal cell systems are available for many animal parvoviruses. For this reason, they are commonly used in validation studies as models for B19V. For an unknown reason, however, B19V has been shown to be more easily inactivated than the other members of the *Parvoviridae* family.[22,23] Therefore, the animal parvoviruses do not mimic the effect of inactivation procedures on B19V.[32] Although different inactivation conditions for B19V have been described, the underlying mechanism of the inactivation and the reason for its higher vulnerability to physicochemical conditions have not yet been elucidated.

In this study we have examined the structural capsid rearrangements occurring during the inactivation of B19V. For this purpose, we have applied two different procedures previously shown to efficiently inactivate B19V.[18,21,27] One is the exposure of the virus to heat (60°C for 10 min) and the other is the exposure to acidic conditions (pH 4 for 2 hr). Our results demonstrated that the first structural transition determining B19V inactivation is not the disintegration of the capsid, which remained intact (Fig. 1), but the loss of the viral DNA (Fig. 2). Interestingly, the heat sensitivity of B19V largely depends on the composition of the buffer. In a recent report, it was shown that a solution containing citrate conferred heat resistance to B19V.[29]

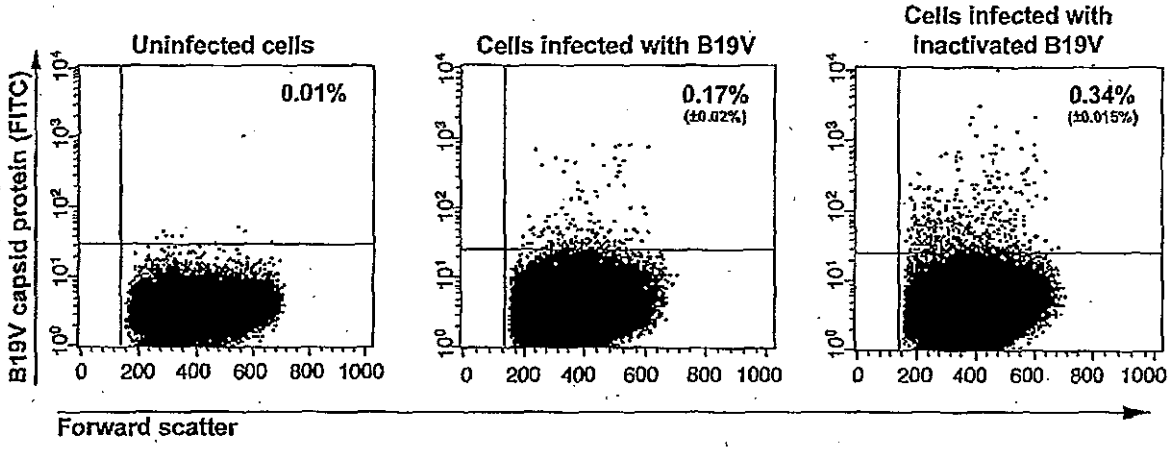

Fig. 4. Capacity of inactivated virus to bind to susceptible cells. UT7 cells were infected with either untreated or heat-inactivated B19V. The proportion of cells with bound virus was determined with FACS and is shown in the upper right quadrant of each panel. The percentages represent the mean ± SD of three separate experiments.

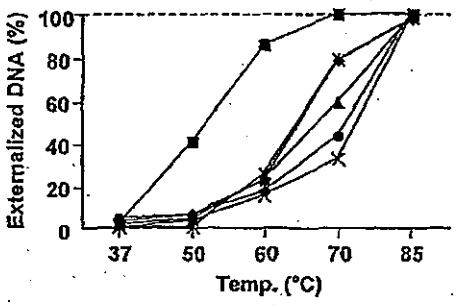

Fig. 5. DNA externalization pattern of different parvoviruses in response to increasing temperatures for 3 minutes. The amount of externalized DNA was quantified with the hybridization-extension assay. Values of DNA (%) refer to the total amount detected after 85°C treatment, which was in the range of 10^5 to 10^6 molecules per microliter for all viruses. (■) B19V; (●) bovine parvovirus; (◆) canine parvovirus; (▲) H1 parvovirus; (×) MVM; (✳) porcine parvovirus.

In our studies, we have confirmed this observation and found that in the presence of citrate, the viral DNA remains encapsidated (Fig. 3).

The release of the viral DNA in response to heat treatment was also detected in other parvoviruses (Fig. 5). Quantitative studies revealed that the kinetics of DNA externalization were surprisingly similar in all tested viruses with the exception of B19V, where it occurred prematurely (Fig. 5). The remarkable instability of the viral DNA in its encapsidated conformation explains the lower resistance of B19V to inactivation treatments.

The mechanism by which the intracellular environment destabilizes the parvovirus particles resulting in the release of the viral DNA is not fully understood. Growing evidence, however, indicates that parvovirus uncoating is performed without the need to disassemble the highly rigid capsid.[26,33-35] A series of capsid transitions triggered by the low endosomal pH seems to play a critical role by rendering the capsid flexible enough to allow the release of the viral DNA.[35] Among these transitions is the exposure of N-VP1. Increasing experimental evidence suggests that the conformational change leading to N-VP1 externalization leads also to DNA externalization.[33,34] Sustaining this notion is the observation that under mild acidification (pH 5), B19V externalizes N-VP1 sequences, and the viral DNA becomes accessible although mostly associated with the capsid.[28,36] In contrast, low pH treatment of MVM externalizes neither the N-VP1[34] nor the viral DNA.[28] Figure 6 represents schematically the progressive capsid rearrangement steps occurring during the inactivation of B19V.

As a result of the inactivation conditions applied in the present study, two major viral components were generated, empty capsids and free viral DNA, which might still have certain biologic activity. It has been recently shown that free genomic Kilham rat virus DNA induces innate immune activation and autoimmune diabetes through the TLR9 pathway;[37] however, whether B19V DNA or capsid proteins stimulate the innate immune system is not known. It has been increasingly acknowledged that pathogenic manifestations of B19V can also be elicited by the virus capsid proteins alone without infection. For instance, it has been shown that VP2 proteins are able to block hematopoiesis in vitro and in vivo.[38] The phospholipase A2 (PLA_2) activity of B19V is thought to contribute to inflammatory and autoimmune manifestations[39,40] and is suspected to be responsible for the arthropathies caused by B19V as well.[41] Although internal in native capsids, the VP1-PLA_2 motif becomes accessible upon exposure to