50 mmol per L KCl, 1.5 mmol per L $MgCl_2$, 0.001 percent (wt/vol) gelatin, 1.25 units AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 100 μmol per L of each dNTP, and 0.2 μmol per L of each of the first-set primers. One microliter of the first amplification product was transferred into a second 50-μL amplification mixture containing the same constituents as the first except that 0.2 μmol per L of each of the second-set primers was present. Nested primer sequences derived from the VP1/VP2 region of the B19-Au genome[21] were used. The first-set primers were 5′-CTTTAGGTATAGCCAACTGG-3′ (nucleotides 2905-2924) and 5′-CCTTATAATGGTGC TCTGGG-3′ (nucleotides 3290-3271), whereas the second-set primers were 5′-CATTGGACTGTAGCAGATGA-3′ (nucleotides 2951-2970) and 5′-GCTTTTGACAGAATTA CTGC-3′ (nucleotides 3193-3174). Amplification was performed in a thermocycler (Model 9600, Perkin Elmer, Foster City, CA) with the following settings: for the first-round amplification, an initial heating at 94°C for 3 minutes was followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds; for the second amplification, 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 50 seconds were performed. A final amplification product of 243 bp was then analyzed by electrophoresis on 1.8 percent agarose gel and visualized by ethidium bromide staining.

For sample extraction and B19 NAT, both the WHO International Standard (NIBSC 99/800, $10^6$ IU of B19 DNA/ mL when reconstituted) and the CBER standard for B19 DNA ($10^6$ IU/mL) were used as controls.[19] Both were diluted $10^3$-fold before use. The amount of B19 DNA expressed as geq was determined by limiting dilution analysis. For 0.1 mL of serum or plasma, the sensitivity of the NAT was 40 geq per mL; for 0.2 mL of AHF, it was 20 geq per mL. Both standards were found to contain $10^6$ geq per mL by our NAT procedure and hence the conversion ratio from IU to geq is 1:1, rather than 1:0.6 to 1:0.8 obtained in a collaborative study.[19]

### Cloning, sequencing, and phylogenetic analysis

The extracted DNA samples were amplified with a seminested B19 NAT procedure for DNA sequencing. Both antisense primers, other NAT constituents, and conditions were similar to those described above for the nested B19 NAT procedure except that a new sense primer derived from the C-terminal NS1 region, that is, 5′-GTGCTTACCT GTCTGGATTG (nucleotides 2408-2427), was used for both rounds of amplification so that the C-terminal NS1 region and the unique VP1 region would be included in the amplified product. The final amplified product, 786 bp, was purified by a polymerase chain reaction (PCR) purification kit (QIAQuick, Qiagen, Hilden, Germany), introduced into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and subsequently was used to transform *Escherichia coli*. For each of the samples, as well as the two NAT controls (WHO and CBER B19 standards), three to six clones were grown and purified by use of a spin miniprep (QIAPrep, Qiagen).

The inserts were sequenced on a sequencer (Model 310, Applied Biosystems) with a cycle sequencing kit containing a high-fidelity DNA polymerase (Big Dye Terminator, Applied Biosystems) with 0.5 μg of plasmid DNA and a universal M13 reverse primer according to the manufacturer's specified procedure. Cloning was not performed on the plasma pool sample that contained high levels of B19 DNA. Instead, the amplified products derived from the pool sample, as well as from both B19 NAT standards, were sequenced directly. The sequencing primers were the same as those used for the seminested NAT procedure. Sequence alignments of the N-terminal unique VP1 region corresponded to nucleotides 2444 through 2666 (nucleotide numbering based on the published Au sequence[21]), and phylogenetic analysis was performed by with a computer program (DNASTAR, DNASTAR, Inc., Madison, WI). Trees were generated by use of a neighbor-joining algorithm in comparison to 12 published B19 sequences adopted from GenBank.

### Anti-B19 detection

Anti-B19 IgM and IgG antibodies were detected by use of B19 IgM and IgG enzyme immunoassay kits (Biotrin International Ltd, Dublin, Ireland) according to the manufacturer's protocols. For serum or plasma specimens, 10-μL aliquots were used as instructed, whereas for AHF, a 100-μL aliquot was used.

## RESULTS

The 4-week postinfusion serum sample from the patient was found to be positive for the presence of anti-B19 (both IgM and IgG) and B19 DNA, whereas the serum sample collected before the infusion of the implicated AHF lots was negative for both (Table 1). The viremic level in the 4-week specimen was $10^3$ geq per mL (equivalent to $10^3$ IU/mL) as determined by limiting dilution analysis with our nested NAT method. Only AHF lot A had detectable B19 DNA, that is, $1.3 \times 10^3$ geq per mL (or $6.5 \times 10^3$ IU/vial of AHF). Anti-B19 IgG was not detected in the product. Hence, the patient received a total of $2 \times 10^4$ geq (or IU) of B19 DNA with no anti-B19 IgG associated with the AHF product. Several plasma pools from which lot A was derived were subsequently tested and all but one contained less than $10^4$ geq per mL B19 DNA (data not shown). That plasma pool contained $10^7$ geq per mL of B19 DNA and was positive for the presence of anti-B19 IgG, as expected for any given large plasma pool.

To investigate the case further, DNA sequencing of the C-terminal NS-1 region and the unique VP1 region was

performed. The unique VP1 sequences obtained from clones derived from the recipient and the implicated AHF product were compared to clones derived from the WHO and CBER B19 DNA standards, direct sequences obtained from the high-titer plasma pool for lot A, the two B19 DNA standards, and 12 published B19 sequences including strains Au[21] and Wi[22] in GenBank. As summarized in Table 2, two unique nucleotide substitutions were identified at positions 88 and 135, equivalent to 2531 (GTT to CTT) and 2578 (TAT to TAC) of B19 Au strain, in all five clones (Pat-1-5) from the patient, in all four clones (VIII-1-4) from the implicated lot A, and in a direct sequence obtained from the high-titer plasma pool (Plasma-P). In contrast, both substitutions were not found in any of the published isolates or in any of the six clones (WHO-1-6) or in the direct sequence derived from the WHO standard (WHO-P). Although one of three clones derived from the CBER B19 standard, that is, CBER-1, had both substitutions, it also had additional sequence variations elsewhere within the region. Identical sequences were obtained from all five (Pat-1-5) clones from the patient, three (VIII-1, -2, and -4) clones from implicated product lot A, and the direct sequence from a plasma pool for lot A, that is, Plasma-P. VIII-3 had two additional substitutions in the VP1 unique region. Variability of the clone sequences from both WHO and CBER standards was also seen; however, it was rarely detected by direct sequencing (Table 2).

**TABLE 1. Anti-B19 and B19 DNA in patient's serum specimens, implicated AHF lots, and a sample of a plasma pool used in manufacturing for lot A**

| Sample | B19 antibodies IgM | B19 antibodies IgG | B19 DNA (geq/mL)* |
|---|---|---|---|
| Patient | | | |
| Preinfusion serum sample | Negative | Negative | Negative |
| 4-week serum sample | Positive | Positive | 1000 |
| Implicated AHF | | | |
| Lot A | ND† | Negative | 1,300 |
| Lot B | ND | ND | Negative |
| Plasma pool for Lot A | ND | Positive | $10^7$ |

* Calculated from geometric mean values of two determinations for the postinfusion sample, four determinations for the AHF sample, and two for the plasma pools. The conversion ratio from geq to IU of B19 DNA by the NAT method is 1:1 (see Materials and methods).
† ND = not determined.

To demonstrate a causal relationship between the implicated product and the patient's infection, phylogenetic analysis of the sequences was performed. Nucleotide sequences from both the patient's postinfusion serum sample and AHF lot A and the predominant sequence of the plasma pool for lot A were highly similar (Fig. 1). In contrast, these sequences were not closely related to those derived from either the WHO and the CBER standards or other known B19 isolates.

## DISCUSSION

Adverse event reports are routinely received by the FDA (often through the MedWatch system), and every effort is made to follow-up reports of disease transmission by FDA-regulated products. Unfortunately, in the case of putative infectivity, preinfusion samples of the recipients' serum samples are rarely available; hence causality is difficult to assess. In the case investigated in this study, a preinfusion specimen obtained immediately before administration of the implicated product was uniquely available for analysis.

The recipient's seroconversion to B19-specific IgM and IgG antibodies, and the appearance of B19 DNA, occurred in the same time as symptoms consistent with acute B19 infection.[1] The low viremic level, $10^3$ geq per mL, found in the patient's 4-week postinfusion specimen was consistent with levels found after seroconversion.[7] Although serum samples of other patients who received the implicated lots at the same hospital were not available, it is reasonable to assume that they had seroconverted at some time in the past because they were all frequent users of AHF products. Evidently, as a result of the protection afforded by their circulating antibodies, they did not experience any B19-associated symptoms.

B19 DNA has been known to be prevalent in plasma-derived AHF products with documented levels as high as $10^7$ geq per mL of reconstituted product.[9,11] In our study, the level of B19 DNA found in the implicated AHF product (lot A) was $1.3 \times 10^3$ geq per mL, which is near the mean level of B19 DNA historically found in US-licensed AHF products derived from plasma unscreened by B19 NAT.[23] As expected, the level of B19 DNA found in the plasma pool from which lot A was derived was much higher, that is, $10^7$ geq per mL, although levels of at least $10^8$ geq per mL have been reported.[11]

B19 is a small nonenveloped virus known to withstand the commonly used virucidal method- for plasma derivatives, such as S/D and heat treatments, although recent findings[24,25] suggest that B19 could be susceptible to inactivation when heated in certain liquid media. Hence, the clearance of B19 for plasma derivatives relies mainly on removal, rather than inactivation, steps, such as chromatography or nanofiltration,[26] the latter being effective only when small pore-size membranes are used. Although the purification procedures for the AHF-implicated product include an immunoaffinity-chromatography step, which has been validated to remove effectively a model virus for B19, the viral load in the manufacturing pool obviously was too high to clear the virus from the final product. Additionally, we found that B19 IgG antibodies, which are considered to be neutralizing antibodies and appear to confer lasting protection,[1] were present in the large plasma pool but not detected in the final product.

## TABLE 2. B19 nucleotide sequences of the VP1 unique region among various isolates

| Nucleotide:* | 9 | 10 | 12 | 34 | 43 | 51 | 53 | 54 | 61 | 71 | 72 | 73 | 76 | 84 | 87 | 88 | 89 | 90 | 96 | 97 | 105 | 106 | 107 | 108 | 129 | 132 | 135 | 138 | 144 | 150 | 151 | 171 | 173 | 174 | 175 | 179 | 180 | 181 | 184 | 203 | 208 | 214 | 217 | 218 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Consensus | A | G | A | G | T | A | C | T | C | T | G | G | T | A | G | G | T | T | A | A | A | G | A | G | T | T | T | T | T | T | A | C | C | T | C | T | T | G | T | T | C | A | T | C |
| B19-Au | | A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | C | | | | | | A | | | | | | | |
| B19-Wi | | | | | | | | | | | | | | G | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Pat-1 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| Pat-2 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| Pat-3 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| Pat-4 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| Pat-5 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| VIII-1 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| VIII-2 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| VIII-3 | | | | | | G | | | | | | | C | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| VIII-4 | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| Plasma-P | | | | | | | | | | | | | | | | C | | | | | | | | | | | C | | | | | | | | | | | | | | | | | | |
| WHO-1 | | | | | | | | | | | | | | | | | | | | | T | | C | | | | | | | | | | | | | | | | | | | | | |
| WHO-2 | | | | | | | | | | | | | | | | | C | C | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WHO-3 | | | | | | | | | | | | | | | | | | | | | C | | G | | | | | | | | | | | | | | | | | | | | | |
| WHO-4 | | | | | | | | | | | | | | | | | | C | | | T | T | G | | | | | | | | | | | | | | | | | | | | | |
| WHO-5 | | | | | | | | | | | | | | | | | | | | T | | | | | | | | | | | | | A | | | | | | | | | | | |
| WHO-6 | | | | | | | | | | C | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| WHO-P | | | | | | | | | | | | | | | | C | | | | | C | | C | | | | C | | | | | | | | | A | | | | | | | | |
| CBER-1 | | | | | | | | | | | | | | | | | | | | | C | | C | | | | | T | | | | | | | | | | | | | | | | |
| CBER-2 | | | | | | | | | | A | | | | | | | | | | | C | | C | | | | | | | | | | | | | | | | | | | | | |
| CBER-3 | | | | | | | | | | | | | | | | | | | | | | | C | | | | | T | | | | | | | | | | | | | | | | |
| CBER-P | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AB126262† | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AB126271† | | | | | | | | | | | | | | | | | | | | | | | | | | | C | | | | | T | | C | | | | | | G | T | G | A | |
| AJ717293† | | A | | | | G | A | C | A | | A | | | | A | | | | G | | | | | | | | | | | | | | | | T | | | | C | | | | | T |
| AY582161† | | | | | | | | | | | | | | | | | | | | | | | | | | | | | C | C | | | | | | | | | | | | | | |
| AY582163† | | | | A | | | | | | | | | | | A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AY582164† | | | | | C | | | | | | | | | | A | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| AY647977† | T | | C | A | C | G | A | C | A | C | A | C | | | A | | C | | T | | T | | | A | A | C | | | C | | | T | A | C | T | | | C | G | T | | | | |
| AY661660† | | | | | | | | C | G | | A | | | | | | | | | | | | | | | | C | | | | C | | | | | | | | G | T | G | | | |
| AY661663† | | | | | | G | A | C | A | | A | | | | A | | | | G | | | | | | | C | | | | | | | T | | C | | | | | | | | | |
| NC000883† | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

* Nucleotide numbering of the consensus sequence is based on the published B19 Au strain, between nucleotide 2444 and nucleotide 2666.

† GenBank accession numbers of the published B19 sequences.

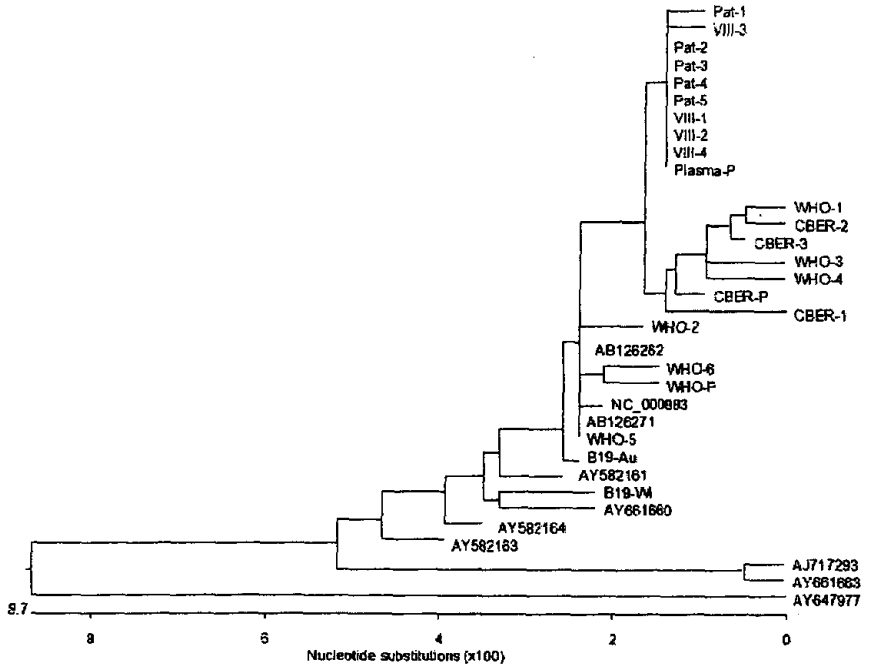


**Fig. 1. Estimated neighbor-joining tree obtained from B19 sequences (223 nucleotides) of the VP1 unique region obtained from the patient's 4-week postinfusion serum sample (Pat clones 1-5), from lot A of AHF (VIII clones 1-4), from the CBER (CBER clones 1-3), and WHO B19 (WHO clones 1-6) standards and sequences determined directly from PCR-amplified products derived from either the plasma pool containing $10^7$ geq per mL B19 DNA for lot A (Plasma-P) or the two corresponding B19 standards (WHO-P and CBER-P). Included for analysis are 12 published B19 sequences including strains Au and Wi in GenBank. Relative molecular distances are indicated as the number of nucleotide substitutions per hundred nucleotides.**

Detection of B19 DNA in both the product and the recipient does not necessarily equate with causality. To further establish a causal relationship,[18] we explored the genetic evidence of B19 sequence similarity among the recipient's serum sample, the infused AHF product, and a plasma pool for the product. In general, the genetic diversity among B19 isolates is very low, with less than 1 to 2 percent nucleotide divergence in the whole genome.[17,27] To confirm two B19-transmission cases by their corresponding coagulation products, Blümel and coworkers[17] sequenced about half of the B19 genome (approx. 2700 nucleotides) consisting of the C-terminal NS-1 region, the VP1-unique region, and the VP2 region. These authors employed direct sequencing of PCR-amplified products, which reflects only the predominant sequence, but not minor variant sequences that may be present in the implicated products. We chose to sequence mainly the unique VP1 region, which appeared to exhibit the most variation in sequences at both the DNA and the protein levels.[17,28] Because there might be many contaminated plasma donations, resulting in a mixture of B19 sequences in the final product, we performed sequence cloning of PCR-amplified DNA from the patient's serum sample and the implicated product to reveal individual sequences present in the original donations.

The sequences of all five clones from the recipient's serum sample were not only identical to three of the four clones from the implicated product, but also to the predominant sequence from the product's plasma pool. Two unique nucleotide substitutions were observed in all sequences from the patient, the product, and the plasma pool. Phylogenetic analysis revealed a close relationship among sequences from all three sources, whereas the sequences determined for the WHO and CBER B19 standards and the published Au, Wi, and 10 other B19 isolates were distinct. In addition, all sequences mentioned in this study were confirmed as genotype 1, distinctively different from strain Lali (genotype 2) and strain V9 (genotype 3) sequences (data not shown).[27]

As a result of the B19 transmission associated with pooled plasma, S/D treated[29-32] in a postmarket surveillance study that correlated product infectivity with a high concentration of virus in the manufacturing pool, testing for B19 DNA by NAT in a minipool format was implemented by the manufacturer to exclude use of plasma donations with high virus titers so that the viral load in the manufacturing pool can be limited to less than $10^4$ geq per mL B19 DNA.[29,32] To reduce the potential risk of transmission by other plasma-derived products, the FDA has since proposed a limit of less than $10^4$ IU per mL for manufacturing pools destined for all plasma derivatives.[32-35] It is neither feasible to exclude all B19 DNA-positive plasma donations nor desirable to remove the high-titer antibody donations associated with low-level viremia.[7] Hence, highly sensitive B19 NAT assays are not suitable for this application because they hold the possibility of removing low viremic, but not infectious, plasma donations and thereby compromising B19 antibody levels in the manufacturing pool.

Some fractionators, mostly those who use source plasma, have begun to use less sensitive, or so-called high-titer, minipool NAT screening. The sensitivity of these screening tests varies with donations identified as B19-positive ranging from at least $10^5$ to at least $10^7$ geq per mL, but they offer a mechanism by which the viral load in manufacturing pools can be limited.[6,34-36] Some final products obtained from minipool-screened plasma have found to be devoid of B19 DNA contamination.[6]

In this study, AHF lot B was derived solely from plasma tested by a high-titer B19 NAT screening procedure and had no detectable B19 DNA. In contrast, AHF lot A was mostly derived from unscreened plasma. The transmission case might not have occurred had B19 NAT screening been performed. That is, if donations with high levels of B19 DNA had been identified, the high-titer plasma pool for the implicated lot, $10^7$ geq per mL, would not have existed. A B19 transmission by a similar S/D-treated, immunoaffinity-purified, AHF product to a seronegative child with mild hemophilia A, who had not been previously infused with any blood product, has been documented.[16] As in most reported cases, however, sequencing analysis was not performed and the amount of B19 DNA infused was unknown.

Little is known regarding the correlation between a product's infectivity and its B19 DNA content. The B19 infectious dose in susceptible individuals, that is, presumably seronegative persons, would be expected to vary depending on whether the product contained anti-B19 IgG antibodies. For example, pooled plasma, S/D-treated, had levels of anti-B19 IgG[11,29] approximately 40 IU per mL in every product lot because each pool of plasma represented up to 2500 plasma donations. Only those seronegative volunteers infused with a 200-mL dose of product lots containing greater than $10^7$ geq per mL B19 DNA were infected, whereas those infused with an equal volume of lots containing less than $10^4$ geq per mL did not seroconvert.[29,30,32]

In a separate transmission case, a seronegative child was infected by infusing a dry heat–treated FVIII concentrate, which contained $4 \times 10^3$ geq per mL B19 DNA, over a period of 52 days.[17] The total infectious dose for this case was equivalent to $4 \times 10^6$ geq of B19 DNA from a product whose anti-B19 content, if any, was unknown. In our study, the seronegative recipient was infected by receiving a total of $2 \times 10^4$ geq of B19 DNA from a product that contained no detectable B19 IgG.

In conclusion, we have confirmed B19 transmission in a recipient of a S/D-treated high-purity AHF product derived from mostly B19 NAT unscreened plasma. The seronegative recipient became infected after receiving $2 \times 10^4$ geq (or IU) of B19 DNA present in the product.

Therefore, to safeguard the viral safety with respect to B19, minipool screening by B19 NAT should be implemented to reduce the level of potentially infectious B19 virus in the resulting products, especially those without the presence of anti-B19.

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**研究報告の概要**

○遺伝子導入マウスモデルにおいて評価したBSEプリオンの垂直感染

本稿では、ウシPrP(boTg)を発現させたトランスジェニックマウスにBSEプリオンを脳内投与した時の母子感染の証拠を示す。脳内$PrP^{res}$沈着がウエスタンブロット解析により検出できる発症少し前の時期に交配させた場合にのみ、感染した母マウスから生まれた新生児マウスの脳に$PrP^{res}$が検出された。boTgマウスの脳内接種後に乳汁中に感染性は検出できず、その他の組織がプリオン伝播キャリアとして関与していることが示唆された。本稿に示す結果から、マウスモデルにおいてBSEプリオンの感染が遠心性に中枢神経系から末梢組織に、また子マウスに広がることが証明される。また、これらの結果によりウシにおけるBSEの垂直感染の発生を支持する過去の疫学データを補足できるかも知れない。

| 報告企業の意見 | 今後の対応 |
|---|---|
| 遺伝子導入マウスモデルにおいてBSEプリオンの感染が遠心性に中枢神経系から末梢組織に、また子マウスに広がることが証明されたとの報告である。 | 今後も引き続き、プリオン病に関する新たな知見及び情報の収集に努める。 |

**使用上の注意記載状況・その他参考事項等**

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血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

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# Vertical Transmission of Bovine Spongiform Encephalopathy Prions Evaluated in a Transgenic Mouse Model

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**In this work we show evidence of mother-to-offspring transmission in a transgenic mouse line expressing bovine PrP (boTg) experimentally infected by intracerebral administration of bovine spongiform encephalopathy (BSE) prions. $PrP^{res}$ was detected in brains of newborns from infected mothers only when mating was allowed near to the clinical stage of disease, when brain $PrP^{res}$ deposition could be detected by Western blot analysis. Attempts to detect infectivity in milk after intracerebral inoculation in boTg mice were unsuccessful, suggesting the involvement of other tissues as carriers of prion dissemination. The results shown here prove the ability of BSE prions to spread centrifugally from the central nervous system to peripheral tissues and to offspring in a mouse model. Also, these results may complement previous epidemiological data supporting the occurrence of vertical BSE transmission in cattle.**

Prion diseases or transmissible spongiform encephalopathies (TSEs) belong to a class of infectious diseases characterized by the presence of an abnormally folded protein ($PrP^{Sc}$) that accumulates in the brains of affected individuals (24). TSEs may be of spontaneous, familial, or infectious origin. While spontaneous and familial etiologies have been described for the disease in humans (22, 23), infectious TSEs have been clustered mainly in domestic animals, from which sheep scrapie was the prototype of disease (17). The epidemic dimension of bovine spongiform encephalopathy (BSE) in the mid-1980s contributed to the spread of the disease to humans in the form of variant Creutzfeldt-Jakob disease (vCJD) (7, 8). It is now generally accepted that the consumption of contaminated meat and/or meat-derived products has been the most probable route of transmission of BSE prions to humans. Natural routes of transmission have been described for scrapie prions (16, 19, 20), although scant information is available regarding BSE natural routes of infection. The ability of scrapie prions to accumulate in placental tissues from genetically susceptible ewes (1, 25, 27) might be a contributing factor in scrapie epidemiology (16). However, this picture still remains diffuse for BSE. No $PrP^{Sc}$ accumulation is detected in placentas from BSE-infected cattle (31), and neither blood nor milk from BSE-infected animals have yet been shown to be infectious, consistent with the apparent absence of the prion agent in peripheral tissues (3). Experiments to test maternal transmission in cattle showed that approximately 10% of calves born to cows with confirmed BSE developed disease (2). This transmission rate, however, was obtained in a scenario of disease prevalence, since some of the calves were born after the feed ban was fully effective.

The ability of prions to move from the central nervous system (CNS) through afferent nerve fibers has been described for several TSEs, including genetic and sporadic human prion diseases (14, 15)and scrapie (28), and was suggested for chronic wasting disease (CWD) (26). Recently, it has been shown how vCJD and Gerstmann-Sträussler-Scheinker syndrome (strain Fukuoka-1) prions retaining full infectivity can be detected in the blood of mice after intracerebral inoculation (6). To test the ability of BSE prions to spread from CNS to peripheral tissues, we studied the efficiency of BSE transmission from intracerebrally BSE-inoculated mothers to their offspring in a transgenic mouse line (boTg110) expressing bovine PrP (4). boTg110 mice express boPrP controlled by the mouse PrP promoter at a level eight times that of the level of bovine PrP in cattle brain as previously described (4). Groups of boTg110 females were intracerebrally infected with a BSE inoculum named $BSE_1$ consisting of a pool from 49 BSE-infected cattle brains (TSE/08/59) supplied by the Veterinary Laboratories Agency (New Haw, Addlestone, Surrey, United Kingdom). The titer of this inoculum was $\sim 10^8$ 50% infective dose units per gram of bovine brainstem when measured in the boTg110 mouse line (data not shown). At different times postinoculation, infected female mice were mated with healthy homologous males (Table 1). Group I female mice (mated at 195 and 223 days postinoculation [d.p.i.]) showed a strong $PrP^{res}$ signal as judged by Western blot analysis of brain extracts (data not shown). In contrast, only mouse 09 from group II (mated at 160 d.p.i.) showed detectable brain $PrP^{res}$ accumulation, in good agreement with the kinetics of $PrP^{res}$ deposition in this mouse model (4).

$PrP^{res}$ was clearly detected by Western blotting in 2 out of 10 mice born from group I females (mated at 195 and 223 d.p.i.) but in only 1 out of 40 in group II (mated at 160 d.p.i.). The $PrP^{res}$ banding pattern observed for group I positive brains was similar to that for brains from Tg110 mice intracerebrally challenged with the $BSE_1$ inoculum, and no differences could be observed in their relative molecular weight mobilities (Fig. 1A)

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TABLE 1. Vertical transmission of BSE in bo-PrP-Tg110 mice after intracerebral inoculation

| Group | Inoculum | Mouse | d.p.i. to: | | | | Clinical signs | $PrP^{res}$ in mothers[a] | No. of offspring with $PrP^{res}$/total no. of offspring (d.p.i.) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mating | Offspring | Milking | Culling | | | |
| I | $BSE_1$ | 01 | 195 | 246 | 256 | 274 | Yes | −++ | 1/5 (622) |
| I | $BSE_1$ | 02 | 223 | 250 | 258 | 274 | Yes | −++ | 1/5 (613) |
| II | $BSE_1$ | 09 | 160 | 182 | 190 | 237 | Yes | + | 1/13 (536) |
| II | $BSE_1$ | 12 | 160 | 182 | 190 | 210 | No | − | 0/14 |
| II | $BSE_1$ | 14 | 160 | 182 | 190 | 210 | No | − | 0/13 |
| III | None (control) | 03 | 220 | 246 | 256 | 276 | No | − | 0/12 |
| III | None (control) | 05 | 220 | 246 | 256 | 276 | No | − | 0/10 |

[a] +++, strong $PrP^{res}$ signal; ++, $PrP^{res}$ accumulation detectable in brain; −, no detectable $PrP^{res}$ in brain.

Deglycosylation experiments with *N*-glycosidase F (PNGase F) confirmed this observation (Fig. 1B). However, differences in the amounts of immunoreactive $PrP^{res}$ were found between group I and II: $PrP^{res}$ levels in mouse 09/02 from group II were found to be clearly lower than those in mice from group I. This fact might be explained by the shorter survival time of this mouse (time to death, 536 d.p.i.) relative to those of mice from group I, which died at 622 and 613 days postinfection. Differences in the percentages of $PrP^{res}$-positive offspring among groups I and II (20% versus 2.5%; $P_{t\ test} = 0.098$) might be related to the time after intracerebral BSE prion inoculation after which mating was allowed. Thus, higher transmission rates, defined by the presence of detectable $PrP^{res}$, are obtained if the accumulation of pathogenic PrP in brain is allowed to reach certain nonpathological levels without disturbing the reproductive competence of female mice. The high percentage of $PrP^{res}$-negative littermates could be attributed to the limited sensitivity of the Western blot technique (5). In addition, exploring the presence of $PrP^{res}$ depositions by immunohistochemistry in brains from mice negative for $PrP^{res}$ by Western blotting was consistently unsuccessful (data not shown). The lack of $PrP^{res}$ detection, however, cannot exclude completely the existence of subclinical infections in the $PrP^{res}$-negative offspring. This assumption can be supported by the statistically significant differences ($P = 0.020$) observed in the survival times between offspring from infected (585 ± 60, 589 ± 71, 583 ± 36, 566 ± 63 and 608 ± 20 d.p.i.) and control (637 ± 57 d.p.i.) mothers (Fig. 2). Moreover, there was no difference between the survival times of $PrP^{res}$-positive and $PrP^{res}$-negative offspring mice. To confirm the fact of subclinical infection, works on second-passage experiments are in progress.

The fact that BSE prions delivered into mice brains can be transmitted to a next generation is indicative of their intrinsic ability to centrifugally spread from the CNS to other peripheral tissues. In fact, the ability of prions to move from CNS through afferent nerve fibers has been also described for other TSEs, including genetic and sporadic human prion diseases (14, 15) and scrapie (28), and was suggested for chronic wast-

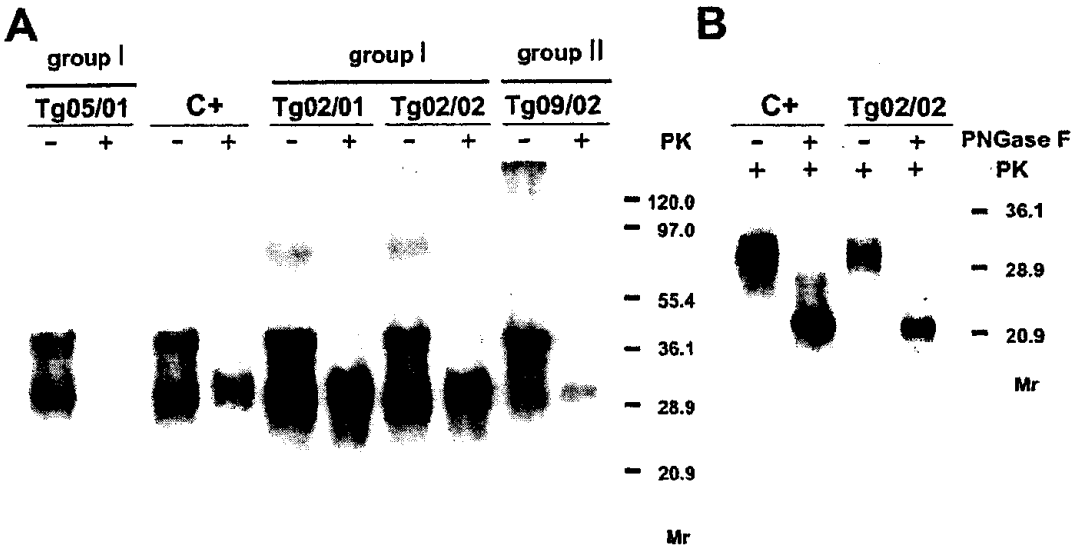


FIG. 1. (A) Comparison of Western blot profiles in brain detergent-insoluble fractions from $PrP^{res}$-positive offspring. Tg05/01, mouse born from the Tg05-uninfected female; Tg01/02 and Tg02/02, mice born from the Tg01 and Tg02 $BSE_1$ inoculum-infected females, respectively; Tg09/02, mouse born from the Tg09-infected female; C+, brain extract from a Tg110 mouse intracerebrally inoculated with $BSE_1$ inoculum; Mr, Relative molecular mass expressed in kilodaltons; PK, proteinase K treatment. Protein loads per lane are equivalent in progeny mice. In the Tg05/01 mouse the PK − lane shows soluble brain fraction. (B) Deglycosylation studies of $PrP^{res}$ from control (C+) and progeny Tg02/02 brain extracts.