withheld to prevent contamination of the urine. Urine was maintained at 4°C during collection. Separate metabolic cages (Rat metabolic cage no. 2100-R; Lab Products, Seaford, DE, USA) were used for each cohort. The urine produced daily was stored at –80°C. The individual collections were then combined into clinical and control pools of ≈60 mL and ≈125 mL, respectively.

Limiting Dilution Titration of Urine

We used the limiting dilution method of titration developed in our laboratory to measure the concentration of TSE infectivity in urine (*1,2*). In this method, a relatively large volume of low-titer sample is injected intracerebrally, 50 µL at a time, into a large cohort of weanling hamsters. Immediately before animal inoculation, aliquots of the clinical and control urine pools were thawed and sonicated on ice with separate sterile ultrasonication probes for each pool. Sonication was for 4 cycles of 15 s on and 10 s off for 1 min of total sonication, using a microtip probe at 40% amplitude (Vibra-Cell 750W; Sonics & Materials, Newtown, CT, USA). Two milliliters of control urine was injected undiluted into 40 hamsters. Clinical urine (urine from hamsters showing clinical signs of disease) was diluted 1:3 with inoculation buffer (phosphate-buffered saline [PBS] supplemented with 1% fetal calf serum and 1× penicillin and streptomycin) to remove concentration-related toxicity. Five milliliters from the clinical urine pool was diluted to 15 mL, and the entire volume was injected into 300 hamsters, 50 µL/animal. Soon after inoculation, 8 animals inoculated with urine from the infected animals died, which left 292 animals in the study. All inoculations were conducted under anesthesia with pentobarbital (40–90 mg/kg). At each step the control urine was processed before the infected urine.

All animals were assessed weekly for early signs of scrapie. At the first signs of disease, animals were separated from their cage mates, observed daily for disease progression, and euthanized after disease was confirmed clinically. After 559 days postinoculation all remaining animals were euthanized. Brains were collected from all animals in the study and assayed for infection-specific, proteinase K–resistant prion protein (PrP^{res}) by Western blot or ELISA, using a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) developed in our laboratory (described below). The infection status of each animal was tabulated, and the probabilities of infection and titer were computed as described (*1,2*; Table 1).

Tissue Collection and Processing

Kidneys and urinary bladders were harvested from each of 12 infected animals that donated urine either 71 or 76 days postinoculation. Animals were euthanized by asphyxiation with CO_2. The bladder was removed first and immediately frozen in liquid nitrogen. The kidneys were collected next; the renal capsule was removed before freezing the tissue in liquid nitrogen. Both tissues were dissected aseptically with a clean, sterile set of instruments for each animal and each organ; particular care was taken to not touch other organs or tissues. The tissues (12 bladders and 19 kidneys) were pulverized with a cryomill by using separate cryo-capsules for each tissue (Cryogenic Sample Crusher, Model JFC-300; JAI, Tokyo, Japan). The tissue powder was stored at –80°C until use.

End-Point Dilution Titration of Tissues

Pooled bladder powder (1.65 g) and pooled kidney powder (0.64 g) were separately mixed with homogenization buffer (PBS, pH 7.2) to make 10% (wt/vol) tissue suspensions before sonication at 40% amplitude, using separate sterile microtip probes for each homogenate. The kidney homogenate was prepared according to the same schedule of sonication used for the urine pools. The bladder homogenate was sonicated for 10 s, repeated 2 times (20 s total sonication time) at room temperature. Longer sonication times or delays in the injection of the bladder homogenate caused the sample to solidify, which made it impossible to dilute and inject. Immediately after sonication the homogenates were serially diluted 10-fold in inoculation buffer, and each dilution was injected into hamsters in 1 to 5 cages (4 hamsters/cage) for titration by end-point dilution (Table 2).

All dilutions were by weight. The study was terminated at 426 days postinoculation, and the infection status of each animal was confirmed by Western blot of the brain for PrP^{res}. The titers were calculated by the methods of Reed and Muench (*13*), Pizzi (*14*), and Spearman and Karber (*15*).

PrP^{res} Detection Procedures

Immunoblotting

Individual brains were homogenized in PBS, pH 7.2, to 10% (wt/vol) by using a FASTH homogenizer (Consul AR; Villenueve, Switzerland) according to the manufacturer's instructions. To test for PrP^{res}, brain homogenate

Table 1. Titer of urine from scrapie-infected hamsters

Hamster	Volume assayed, mL	Fold dilution	Volume inoculated, mL	Total no. hamsters	No. infected hamsters	Titer, ID/mL*	SD†
Infected	4.87	3	14.6	292	18	3.8	0.9
Noninoculated	2	None	2	40	1	–	–

*ID, infectious dose. Titer = –ln(P(0)) × (1/v), where P(0) = (noninfected animals)/(total animals inoculated) and v = inoculation volume, 0.05 mL.
†SD = square root (titer/V), where V = 4.87 mL, the total volume of the undiluted urine inoculated (*1*).

Table 2. End-point dilution titration of urinary bladder and kidney from scrapie-infected hamsters

Dilution	Total/no. infected	
	Bladder	Kidney
10^{-1}	19/19	4/4
$10^{-1.3}$	8/8	20/20
$10^{-1.7}$	8/8	8/8
10^{-2}	4/4	8/8
10^{-3}	4/4	4/3
10^{-4}	4/2	4/1
10^{-5}	4/1	4/0
10^{-6}	4/0	4/0
Titer (log_{10} ID_{50}/g)*	5.5	5.0
Standard error	0.5	0.4

*ID_{50} 50% infectious dose. Titers calculated by the Reed and Muench method (*13*); standard errors by the Pizzi method (*14*).

was digested with proteinase K at 0.1 mg/mL final concentration as described by Gregori et al. (*1*). Sample buffer containing 2% sodium dodecyl sulfate was added, and the samples were heated at 100°C for 10 min and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blots of the samples were developed by using anti-PrP 3F4 monoclonal antibody (Covance, San Diego, CA, USA) for PrP detection (*1*).

ELISA

After proteinase K digestion and heat denaturation as described for immunoblotting, the samples were diluted 100-fold in assay buffer (DELFIA Assay Buffer; PerkinElmer, Waltham, MA, USA). They were then assayed for PrP concentration by DELFIA by using a Wallac Victor V instrument (PelkinElmer) for signal detection, with purified 3F4 monoclonal antibody (Covance) as the capture antibody and purified 7D9 monoclonal antibody (Covance) labeled with Europium according to the manufacturer's instructions (PerkinElmer) as the detection antibody. The molar ratio of Europium:7D9 antibody was 7.4:1 (*2*).

Histologic and Immunologic Tissue Preparation

Formalin-fixed brains were cut and divided on the midline; 1 hemisphere was cut in the sagittal plane; the other was cut coronally at the anterior basal ganglia, the middle of the thalamus, and the brainstem with cerebellum. Spleens, kidneys, and bladders were divided in the middle. All blocks were embedded in paraffin and processed for conventional staining with hematoxylin and eosin and Luxol fast blue/nuclear fast red (for brain) as well as for immunohistochemical detection of PrP with monoclonal anti-PrP antibody 3F4 (1:1,000; Covance). For detection of PrP^{d}, sections were pretreated with 30 min of hydrated autoclaving at 121°C followed by 5 min in 96% formic acid. Immunostained sections were counterstained with hematoxylin.

Animal Husbandry and Decontamination Procedures

Animals were maintained in a Biosafety Level 3 (BSL-3) animal facility at the Veterans Affairs Medical Center in Baltimore, Maryland, USA. Standard operating procedures specifically designed for TSEs, including TSE select agents, were followed. The operation of this facility has been described in detail (*16*). Animal cages were changed once a week, and cages and bedding were decontaminated by autoclaving for 1 h at 134°C. The sonicator probes and dissection instruments were decontaminated by autoclaving for 2 h at 134°C immersed in 2 N NaOH, followed by cleaning, repackaging, and sterilizing. All laboratory surfaces were decontaminated before use with either 2 N NaOH or LpH (Steris Corporation, Mentor, OH, USA) (*16*).

Results

Urine Titration

Urine collections from infected and control animals were combined into separate pools. Pools minimized the possibility of an idiosyncratic measurement from an individual and serve as a resource for future experiments once the titer has been determined. Clinically affected animals consumed lower amounts of water and produced 4–5-fold less urine than control animals. This resulted in slightly elevated specific gravity, proteins, glucose, and ketones as measured with a standard urine dipstick. Elevated urine ketones may also have been caused by fasting. The higher concentration of the urine pooled from infected animals resulted in a toxicity that required a 3-fold dilution in buffer before it could be injected.

TSE developed in 18 of the 292 animals that survived the injection of the 3-fold diluted infected pool. Incubation times are shown in Figure 1. As observed in other studies (*1*;*2*), scrapie incubation times for animals infected with low-titer samples begin at ≈150 days and rarely extend past 500 days. None of the animals from either the infected or noninfected cohorts that survived to the end of the experiment were positive by DELFIA. None of the 24 animals that died during incubation without clinical evidence of scrapie were positive for scrapie infection by Western blot. Only those animals with clinical scrapie had the typical PrP^{res} signal in the brain as assessed by Western blot. The infectivity titer of the urine as calculated from the Poisson distribution was 3.8 ± 0.9 infectious doses (ID)/mL (Table 1).

Scrapie developed (at 425 days postinoculation) in 1 of the 40 hamsters inoculated with control urine. Because none of the control donor animals contracted scrapie and because their brains were negative for PrP^{res}, it is clear that this infection resulted from contamination. However, the contamination was unlikely to have been environmental. Our BSL-3 is managed under a strict regimen of continuous decontami-

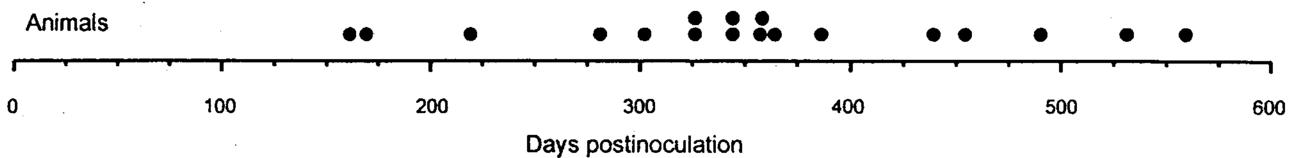

Figure 1. Distribution of incubation times of hamsters infected by injected urine. Each dot represents 1 animal with clinical scrapie that was euthanized at the corresponding day postinoculation. The 22 additional animals that died during the incubation period and the 252 animals that survived to the end of the experiment (559 days) showed no clinical or immunochemical evidence of scrapie and were scored as scrapie negative.

nation and precautionary cleaning (*16*). As evidence of the effectiveness of these measures, we have conducted several titrations, involving hundreds of animals each, in which there were no infections at all during ≥540 days of incubation. One such study was ongoing during the titration of the urine pools reported in this study (*2*). If there are environmental sources of infectivity, the concentration is below the level of detection by the data accumulated in infection-free titrations to date. Instead, after an intensive review of our procedures, we concluded that the most likely source of this contamination was a technical lapse during collection of the urine pools. The level of contamination (1 infection/2 mL of control pool injected vs. 18 infections/4.87 mL of clinical pool injected) is consistent with a pooling error at the time of collection. Nevertheless, had it been an environmental contamination, the associated titer (0.51 ID/mL SD = 0.50 ID/mL) would have had a negligible effect on the value determined for the infected urine.

Tissue Titrations

The concentration of scrapie infectivity in hamster urine is similar to that in plasma of scrapie-infected hamsters at the same stage of disease, which suggests plasma as a possible source of the infectivity. To investigate other possible sources, we also measured the concentration of TSE infectivity in separate pools of kidneys and bladders collected from the same donor animals. The titrations were by the end-point dilution method. The titers calculated by the methods of Reed and Muench (*13*) and Pizzi (*14*) were $10^{5.5 \pm 0.5}$ 50% infectious doses (ID_{50})/g of bladder and $10^{5.0 \pm 0.4}$ ID_{50}/g of kidney. The Spearman and Karber method gave almost identical values (*15*).

Histologic and Immunohistochemical Examination of Tissues

Others have reported TSE infectivity in the urine of scrapie-infected mice with nephritis but not in infected mice without nephritis (*11,12*). In contrast, our hamster colony in general, and the animals in this experiment, showed no evidence of inflammation, as indicated by clinical assessments or urine parameters. Nitrates were within normal limits, and no leukocyturia was noted. Proteinuria in the clinical hamsters was likely the consequence of low-volume urine excretion. To further assess whether hamsters infected with scrapie were also affected by kidney inflammation or other abnormalities of the urinary system, we examined the kidneys and the urinary bladders of 8 scrapie-affected hamsters at 84 days postinoculation and 4 preclinically infected hamsters at 49 days postinoculation for PrP^d by immunohistochemical and histologic methods (Figure 2). We also examined control tissues from 10 age-matched uninoculated animals as well as brain and spleen tissues from infected and control animals.

All tissues were evaluated for signs of inflammation and for the pattern of PrP^d immunoreactivity; brains were also examined for spongiform change. No inflammatory changes were found in any tissue examined. In 9 infected animals (clinical and preclinical), we noted nidus formation in the lumina of the bladder with a few neutrophilic granulocytes. However, leukocytes had not invaded the wall of the bladder. Nidus formation is often associated with dehydration.

PrP immunoreactivity was not observed in the bladder wall of scrapie-infected or control animals (data not shown). Spongiform change and deposition of PrP^d was lacking in control animal brains (Figure 2, panel A) and was noted to various extents, according to the stage of the disease, in all scrapie-infected animal brains (Figure 2, panel E). We observed fine synaptic PrP^d immunoreactivity with focal patchy or plaque-like appearance in gray matter structures, but we also noted ependymal, subependymal, perivascular, and white matter PrP^d deposits (data not shown). PrP^d immunoreactivity was observed in the germinal centers of the spleen of all scrapie-infected animals (Figure 2, panel F) but not in those of controls (Figure 2, panel B). None of the control animals exhibited immunoreactivity for PrP^d in the kidneys (Figure 2, panels C, D). PrP^d immunostaining showed fine granular deposits in the collecting tubules of the medulla (Figure 2, panels G, H) in 4 (50%) of 8 animals in the clinical stage of scrapie and in 3 (75%) of 4 animals in the preclinical stage, for a total of 7 (58.3%) of 12 scrapie-infected animals.

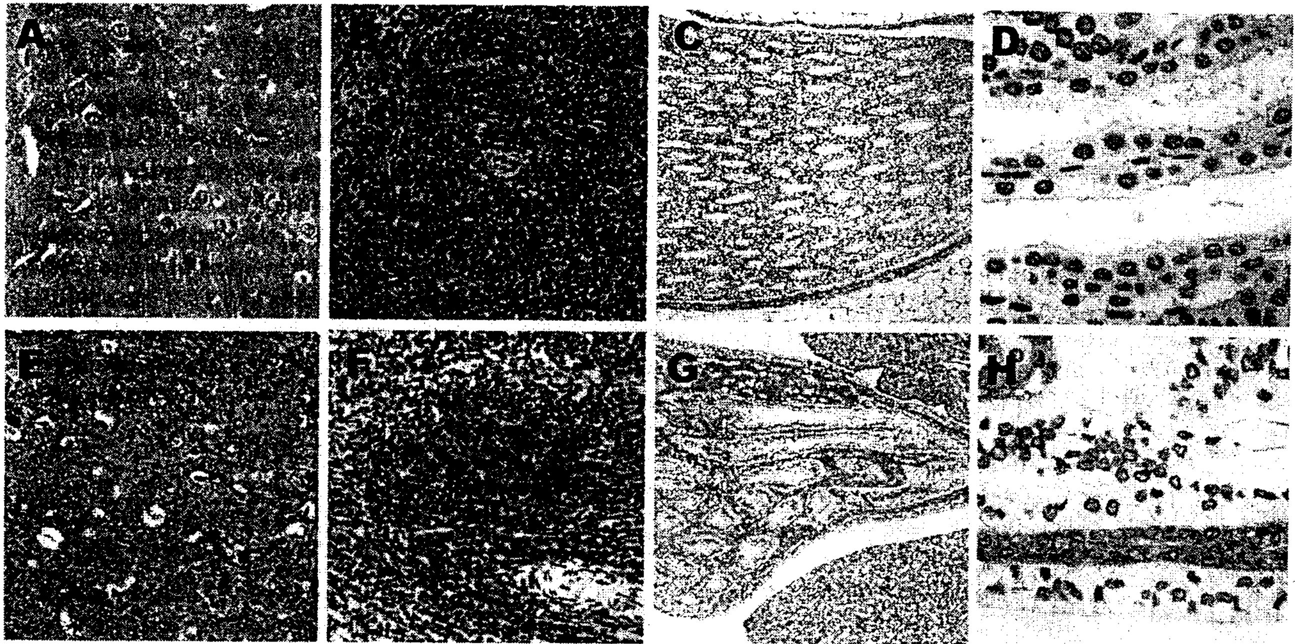

Figure 2. Immunostaining for prion protein (PrP) in control and scrapie-infected hamsters. Deposition of disease-associated PrP is lacking in the brain (A), spleen (B), and kidneys (C,D) of control hamsters. Fine synaptic and plaque-like PrP immunoreactivity in the frontal cortex (E), granular immunoreactivity in the germinal center of spleen (F) and in the collecting tubules of kidneys (G,H) in a representative scrapie-infected animal. Original magnification ×200 for panels A, B, D, E, F, and H and ×40 for panels C and G.

Discussion

Anticipating that the titer of scrapie infectivity in excreted urine would be low, we measured concentration by using limiting dilution titration, a method with which we have extensive experience quantitating TSE infectivity in blood and blood components. In a limiting dilution titration, all animals in the bioassay are inoculated with the highest concentration of inoculum that is tolerated by the intracranial (most efficient) route. Infectivity assorts randomly into the inoculated animals; provided that at least some, but not all, of the animals are infected, the concentration can be calculated from the Poisson distribution of the infections (*1*). The method is highly sensitive and far more precise than other methods of TSE titration. We considered concentrating the urine before bioassay, but to circumvent uncertainties about the recovery of endogenous infectivity, we decided to inject the urine as collected.

We found TSE infectivity in the urine of hamsters that had no evidence of kidney or bladder inflammation. In contrast, Seeger et al. did not detect infectivity in the urine of scrapie-infected mice (*11*) unless the mice were also affected by nephritis, in which case they found low levels of infectivity. Whether the bioassay they used was capable of detecting infectivity at the concentration we observed for hamsters is not clear. If it was not capable, then detection of infectivity in mice with nephritis implies a higher concentration of infectivity in urine excreted by a nephritic kidney. In another study, urine and feces from deer with chronic wasting disease failed to demonstrate infectivity when orally given to the same susceptible species (*17*). Although usually an inefficient route of inoculation, the oral route did successfully transmit chronic wasting disease infectivity in saliva. The authors identified several possible reasons for the unsuccessful transmission by excreta, including incubation time, genotype, or sample size.

In our experiments, cross-contamination by feces can not be excluded as a source of infectivity. Although the metabolism cage effectively separated urine and feces, some contact is possible because of the anatomy of the hamster.

Protein misfolding cyclic amplification uses sonication to generate PrP^{res} and infectivity in vitro. Although we routinely disperse all samples by ultrasonication before injection, our conditions are much harsher than those used to generate PrP^{res} de novo (*18*) and do not support protein misfolding cyclic amplification of PrP^{res}, or presumably infectivity (L. Gregori and R.G. Rohwer, unpub. data).

The kidney and bladder titers were far greater than expected compared with findings of historical studies in which, with only rare exceptions (*19–21*), most attempts at transmission have been unsuccessful. These titers cannot be explained by the infectivity in residual blood (10 ID/mL) (*1,2*). In addition, we observed PrP^d in the kidneys of scrapie-infected animals that had no indications of tissue inflammation. Heikenwalder et al. found PrP^d staining within

follicular infiltrates only in kidneys of mice affected by nephritis and not in control mice with noncomplicated scrapie (*12*). These data together with those by Seeger et al. (*11*) suggested that renal inflammation might be a prerequisite for TSE infectivity in renal tissue and its excretion in urine. In contrast, our results indicate that renal inflammation is not necessary for the deposition of PrP[d] in kidneys or for excretion of infectivity. One interpretation is that nephritis enhances the accumulation of PrP[d] at sites of inflammation, consistent with the excretion of higher levels of infectivity inferred above for this same condition (*11*).

Two studies of scrapie in naturally and experimentally infected sheep reported PrP[d] depositions in the renal papillae (*22*) and in the intraepithelial cortex, medulla, and papillae (*23*). Similar to our findings, both studies indicated that not all scrapie tissues examined were positive for PrP[d]. In chronic wasting disease, PrP[d] staining was uniquely localized in the ectopic lymphoid follicle of the kidney of a whitetail deer (*24*). All studies indicated either no changes (*22,24*) or mild to no inflammatory changes of the kidney (*23*). Thus, our histologic and immunohistochemical results for scrapie-infected hamsters are consistent with results found for sheep and deer and suggest that under normal conditions TSE diseases do not have concomitant inflammatory changes in the kidney.

That urine titer is similar to that of plasma suggests that urine infectivity may originate from blood (*25*), but how the infectivity would be excreted is not clear. In general, proteins >40 kDa are not excreted and smaller proteins crossing the glomeruli are reabsorbed in the renal tubule and returned to the blood. If TSE infectivity is particulate (>40 kDa), its presence in urine might indicate abnormalities in renal filtration, perhaps related to the accumulation of PrP[d] in the collecting tubules of the medulla. The accumulation of immunoglobulins in the urine of TSE-infected hamsters and humans may also indicate malfunction of the urinary system (*9,26*). Excretion of a small C-terminal fragment of the normal cellular form of the prion protein in urine of infected and noninfected animals has been reported (*27*), but PrP[res] or PrP[d] forms can only be inferred from the presence of infectivity. Nevertheless, excretion of proteins similar to PrP[res] or PrP[d] forms has been documented. Follicle-stimulating hormone is a glycosylated protein of 203 amino acids organized mostly as a β-sheet, which bears some remarkable similarities to β-rich forms of the prion protein. Follicle-stimulating and several similar hormones are excreted in urine at great enough concentration to be extracted commercially. Alternatively, TSE infectivity may be excreted by processes analogous to those responsible for the low-level virurias that occur during infections of the nervous system by mumps, measles, and West Nile virus (*28–30*).

To the extent that results from the hamster model can be generalized to other TSE infections (and it has so far proven highly predictive), then even the very low concentrations of infectivity measured here could result in substantial environmental contamination. Several liters of urine and several thousand doses of TSE infectivity may be excreted daily over the course of the illness; even higher titers might be excreted by an animal with nephritis. The high stability of TSE infectivity would account for its persistence in pasture years after infected animals are removed (*31*). Recent studies have shown that infectivity that is adsorbed and immobilized by soil minerals (*32*) can still infect hamsters by oral exposure 29 months later (*33*). Our study also warns of a possible risk from TSE contamination to fertility hormones and other medicinal products extracted from human urine.

Acknowledgments

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Dr Gregori is deputy director of the Molecular Neurovirology Laboratory in the Veterans Affairs Medical Center in Baltimore and a faculty member of the Department of Neurology at the University of Maryland in Baltimore. Her primary research interest is TSEs, with particular focus on TSE transmission by secondary exposure such as blood transfusion.

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研究報告の概要

○輸血による二次感染後のvCJD病原体に重大な変化はない
背景:輸血による変異型クロイツフェルトヤコブ病(vCJD)の伝播が同定されたことから、輸血経路による二次伝播後のvCJD病原体に変化があるかを調べることとした。病原性や宿主適応性が増加すれば、vCJDの重大な二次アウトブレイク発生の可能性に関するリスク分析の再評価が必要である。一般集団にvCJDキャリアがいる可能性が高いため、輸血や汚染された外科用器具などの経路から更に感染する可能性がある。
方法:我々は、野生マウスおよびトランスジェニックマウスに、輸血関連vCJD感染第1号症例由来材料を接種した。
主な知見:輸血関連vCJD感染株の伝播の特性は、ウシ海綿状脳症(BSE)からの伝播に関連したvCJD株と著しい類似を見せている。
結論:ヒトにおいて第2の感染経路を通してBSE病原体が適応することにより、ヒトに対する病原性の毒性が増加し、その後の伝播リスクがより大きくなるという仮説が立てられたが、本稿に示した2匹のマウスモデルのデータからは、vCJDのヒト-ヒト伝播後の病原体の伝播効率に重大な変化はないことが示される。

使用上の注意記載状況・その他参考事項等

新鮮凍結血漿「日赤」
新鮮凍結血漿-LR「日赤」

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

報告企業の意見	今後の対応
野生マウスおよびトランスジェニックマウスに、輸血関連vCJD感染第1号症例由来材料を接種したところ、輸血による二次感染後のvCJD病原体に重大な変化はないことが明らかになったとの報告である。	日本赤十字社は、vCJDの血液を介する感染防止の目的から、献血時に過去の海外渡航歴(旅行及び居住)を確認し、欧州36ヶ国に一定期間滞在したドナーを無期限に献血延期としている。また、英国滞在歴を有するvCJD患者が国内で発生したことから、平成17年6月1日より1980~96年に1日以上の英国滞在歴のある人の献血を制限している。今後もCJD等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。

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No Major Change in vCJD Agent Strain after Secondary Transmission via Blood Transfusion

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Abstract

Background: The identification of transmission of variant Creutzfeldt-Jakob disease (vCJD) by blood transfusion has prompted investigation to establish whether there has been any alteration in the vCJD agent following this route of secondary transmission. Any increase in virulence or host adaptation would require a reassessment of the risk analyses relating to the possibility of a significant secondary outbreak of vCJD. Since there are likely to be carriers of the vCJD agent in the general population, there is a potential for further infection by routes such as blood transfusion or contaminated surgical instruments.

Methodology: We inoculated both wild-type and transgenic mice with material from the first case of transfusion associated vCJD infection.

Principal Findings: The strain transmission properties of blood transfusion associated vCJD infection show remarkable similarities to the strain of vCJD associated with transmission from bovine spongiform encephalopathy (BSE).

Conclusions: Although it has been hypothesized that adaptation of the BSE agent through secondary passage in humans may result in a greater risk of onward transmission due to an increased virulence of the agent for humans, our data presented here in two murine models suggest no significant alterations to transmission efficiency of the agent following human-to-human transmission of vCJD.

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Introduction

Variant Creutzfeldt-Jakob disease (vCJD) is an acquired form of human transmissible spongiform encephalopathy (TSE) caused by infection by the bovine spongiform encephalopathy (BSE) agent that entered the human food chain in the United Kingdom during the 1980s and early 1990s. [1,2] 164 cases of vCJD have been identified in the United Kingdom and a further 41 cases in other countries worldwide. Annual mortality rates indicate that the vCJD outbreak is now in decline in the UK following a peak in 1999/2000. [3] In 2003 the first case of human-to-human secondary transmission of vCJD via blood transfusion was identified through a collaborative study between the UK National Blood Services, the National CJD Surveillance Unit, and the Office of National Statistics (Transfusion Medicine Epidemiology Review, TMER). [4,5] Statistical analysis showed that the possibility of this case being due to BSE infection was in the order of 1:15,000 to 1:30,000. [4] This patient had received a transfusion of non-leucodepleted red cells that had originated from a donor who 3 years 4 months later developed clinical vCJD. The blood recipient was methionine homozygous at codon 129 of the prion protein (PrP) gene (*PRNP*), the same genotype as all tested vCJD cases. [6]

Two further cases of vCJD linked to blood transfusion, in MM genotype individuals, have subsequently been identified through the TMER study. [7,8] Following the discovery of these cases policy changes were made in relation to blood donation in the UK and elsewhere. In 2004 the UK Blood Service deferred transfusion recipients from acting as blood donors.

A fourth case, of asymptomatic infection following blood transfusion, was described in 2004 and this individual was heterozygous (MV) at codon 129. [9] This case was the first indication that individuals with *PRNP* genotypes other than MM could be infected by the vCJD agent. All three codon 129 genotypes are now thought to be susceptible to vCJD infection following the identification of two VV genotype appendix tissues positive for vCJD associated PrP (PrP^{Sc}) in an anonymous screening study, and the successful transmission of vCJD to 'humanised' transgenic mice of each genotype. [10–12]

The implications of these findings are that a significant number of the UK population may be carriers of vCJD infectivity, that some of the individuals may be donating blood, and that not only

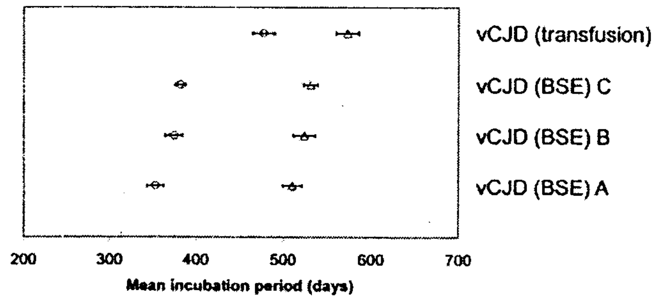

Figure 1. Comparison of incubation periods in wild-type mice. Incubation period plot comparison of vCJD (transfusion) case versus transmissions in wild-type mice of vCJD (BSE) from three sources. (Data shows mean incubation period±standard error of the mean. Open circles RIII line and open triangles VM line.)
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those with an MM genotype may be susceptible to infection from this source. Our research in transgenic models indicates that MV and VV individuals are likely to remain in an infectious preclinical state for a significant period of time with incubation periods potentially longer than average lifespan. [12] The identification of four instances of secondary transmission of vCJD infection from a group of 66 individuals known to have received blood products from vCJD donors, including only 28 who survived at least five years post transfusion indicates that blood transfusion is a significant risk factor for vCJD. This is likely to be due to either the route of transmission being more efficient of the agent being more infectious on human-to-human transmission or a combination of both.

TSE transmission by the blood transfusion route has been investigated in a sheep model. [13,14] These studies used intravenous (i.v.) transfusion of whole blood and blood fractions from clinical and preclinical sheep infected with BSE or scrapie. Preliminary data showed that the i.v. route gave relatively short and consistent incubation periods suggesting an efficient transmission route, with success rates of 60% for sheep infected with BSE and 40–45% for natural scrapie. [14,15]

Strain characterisation using a standard panel of inbred lines of wild-type mice originally demonstrated that BSE and vCJD agents had similar biological properties following transmission. [2,16] Similar work in other murine models has also been undertaken to study other human TSEs (genetic and iatrogenic CJD [17], and sporadic CJD [2]), and has been used to examine emerging TSEs (atypical BSE [18] and chronic wasting disease in deer and elk [19]). [20] The development of transgenic mice expressing human PrP has lead to further dissection of the nature of human TSE strains, including transmission of vCJD to gene targeted human transgenic mice. [12,17,21,22] Extensive data from studies in both wild-type and transgenic models at the Neuropathogenesis Division provide an essential background which will allow us to identify any change in the transmission characteristics of vCJD following secondary transmission. [2,12,23]

To investigate the nature of the transmissible agent following secondary transmission from human-to-human following blood transfusion we have examined the biological properties of brain material from the first case of transfusion-associated vCJD inoculated into panels of both wild-type, and transgenic mice expressing human PrP.

Results

Clinical signs of a TSE in the transgenic mice were rare and occurred after long incubation periods (IP) as found in our previous study. [12] Inoculation of the vCJD (transfusion) case produced one clinically positive HuMM mouse (at 659 days post inoculation), two positive HuMV mice (at 596 and 638 dpi) and no positive HuVV mice. Transmission of the vCJD (transfusion) case to the RIII and VM lines showed extended incubation periods compared to the three vCJD (BSE) cases. However, the hierarchy of incubation periods in the two wild-type lines was identical. (Figure 1 and Table 1) These data also show close similarities to previously published vCJD (BSE) transmission to wild-type mice despite different methodologies. These earlier studies used cerebellar material for the inoculum which was injected by simultaneous intracerebral and intraperitoneal routes. [2,23,24]

The frequency of transgenic mice positive for TSE associated vacuolation was similar between the vCJD (transfusion) case and the published vCJD (BSE) case [12], with positive results in 8/15 HuMM, 0/17 HuMV, and 0/17 HuVV mice and 6/16 HuMM, 1/15 HuMV, and 1/15 HuVV mice respectively. Regional distribution of TSE vacuolation in the brain was assessed through lesion profiling. All wild-type and the HuMM transgenic lines had sufficient positive mice to generate a profile (n≥6 mice). The overall pattern of the lesion profiles was the same in the vCJD (transfusion) and vCJD (BSE) cases for all lines of mice, however,

Table 1. Clinical and pathological assessment of wild-type mice.

Inoculum	Mouse Line	Mice Inoculated[a]	Positive for Clinical TSE Signs	Positive for TSE Vacuolation	Incubation Period (days±SEM)
vCJD(BSE) A	RIII	20	17	17	352.76±9.78
vCJD(BSE) B	RIII	20	18	17	374.35±9.98
vCJD(BSE) C	RIII	21	17	16	381.88±6.07
vCJD (transfusion)	RIII	23	18	18	477.33±12.68
vCJD(BSE) A	VM	22	15	22	510.20±10.97
vCJD(BSE) B	VM	22	20	21	523.75±12.57
vCJD(BSE) C	VM	21	13	18	530.69±8.16
vCJD (transfusion)	VM	22	15	18	572.90±12.96

Wild-type mouse lines RIII and VM, inoculated with vCJD(BSE) and vCJD(transfusion) were assessed clinically and pathologically for signs of TSE and mean incubation periods calculated.
[a]The group of 24 was reduced due to unavailability of some brain material for analysis.
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for the former case the VM and HuMM mice scores were lower. (Figure 2)

Immunocytochemical (ICC) detection of disease associated abnormal PrP in paraffin sections was also used as a method of assessing whether mice were transmission positive. There were 13/14 HuMM, 8/17 HuMV, 1/17 HuVV positive mice in the vCJD (transfusion) case, which was similar to the frequency of positives in the published vCJD (BSE) case: 11/15 HuMM, 11/13 HuMV, 1/15 HuVV mice. ICC data can be used to show variation in targeting of abnormal PrP deposition in the brain and variation in the nature of deposits. The ICC pattern in transgenic mice inoculated with the vCJD (transfusion) case matched that reported for vCJD (BSE) [12]. The thalamus was specifically targeted with deposition of abnormal PrP, and for the HuMM mice the hippocampus contained many intensely stained plaques including vCJD transmission associated florid plaques. ICC pattern in wild-type mice also showed similarities between the data sets with abnormal PrP deposition targeted to the thalamus and hippocampus, and large aggregates in the white matter of the corpus callosum. (Figure 3)

Biochemical analysis of disease-associated PrP by Western blot can discriminate between human cases of vCJD and sporadic CJD. [25] In the vCJD (transfusion) case the HuMM mice had a type 2B gel mobility and glycoform ratio identical to that found in vCJD (BSE) transmission to HuMM mice, and in vCJD itself. (Figure 4) Brain tissue from both vCJD (transfusion) [4] and published vCJD (BSE) [26] patients showed the type 2B pattern. The levels of PrP^{Sc} seen in the HuMV and HuVV were too low to allow typing by this standard Western blot method.

Discussion

Secondary passage of vCJD infection via blood transfusion in an MM codon 129 genotype individual results in a clinical disease phenotype and pathological characteristics that are similar to vCJD derived from BSE. [4] In this paper we confirm that the agent strain properties of primary and secondary vCJD cases are similar in transmission studies in transgenic and wild-type mice. Strain characteristics can be assessed by the frequency of clinical signs in recipient animals, the incubation period, neuropathological features, and PrP typing. All these parameters were similar in the transmission studies of primary and secondary vCJD in transgenic mice, indicating that the strain properties of the vCJD agent have not changed significantly following secondary passage in humans.

There were some differences in the results of the transmission studies which deserve further comment. The incubation period in wild-type mice was relatively extended in the vCJD(transfusion) case. However, the hierarchy of incubation periods in different inbred mouse strains was unchanged and the most plausible explanation for these findings is that, rather than implicating a change in agent characteristics, the titre of infectivity was less in the brain sample from the vCJD(transfusion) case. The distribution and degree of vacuolation was identical in the RIII mice. (Figure 2) While the distribution was identical in the VM and HuMM mice the degree of vacuolation intensity was lower for the vCJD(transfusion) case. This variability could be due to the much longer incubation times observed in these lines of mice or due to minor changes of the strain properties.

Preliminary investigation of the individuals diagnosed with vCJD following blood transfusion does not indicate a change in the neuropathological characteristics of vCJD following secondary transmission, although further studies are required to confirm this observation.

The level of infectivity in peripheral tissues in secondary cases of vCJD is unknown, although spleen and a lymph node were PrP positive in the sub-clinical case linked to blood transfusion. Evidence from BSE inoculation of primates indicates similar peripheral distribution of disease associated PrP following either oral or intravenous infection. [27] Further studies are required to assess the anatomical distribution, strain properties and level of infectivity in peripheral tissues in secondary vCJD infection. This may be important for accurate assessment of the public health risks associated with the potential for iatrogenic transmission of vCJD, which are not solely defined by the agent characteristics in brain.

Blood transfusion appears to be a relatively efficient means of secondary transmission of vCJD. To date, there have been four such transmissions in a cohort of 28 individuals who survived at least five years following transfusion of blood derived from individuals incubating vCJD. Despite extensive exposure of the UK population to the BSE agent in the food chain, there have been a relatively limited number of primary cases of vCJD (164 in the UK) and the outbreak has been in decline since 1999/2000. An important question is why there should be a disparity in the apparent efficiency of infection between primary and secondary vCJD. Transmission is generally more efficient within species than between species which may explain this observation. [28,29] Inoculation of wild-type mice with material from primary and secondary BSE passage in macaques showed that the BSE agent retained a characteristic lesion profile even though the second passage incubation period in the macaques was reduced by 50%. [30] This suggests that efficiency of transmission may increase without obvious changes to the agent strain.

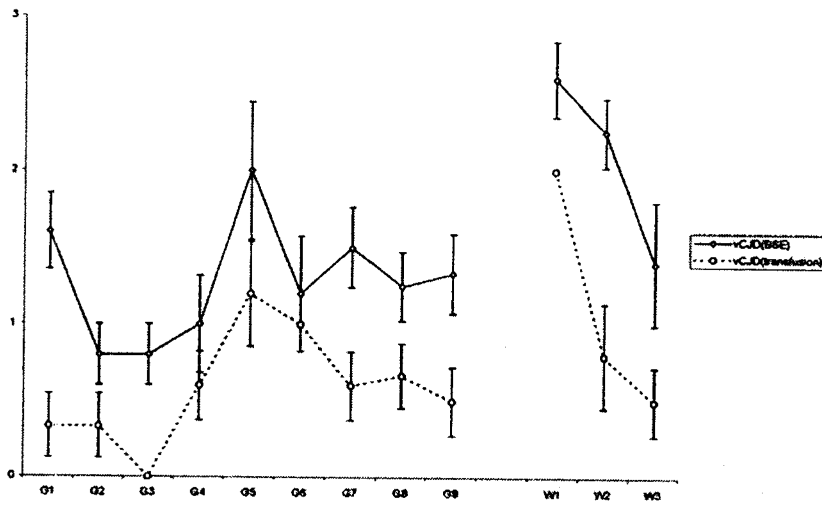
Another factor is that the intravenous route of infection is very much more efficient than the oral route, as shown in experimental models. [27,31,32] Results from this study suggest the major factor here is likely to be the route of infection rather than any changes in the strain of agent. Future studies, including those using experimental oral exposure to infectivity in transgenic mice, will further address this issue.

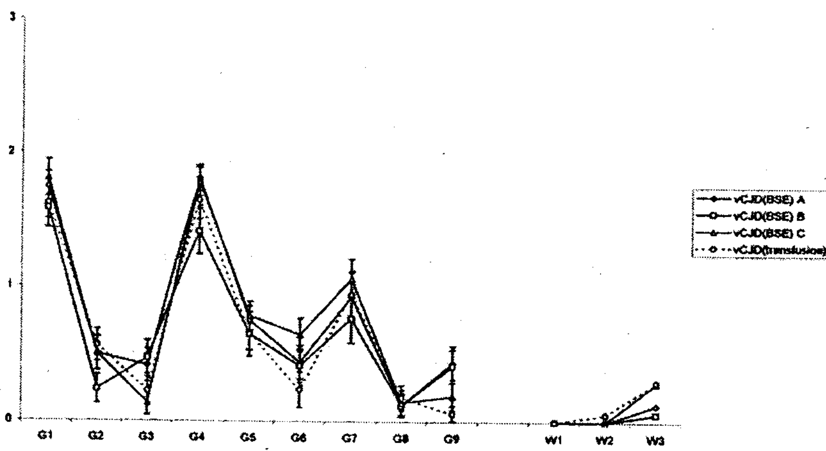
All the primary and secondary clinical cases of vCJD have occurred in individuals with a MM genotype. The sub- or pre-clinical transfusion related infection was in a codon 129 heterozygote and genotyping of positive appendix samples identified in a screening study confirmed valine homozygosity in 2 of 3 samples tested. [10] This indicates that individuals with all codon 129 genotypes may be infected with the vCJD agent and the effect of the MV or VV background on the characteristics of the vCJD agent have not been addressed by the data in this paper.

In conclusion, transmission studies indicate that the strain characteristics of vCJD have not been significantly altered by secondary transmission through blood transfusion. This suggests that the risk of onward transmission of vCJD through other routes, for example contaminated surgical instruments, have not been increased by adaptation of the infectious agent to humans following secondary passage. However the characteristics of the infectious agent in different genetic backgrounds has not yet been defined and the prevalence of vCJD infection in the general population remains uncertain. There is need to continue to implement appropriate policies to protect against the risk of secondary transmission of vCJD until many of the remaining uncertainties are resolved.

Materials and Methods

The transgenic mice (HuMM, HuMV, HuVV) used in these experiments have been described previously. [12] These mice express human PrP under the regulation of the murine promoter


HuMM
vCJD(BSE)
vCJD(transfusion)
G1
G2
G3
G4
G5
G6
G7
G8
G9
W1
W2
W3


RIII
vCJD(BSE) A
vCJD(BSE) B
vCJD(BSE) C
vCJD(transfusion)
G1
G2
G3
G4
G5
G6
G7
G8
G9
W1
W2
W3

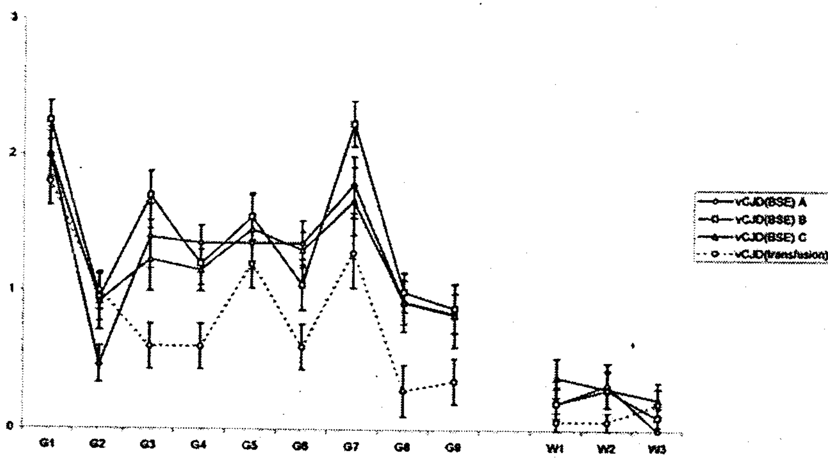

VM
vCJD(BSE) A
vCJD(BSE) B
vCJD(BSE) C
vCJD(transfusion)
G1
G2
G3
G4
G5
G6
G7
G8
G9
W1
W2
W3

Figure 2. Vacuolation scoring in the mouse brain. Lesion profile comparison of vCJD (transfusion) case versus vCJD (BSE) transmissions to identify similarities in vacuolar pathology levels and regional distribution in mouse brains. (mean score ±SEM; dashed line - vCJD (transfusion) case; solid lines – 3x vCJD (BSE) cases for wild-type mice (diamonds – vCJD(BSE) A; squares – vCJD(BSE) B; triangles – vCJD(BSE) C) and published vCJD (BSE) for HuMM transgenic; G1–G9 grey matter scoring regions; W1–W3 white matter scoring regions)
doi:10.1371/journal.pone.0002878.g002

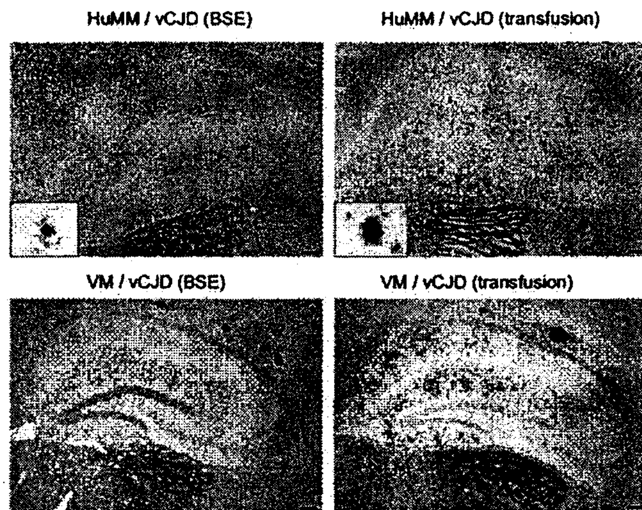


Figure 3. Detection of abnormal PrP in the mouse brain. Immunocytochemical detection of abnormal PrP deposition in hippocampus and thalamus (lateral posterior nucleus) of HuMM transgenic (with additional 40× magnification of florid plaque structure, see box lower left) and VM wild-type mice following inoculation with vCJD (BSE) and vCJD (transfusion) material. (Scale bar 200 μm, anti-PrP antibody 6H4)
doi:10.1371/journal.pone.0002878.g003

sequences, and survive for the same lifespan as non-transgenic mice of the same genetic background (129Ola) with no adverse effects and no features of spontaneous TSE disease. Wild-type mice (lines VM and RIII) are inbred lines used routinely for strain typing of TSEs. RIII is a *Prnp*-a genotype line and VM is a *Prnp*-b genotype line. [33] Use of mice for this work was reviewed and approved by the Neuropathogenesis Division Ethics Committee for Animal Experimentation.

Mice were inoculated as described previously. Groups of 24 wild-type mice received a 0.02 ml dose at 10^{-1} dilution by the intracerebral route, for vCJD (transfusion) and vCJD (BSE). Groups of 18 transgenic mice were injected with inoculum at a higher dilution of 10^{-2} as in previous experiments more concentrated inocula had been found to be toxic to the mice. Inoculum was prepared as a homogenate in sterile saline from frozen frontal cortex (with full consent from the patient's relatives, and approved by the Lothian NHS Board Research Ethics Committee (Reference: 2000/4/157)) to allow accurate comparison with previous data. Cases used for transmission were: the first

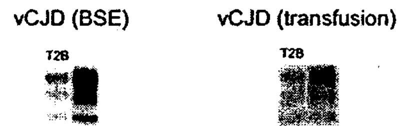


Figure 4. PrP^{Sc} typing by Western blot. Brain homogenates from HuMM mice inoculated with both vCJD (BSE) and vCJD (transfusion) show similar mobility and glycosylation profile (type 2B) as material from vCJD patients. (T2B: control vCJD material; antibody: 6H4)
doi:10.1371/journal.pone.0002878.g004

blood transfusion associated case, designated here as vCJD (transfusion), and three historical vCJD cases designated here as vCJD (BSE) A, B, and C. The historical vCJD cases were not inoculated into the transgenic mice. Data from vCJD (transfusion) inoculation of the transgenic mice was compared with that already published for vCJD (BSE). [12] Data from vCJD (transfusion) inoculation of the wild-type mice was compared with data from the three historical vCJD cases.

Mice were housed in independently ventilated cages in a Category 3 facility, monitored daily and scored for signs of TSE disease weekly from 100 days post inoculation. Mice were culled, when clinical TSE was evident or for animal welfare reasons, by cervical dislocation and the brain bisected sagittally; one half frozen for biochemical analysis of disease-associated prion protein and the other half fixed in formalin for histology.

Vacuolation scoring was performed according to published protocols and lesion profiles generated. [34,35] Immunocytochemical detection of abnormal PrP deposition was performed as published and Western blotting of disease-associated PrP from the frozen half-brain carried out according to Head *et al.* [12,25]

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Author Contributions

Conceived and designed the experiments: MTB RGW MB JCM. Performed the experiments: MTB DLR VT. Analyzed the data: MTB DLR MWH. Contributed reagents/materials/analysis tools: JWI MWH. Wrote the paper: MTB RGW JWI JCM.

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研究報告の概要

<背景>
ヒトの vCJD は、古典的 BSE に罹った畜殺牛のプリオンを食料として摂取することで感染する。非定型 BSE は、高齢牛では殆どが無症候であるが、最近ヨーロッパと北米の畜殺場で確認され、これらの新しいプリオン株に対するヒトの感受性についての問題が提起されている。

<方法/主な所見>
古典的 BSE と非定型 BSE に感染した牛の脳のホモジネートを、以前に古典的 BSE のオリジナル株に感受性が高いことを示したヒト以外の霊長動物モデルであるカニクイザルに脳内接種した。こうして発現させた疾患を、臨床兆候、組織学、異常プリオンたん白の生化学の点から比較した。非定型 BSE に感染した 1 頭のサルは生存期間が短く、古典的 BSE または vCJD 接種動物のいずれとも異なる臨床的展開、組織変化、プリオン蛋白(PrPres)パターンを示した。加えて、非定型 BSE の接種動物における PrPres の生化学的特徴として、octa-repeat 領域のプロテイナーゼ K への高い感受性を有していることが判明した。我々は、感染牛と同じ郡に住んでいた孤発性 CJD および MM type 2 PrP 遺伝型の 4 人の患者のうちの 3 人に、同じ生化学的特徴があるのを見出した。

<結論>
我々の結果は、霊長動物において、古典的 BSE よりも非定型 BSE の方が病原性が高い可能性があることを示し、加えて外見上孤発性 CJD に見えるまれな症例群と結びついている可能性についての問題を提起している。これより、古典的 BSE の流行が衰えているにも関わらず、非定型株の発生によって、BSE 汚染製品による偶発的汚染から公衆衛生を保護するために現在実施されている措置を緩和することを推進することは抑えるべきである。

使用上の注意記載状況・その他参考事項等

代表としてノイアート（献血）の記載を示す。
2. 重要な基本的注意
(1) 略
1) 略
2) 現在までに本剤の投与により変異型クロイツフェルト・ヤコブ病（vCJD）等が伝播したとの報告はない。しかしながら、製造工程において異常プリオンを低減し得るとの報告があるものの、理論的な vCJD 等の伝播のリスクを完全には排除できないので、投与の際には患者への説明を十分行い、治療上の必要性を十分検討の上投与すること。

報告企業の意見	今後の対応
霊長動物では非定型 BSE の方が古典的 BSE よりも病原性が高く、孤発性 CJD に見える症例と結びついている可能性があるとの報告である。 これまで血漿分画製剤によってvCJD、スクレイピー及びCWDを含むプリオン病が伝播したとの報告はない。しかしながら、万一vCJD感染者の血漿が本剤の原料に混入した場合には、製造工程においてプリオンを低減し得るとの報告があるものの、製剤から伝播する可能性を完全には否定し得ない。そのため、弊社の血漿分画製剤の製造工程におけるTSE感染性低減に関する検証実験を加速し、自社データを早期に取得し、工程評価を行い、必要に応じて工程改善を実施する予定である。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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Atypical BSE (BASE) Transmitted from Asymptomatic Aging Cattle to a Primate

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Abstract

Background: Human variant Creutzfeldt-Jakob Disease (vCJD) results from foodborne transmission of prions from slaughtered cattle with classical Bovine Spongiform Encephalopathy (cBSE). Atypical forms of BSE, which remain mostly asymptomatic in aging cattle, were recently identified at slaughterhouses throughout Europe and North America, raising a question about human susceptibility to these new prion strains.

Methodology/Principal Findings: Brain homogenates from cattle with classical BSE and atypical (BASE) infections were inoculated intracerebrally into cynomolgus monkeys (*Macacca fascicularis*), a non-human primate model previously demonstrated to be susceptible to the original strain of cBSE. The resulting diseases were compared in terms of clinical signs, histology and biochemistry of the abnormal prion protein (PrPres). The single monkey infected with BASE had a shorter survival, and a different clinical evolution, histopathology, and prion protein (PrPres) pattern than was observed for either classical BSE or vCJD-inoculated animals. Also, the biochemical signature of PrPres in the BASE-inoculated animal was found to have a higher proteinase K sensitivity of the octa-repeat region. We found the same biochemical signature in three of four human patients with sporadic CJD and an MM type 2 PrP genotype [illegible] as the infected bovine.

Conclusion/Significance: Our results point to a possibly higher degree of pathogenicity of BASE than classical BSE in primates and also raise a question about a possible link to one uncommon subset of cases of apparently sporadic CJD. Thus, despite the waning epidemic of classical BSE, the occurrence of atypical strains should temper the urge to relax measures currently in place to protect public health from accidental contamination by BSE-contaminated products.

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Competing Interests: CEA owns a patent covering the BSE diagnostic tests commercialized by the company Bio-Rad.

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Introduction

Classical Bovine Spongiform Encephalopathy (cBSE), the first prion disease identified in cattle, was initially reported in 1986 in the UK. Food-borne transmission of cBSE to humans was observed ten years later as a variant form of Creutzfeldt-Jakob Disease (vCJD) [1], leading to a major public health crisis.

This strain of cBSE is now rapidly disappearing as a result of appropriate containment measures. However, atypical forms of BSE have recently been identified in Europe and North America as a consequence of cBSE testing performed in these countries [2–4]. Because these cases are only found sporadically in older animals (≥8 years) coming to slaughter with few or no signs of disease, it would be plausible to suppose that atypical forms of BSE may have a lower virulence than cBSE and be innocuous to humans. However, recent studies suggest that one of the two main forms of atypical BSE, initially discovered in Italy and referred to as the bovine amyloidotic spongiform encephalopathy (BASE), might be at the origin of the cBSE epidemic: inoculation of the BASE strain into transgenic and inbred mice showed an apparent natural evolution towards the typical BSE strain [5,6]. Moreover, a possible link has been suggested between BASE and one subtype (MV2) of human sporadic CJD (sCJD) on the basis of biochemical similarities [2,7]. In contrast to vCJD, sCJD is believed to occur de novo without food-borne transmission. However, specific contaminating events by ingestion are difficult to rule out because human prion diseases can have silent incubation periods exceeding 50 years, as demonstrated for kuru [8].

One strategy to evaluate the risk of BASE for humans consists in assessing the susceptibility to disease transmission and the degree of pathogenicity in a non-human primate model that has already been shown to have characteristic clinical signs, histopathological lesions and PrPres profiles following infections with either BSE or vCJD [9,10]. We therefore inoculated cynomolgus macaque monkeys (*Macacca fascicularis*) intracerebrally with BASE, cBSE and vCJD prion strains. The BASE strain, prepared from brain extract of a 15-

Table 1. Survival times of macaques inoculated intracerebrally with brain homogenates from cattle with BASE or BSE, and from humans with vCJD.

Strain	Source	Dose*	Survival time (months)
BASE	cattle	25 mg	[illegible]
BSE	cattle	100 mg	40
BSE	cattle	100 mg	[illegible]
vCJD	human	40 mg	25
vCJD	human	40 mg	[illegible]
vCJD	human	40 mg	32
vCJD	human	40 mg	[illegible]

*Amount of crude brain in 10% brain suspension inoculated intracerebrally. BSE brain had a 10-fold greater concentration of PrPres than the BASE brain). Animals inoculated with vCJD also received the equivalent of 8 mg of brain by intra-tonsillar injection.

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year-old asymptomatic cow induced a distinctive and more rapidly fatal disease than cBSE, and showed a biochemical signature similar to that of the MM2 cortical subtype of human sCJD.

Methods

Cattle and human samples

The BASE inoculum (mix of brainstem and thalamus) from an asymptomatic 15 year-old Italian Piemontese cow [2]: 250 µl of a 10% brain homogenate in 5% glucose were inoculated intracerebrally (i.c.) to a single macaque monkey. As controls, we used two macaques inoculated i.c. with cBSE (brainstem from infected UK cattle) and 4 macaques inoculated i.c. with human vCJD [9,11]. Twenty-one subjects with a diagnosis of definite sCJD were referred to the Medical Center in Verona, Italy during the period 2000–2004. Tissues were processed 4–18 hours post-mortem according to established guidelines regarding safety and ethics. Brains were cut longitudinally into two halves. Hemi-brains were frozen and stored at −80°C until biochemical studies were performed. The patient group encompassed all of the different

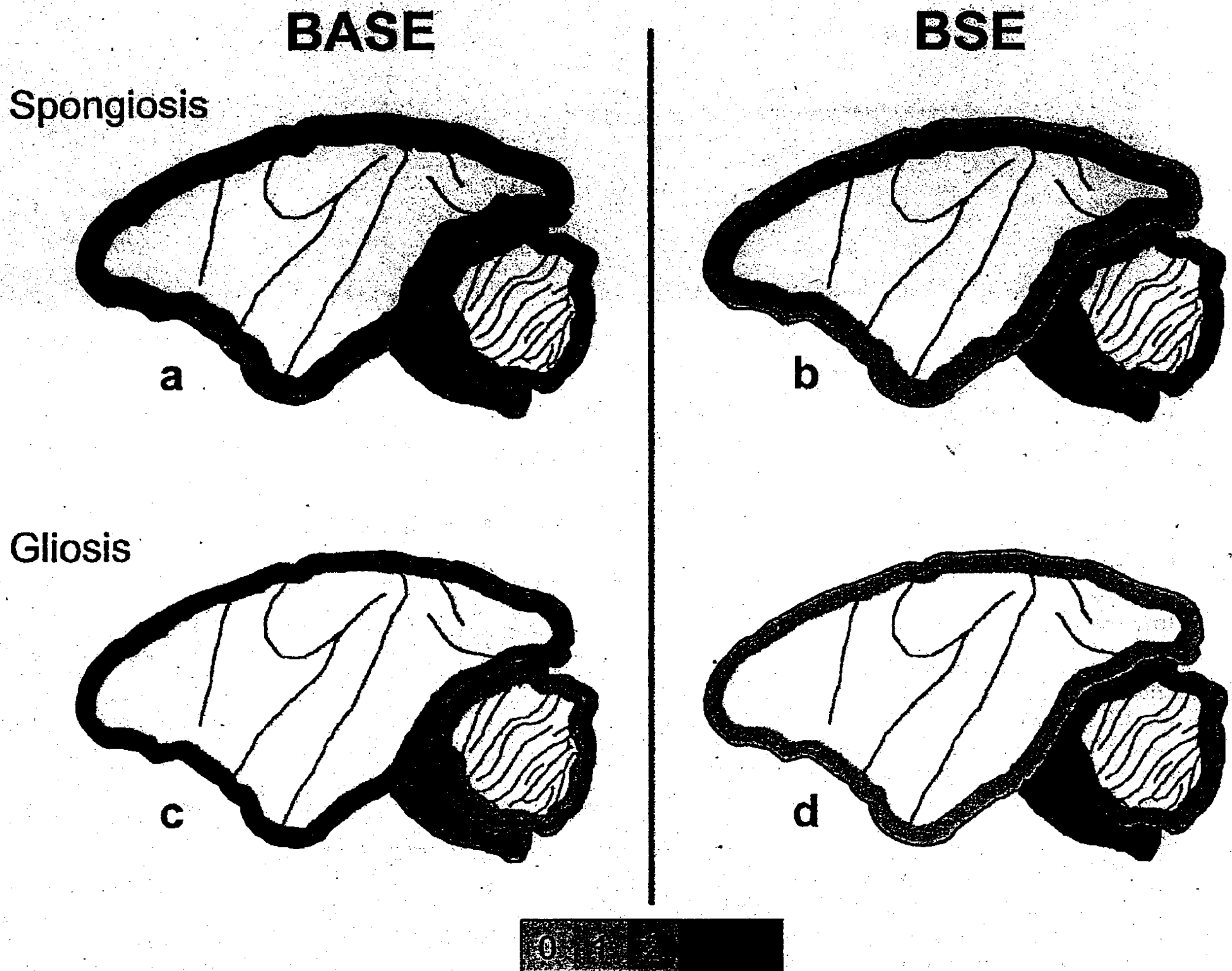

Figure 1. Diagrammatic representation of histologic lesions. Topographic distribution of spongiosis (a and b) or gliosis (c and d) in BASE and cBSE-infected primates. The lesions were scored from 0 to 4 (negative, light, mild, moderate, and severe).
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Western blot subtypes of sCJD described by Parchi et al [7]: MM1 (5 cases), MV1 (2), VV1 (1), MM2 (4), MV2 (6) and VV2 (3).

Non-human primate model

Cynomolgus macaques (*Macacca fascicularis*), captive-bred from the Centre de Recherche en Primatologie (Mauritius), were checked for the absence of common primate pathogens before importation and handling in accordance to national guidelines. Animals were maintained in biological security level 3 animal facilities and clinical examinations were performed regularly. They were humanely euthanized at the terminal stage of the disease, and tissues were either fixed in Carnoy's fluid for histological examination or snap-frozen in liquid nitrogen and stored at −80°C for biochemical analyses.

Neuropathology and immunochemistry

Neuropathology and immunochemical detection of proteinase-resistant prion protein (PrPres) and Glial fibrillary acidic protein (GFAP) was performed on brain sections as previously described [12].

PrPres analysis

Tissues were homogenized to 20% (w/v) final concentration in a 5% sterile glucose solution. PrPres was purified according to a protocol optimized for strain discrimination in ruminants [13,14] (Discriminatory kit ref 3551177, BioRad, Marnes la Coquette, France). Briefly, brain homogenates were first subjected to proteolysis using either 0.4 µg ("low" concentration) or 4 µg ("high" concentration) of proteinase K/mg of brain (final concentration) in a special buffer that partially protects the N-terminal part of PrPres in order to increase strain discrimination, and then purified PrPres was concentrated by centrifugation. Purified, non-human primate and human samples were processed for Western blot analysis as previously described: briefly, samples were separated by electrophoresis on a 12% SDS polyacrylamide gel, blotted onto a nitrocellulose membrane and detected by two mouse monoclonal antibodies: the antibody from the BioRad Discriminatory kit, which targets the epitope WGQPHGGX within the N-Terminal octarepeat region at position 57–88, and 3F4, which targets the epitope MKHM in the hydrophobic core at position 109–112. The protein bands were visualized using a peroxidase-conjugated goat anti-mouse antibody and chemiluminescence.

Results

Transmission characteristics of BASE and BSE

Clinical features. The BASE-inoculated macaque developed clinical signs after a 21 months incubation period. Clinical signs evolved slowly during the first four months, being limited to mild tremor and myoclonus, without impairment in coordination or locomotion, and without anxiety or aggressiveness. In the last month, the clinical picture rapidly worsened with evidence of major spatial disorientation (the animal did not recognize its environment and seemed lost in its cage), cognitive troubles (no recall of food location and at intervals unaccountably stopped eating) and the appearance of incoordination and disequilibrium; however, appetite and general fitness were maintained. Euthanasia was performed at the terminal

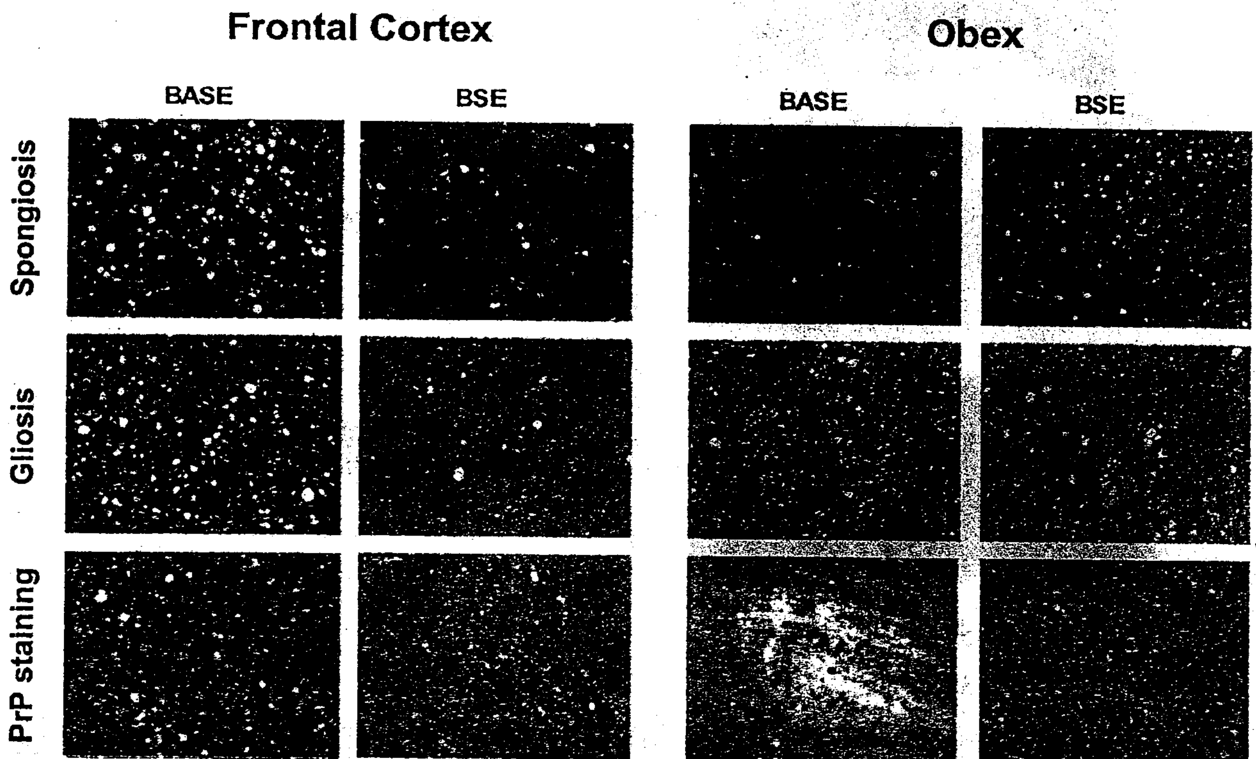

Figure 2. Histopathology and PrPres immunostaining. Spongiosis, gliosis (GFAP staining) and PrPres deposition in frontal cortex and obex in BASE- and cBSE-infected primates (original magnification ×200 for spongiosis and gliosis, ×400 for PrPres staining). Immunostaining of PrPres was performed with 3F4 monoclonal anti-PrP antibody after proteinase K treatment as previously described [11]. No staining was observed in the brain of control healthy primates (data not shown) in these conditions.
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stage of illness at 26 months post inoculation (Table 1). The two cBSE-inoculated animals had longer incubations periods (37.5 months) and survivals (40 months) despite a presumably larger infecting dose (100 mg containing a 10-fold higher PrPres concentration). Moreover, the clinical presentation was very different: animals exhibited aggressiveness and anxiety in combination with incoordination, severe ataxic tremor, and loss of appetite to the point of near starvation. The four animals inoculated with human vCJD had a clinical evolution similar to that of animals inoculated with BSE, though with less prolonged survivals (25 to 37 months).

Histopathology (Figures 1 and 2). In the BASE-inoculated animal, the cortex showed widespread spongiosis and gliosis that were especially prominent in the fourth and fifth layers. Spongiosis was intense in the frontal cortex, with a loss of pyramidal cells in the third and fifth layers. Lesions in the parietal cortex were even more severe, with a complete disappearance of neurons in the fourth layer. In the cBSE-inoculated animals, spongiosis and gliosis were more discrete, and mainly affected the occipital cortex. In the obex and cerebellum, the lesions (spongiosis and loss of Purkinje and granular cells) were less pronounced in BASE than cBSE-infected animals.

Immunohistochemistry (Figure 2). In the BASE-infected animal, PrPres was distributed in a diffuse synaptic pattern (either fine and sandy or roughly granular) with laminar enhancement in the parietal cortex but no evidence of plaques, even when stained with thioflavine T (data not shown), whereas cBSE-infected animals had weak diffuse synaptic labeling but multiple intensely-stained PrPres aggregates and characteristic plaques [9].

Strain discrimination by proteinase K sensitivity and antibody reactivity

We made use of a technique developed to discriminate and classify prion strains in small ruminants [14], based on the differential sensitivity of the octapeptide and core regions of PrPres proteins to proteinase K (PK) digestion. Controlled conditions of proteolysis allowed a strain-dependent threshold of removal of the octapeptides. This method, illustrated in Figure S1 (supplementary data), was successfully applied for the diagnosis of the first case of cBSE in a goat [15] and has now been validated by the European Commission for regular use on field. We adapted this test to primate prion strains, using only the higher PK concentration and substituting the monoclonal antibody 3F4 as the anti-core antibody to macaque and human PrP.

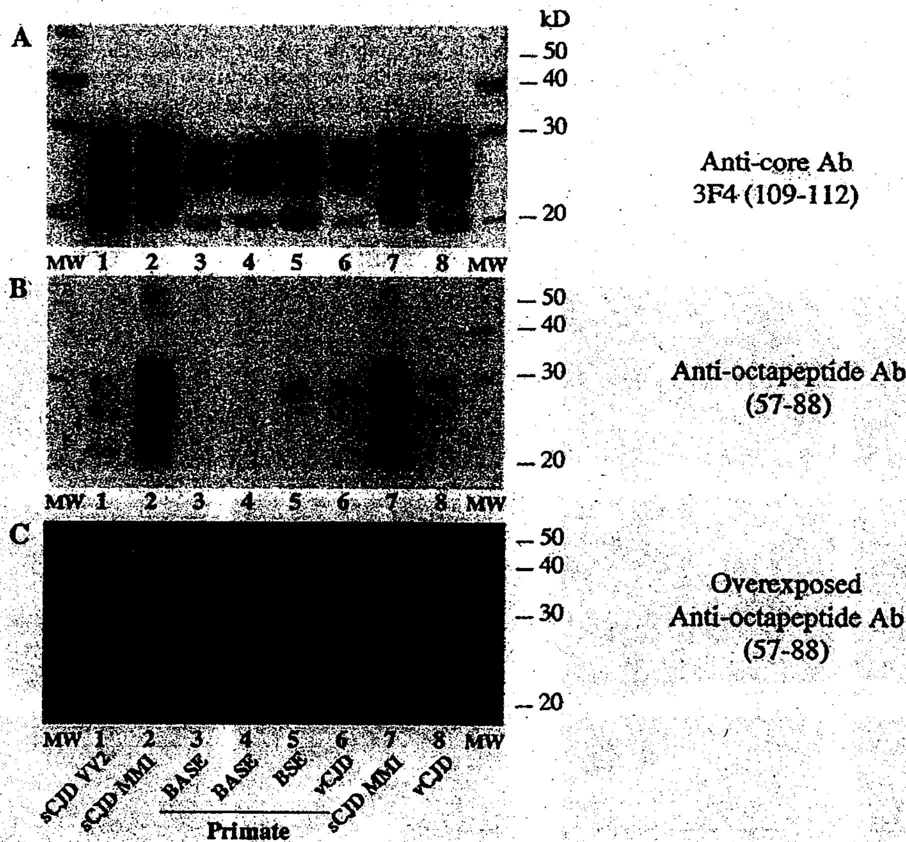

Figure 3. Electrophoretic analysis and differential sensitivity to proteolysis of PrPres in various prion diseases of primates and humans. PrPres from brain homogenates (MM1 or VV2 sCJD, vCJD in humans, or primates experimentally infected with BASE, cBSE or vCJD inocula) were purified under high concentrations of proteinase K, and detected with monoclonal antibodies recognizing either the core (3F4, panel A) or the octapeptide region (panel B and C) of the protein. Frontal cortex and obex regions of BASE-infected primate were both analysed (lanes 3 and 4 respectively). Panel C is an overexposure of the autoradiography of Panel B to detect weak signals. The absence of octarepeat region reactivity in the PrPres of the BASE in Panel C indicates a proportion at least ten fold lower than that of the vCJD or cBSE samples on the basis of a quantitative analysis of chemiluminescence signal intensities.
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Banding patterns in Western blots following pre-treatment with a high PK concentration are shown in Figure 3. Both vCJD/cBSE and BASE reacted strongly to anti-core antibody (Panel A). In contrast, vCJD/cBSE also reacted weakly to anti-octapeptide antibody (Panel B), whereas BASE reactivity was abolished (Panels B and C), indicating a gradient of resistance to proteolysis of the N terminal part of the PrPres among these strains. In cattle, the signal was abolished for both cBSE and BASE strains (data not shown).

The method also revealed notable differences of octapeptide sensitivity to PK in different types of human prion disease (figures 3 and 4). Comparisons of the relative signals with both anti-core and anti-octapeptide antibodies for each sample indicated that the N terminal part of PrPres from vCJD and the VV2 subtype of sCJD were far more sensitive than either the MM1 or VV1 subtypes (Panel B). The MV2 subtype showed a strong resistance to proteolysis that was clearly different from the BASE-infected primate; however, three of the four MM2 subtype cases exhibited the same signature as BASE, and the fourth case had a significant proportion of PrPres with an intact octapeptide region, as shown in figure 5, indicating the coexistence of two types of PrPres (the majority being type 2).

All four cases had clinical features consistent with the MM2 subtype as described by Gambetti et al [16]: comparatively long illnesses dominated by cognitive impairment followed by aphasia, and later in the course of illness the appearance of pyramidal and extrapyramidal signs together with myoclonus, but no cerebellar signs. Neuropathology was also typical of the MM2 subtype, with major cortical spongiosis and little or no involvement of the cerebellum (Table 2 summarizes the clinical, laboratory, and neuropathological features of each case).

Discussion

We have shown that BASE, the first identified atypical strain of BSE [2], originating from asymptomatic cattle, is transmissible by i.c. inoculation to a species of non-human primate. Although this observation concerned only one animal, its survival was substantially shorter than for all the macaques inoculated with classical BSE as well as the majority of those inoculated with human vCJD. Moreover, in earlier experiments by others on a total of 6 macaques inoculated i.c. with 50 mg of cBSE brain, none had an incubation period of less than 30 months [17], and humanized transgenic mice have been found to be highly susceptible to infection with BASE, and completely resistant to infection with cBSE [18]. If BASE is more pathogenic than classical BSE for primates, it could indicate a more readily transmissible infection from cattle to humans than previously suspected. A preliminary trial of oral transmission is currently ongoing for alimentary risk assessment: 49 months after oral dosing there is no indication of transmission; however, the incubation period following similar oral challenge with cBSE in an already completed experiment was 60 months.

The disease induced by BASE was different in all respects from that induced by classical BSE. The clinical presentation was characterized by mild tremors and myoclonus, progressing to a marked cognitive disorder, including spatial disorientation but without anxiety, aggressiveness or loss of appetite. In contrast, cBSE presented signs of anxiety and aggressiveness together with progressive difficulties in locomotion as well as cerebellar signs (major ataxia), and severe decrease of appetite with concurrent weight loss. The widespread spongiform lesions and loss of pyramidal cells in the third and fifth layers of the frontal cortex together with the severe parietal lesions could explain the prominent cognitive signs and the spatial disorientation seen in the BASE-infected monkey, contrasting with the severity of lesions in the obex and cerebellum consistent with the incoordination seen in animals inoculated with cBSE. Amyloid plaques, the hallmark of BASE in cattle, are not produced in the Macaque monkey, and conversely, cBSE does not produce plaques in cattle, but does so in the Macaque [9], a clear indication that plaque deposition depends as much on the host as the prion strain.

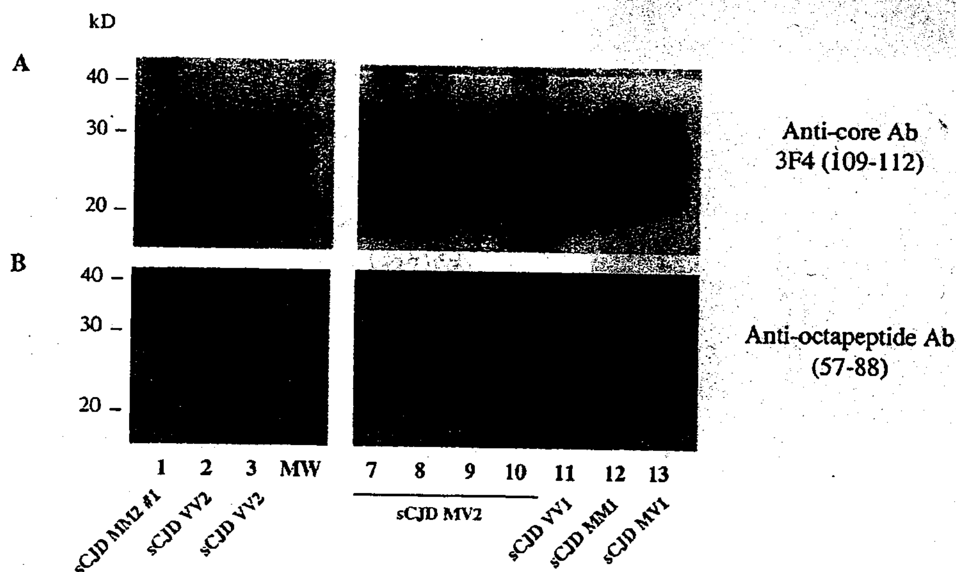

Figure 4. Electrophoretic analysis and differential sensitivity to proteolysis of PrPres in different subtypes of CJD. PrPres from human brain homogenates (MM1, MV1, VV1, MM2, MV2 or VV2 subtypes of sCJD, and vCJD) were purified under high concentrations of proteinase K, and detected with monoclonal antibodies that recognize either the core (3F4, Panel A) or the octapeptide region (Panel B) of the protein. The proportion of PrPres with an intact octarepeat region after PK exposure in VV2 and human (or macaque) vCJD was estimated to be only one-tenth and one-twentieth as high as in an MM1 sub-type of sCJD, respectively.
doi:10.1371/journal.pone.0003017.g004

At the molecular level, under conditions of high proteinase pre-treatment and detection using two antibodies reacting with either an epitope in the N terminal octapeptide repeat region or the core of PrP, BASE and cBSE were clearly distinguishable in primate. BASE was detectable only by the core antibody, whereas cBSE was detectable by both antibodies. We estimated that the proportion of octapeptide-resistant PrPres molecules in the BASE brain homogenate was only a small fraction (≤1/10) of that of the cBSE brain homogenate. The difference in octapeptide sensitivity to PK between cBSE and vCJD in macaques on the one hand, and Type 1 sporadic CJD in humans on the other hand, is similar to what was observed between cBSE and classical scrapie in sheep. This method can now be used to test both ruminant and human samples to identify similarities and differences in their molecular protein signatures, and to implement the classification of ruminant and possibly human strains.

Although classical epidemiological studies have not found any link between scrapie in sheep and goats and human CJD, newer molecular biological studies now indicate that about half of all cases of scrapie are due to previously undetected atypical strains [19] that are experimentally transmissible to sheep and mice [20]. Their risk for humans is unknown and is the subject of current studies in experimental models, including primates. cBSE has been shown to be responsible for human cases of vCJD, but the comparative risk for humans of BASE and other atypical strains of BSE is still unknown, and its clarification will require many years of epidemiological surveillance and molecular biological testing of both bovine and human populations.

The first cases of BASE in cattle had PrPres electrophoretic profiles similar to the MV2 subtype of sporadic CJD patients [2] that, together with the presence of amyloid plaques in both the cattle and the patients, suggested a possible link between BASE and this subtype of sCJD. However, our PrPres typing technique has shown that, in the primate, PrPres of other MV2 sCJD patients exhibited a resistance to proteolysis different from the BASE-infected primate, whereas PrPres from vCJD-infected patients and primates behave similarly. This observation, together with the absence of amyloid plaques in the BASE-infected primate, weakens the likelihood of a direct link between BASE and MV2 subtype sCJD patients.

In contrast, the specific signature of PrPres in the BASE-infected primate was similar to that seen in three of four patients with the MM2 cortical subtype of sporadic CJD [7]. It is interesting that an

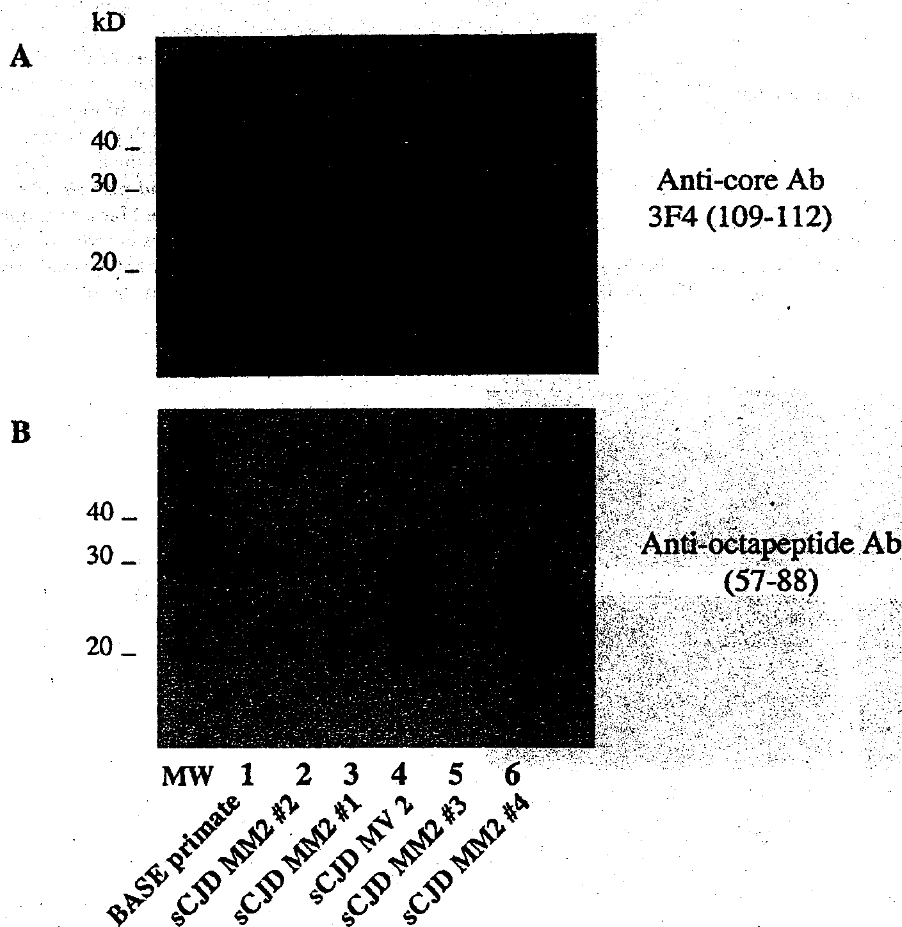

Figure 5. Electrophoretic analysis and differential sensitivity to proteolysis of PrPres in different MM2 CJD patients. PrPres from human brain homogenates (MM2 or MV2 subtypes of sCJD) and from primate experimentally infected with BASE were purified under high concentrations of proteinase K, and detected with monoclonal antibodies that recognize either the core (3F4, Panel A) or the octapeptide region (Panel B) of the protein.
doi:10.1371/journal.pone.0003017.g005

Table 2. Summary of MM2 subtype sporadic CJD patients.

Case #	1	2	3	4
Sex	Female	Male	Male	Female
Age at onset	60 years	59 years	59 years	69 years
Duration of illness	24 months	9 months	6 months	16 months
Onset	Progressive memory impairment beginning with episodic memory disturbance, then attention loss, spatial and temporal orientation	Depression, insomnia, headache. Episodic memory impairment and poor language	Memory disturbance	Depression, memory impairment
Evolution	Progressive cognitive decline, motor and sensory aphasia, progressive global impairment of all cortical functions, with assistance needed for self care. Pyramidal signs mainly characterized by hyperreflexia	Worsening of memory impairment, motor aphasia, intellectual decline, progressive loss of all cortical functions. Myoclonus, urinary incontinence, loss of self-care	Confusional state, global impairment of higher cortical functions	Progressive cognitive decline characterized by global impairment of all cortical functions. Myoclonus
Terminal stage	Diffuse spastic rigidity; Pyramidal signs; dystonic movements and sporadic myoclonic jerks	Akinetic mutism	No information available	Akinetic mutism
MRI	Cortical atrophy with hyper-intensity of the head of Caudate nucleus and Putamen in T2-weighted images	Normal (early stage of illness)	Normal (early stage of illness)	Hyperintensity on T2-weighted images of fronto-temporal cortical lobes
SPECT 99mTc-ECD	Not done	Hypo-perfusion of frontal, parietal and temporal cortices, bilaterally. Normal perfusion of subcortical ganglia and cerebellum	Hypo-perfusion of frontal, parietal and to a lesser extent temporal cortices	Not Done
MMSE	10/30	Not done	Not done	Not reported
CSF 14-3-3 protein	Positive	Positive	Not done	Negative
EEG	Diffuse slowing	Diffuse slowing	Diffuse slowing	Periodic sharp waves
Neuropathology	Cortical spongiosis, Cerebellum relatively spared	Cortical spongiosis, Cerebellum relatively spared	Cortical spongiosis, normal cerebellum	Cortical spongiosis, cerebellum relatively spared
Type PrPres	Type 2	Type 2	Type 2	Type 1+2
Resistance of N-terminal part to proteolysis	No (BASE infected primate-like)	No (BASE infected primate-like)	No (BASE infected primate-like)	Yes

doi:10.1371/journal.pone.0003017.t002

important feature of the clinical-pathological syndrome in this BASE-infected macaque – the absence of cerebellar involvement – is also a common element in patients with the MM2 subtype of human sporadic CJD (Supplementary Figure S2). However, as illustrated by the clinical details of our four tested MM2 cases, there is considerable patient-to-patient variation, just as there can be variation among individual animals experimentally inoculated with a given strain of TSE. [21,22].

It is not known whether atypical strains of BSE have been circulating for years, or represent new forms of disease, and continuing research is clearly needed to answer both this and the equally important question about a possible relationship to at least certain forms of what are presently regarded as sporadic cases of human disease (sCJD) [4,23]. Moreover, the BASE strain has been described to evolve naturally towards BSE after successive transmissions in inbred mice [6]. The stability and pathogenicity of this strain in humans remains to be determined, and it is worth recalling that the stability of the cBSE/vCJD strain, which retains its specific molecular signature in different infected hosts, is the exception rather than the rule. As has been previously observed [24–26], one patient (Case No. 4, cf. figure 5 sample MM2#4) exhibited both types of PrP, i.e. type 2 typical of the MM2 subtype and type 1 observed in the MM1 subtype. On the one hand, this demonstrates the interest of such a simple biochemical test to refine PrP analysis, and on the other hand it raises a question about the existence of different PrPres signatures in the same patient, i.e., different prion strains linked to multiple infections or to variants selected by the host.

In summary, we have transmitted one atypical form of BSE (BASE) to a cynomolgus macaque monkey that had a shorter incubation period than monkeys infected with classical BSE, with distinctive clinical, neuropathological, and biochemical features; and have shown that the molecular biological signature resembled that seen in a comparatively uncommon subtype of sporadic CJD. We cannot yet say whether BASE is more pathogenic for primates (including humans) than cBSE, nor can we predict whether its molecular biological features represent a clue to one cause of apparently sporadic human CJD. However, the evidence presented here and by others justifies concern about a potential human health hazard from undetected atypical forms of BSE, and despite the waning epizoonosis of classical BSE, it would be premature to abandon the precautionary measures that have been so successful in reversing the impact of cBSE. We would instead urge a gradual, staged reduction that takes into account the evolving knowledge about atypical ruminant diseases, and both a permanent ban on the use of bovine central nervous system tissue for either animal or human use, and its destruction so as to eliminate any risk of environmental contamination.

Supporting Information

Figure S1 Resistance to proteolysis of different prion strains in sheep. PrPres from brain homogenates of sheep infected with classical scrapie, experimental cBSE, or atypical Nor-98 scrapie, and of an uninfected control sheep. Samples were purified using low (odd lanes) or high (even lanes) concentrations of proteinase K, and visualized with monoclonal antibodies that recognize either the core region (Panel A) or the octapeptide region (Panel B) of the protein. With the lower concentration of PK used in the purification step (in order to maximize test sensitivity) of one widely utilized BSE screening test [13], all three strains gave a positive result with both the anti-core and anti-octapeptide antibodies (odd lanes). Using a higher concentration of PK (even lanes) did not alter the positivity with either antibody for classical scrapie, but the cBSE strain no longer reacted with the anti-octapeptide antibody while Nor98 did not react with either antibody. Thus, by using the higher concentration of PK and two different antibodies, it is possible to discriminate between all three strains.
Found at: doi:10.1371/journal.pone.0003017.s001 (2.51 MB TIF)

Figure S2 Lesion profiles in cBSE- and BASE-infected macaque, and in MM2 sporadic CJD patients. The lesions were scored from 0 to 4 (negative, light, mild, moderate, and severe) for the different following gray matter regions: frontal (FC), temporal (TC), parietal (PC) and occipital (OC) neocortices, hippocampus (HI), parasubiculum and entorhinal cortex (EC), neostriatum (ST) (nuclei caudatus and putamen), thalamus (TH), substantia nigra (SN), midbrain periventricular gray (PG), locus ceruleus (LC), medulla (ME) (periventricular gray and inferior olive), and cerebellum (CE). Scoring for MME sCJD patient was issued from Parchi et al. [7].
Found at: doi:10.1371/journal.pone.0003017.s002 (1.52 MB TIF)

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Author Contributions

Conceived and designed the experiments: CC CIL. Performed the experiments: NLE SF DM FA. Analyzed the data: EEC NLE MMR SF JPD. Contributed reagents/materials/analysis tools: CC GZ MMR MC. Wrote the paper: EEC GZ PB JPD. Participated to the final reviewing of the manuscript: CC SM NS MC PL CIL.

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別紙3-4

医薬品
医薬部外品　研究報告　調査報告書
化粧品

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008年5月8日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Estimating the pathogen safety of manufactured human plasma products: application to fibrin sealants and to thrombin. Horowitz, B. and Busch, M. Transfusion, 48, 1739-53 (2008)	公表国 米国	
販売名（企業名）						

研究報告の概要	使用上の注意記載状況・その他参考事項等
本稿では，米国で最近上市された2種類の血液製剤（トロンビン及びフィブリノゲン）を用いて，パルボウイルスB19（B19V），A型，B型及びC型肝炎，HIVならびに変異型クロイツフェルト・ヤコブ病（vCJD）関連プリオンの病原体感染リスクを評価した。特に，A型肝炎ウイルス（HAV）及びB19Vは，エンベロープを持たないため不活化がより困難であることから本試験の対象とした。これら血液製剤の製造過程では2つの異なるウイルス除去工程が使われている。各病原体の安全域は，出発原料内の最大ウイルス量と製造工程の除去能との比較によって決定した。フィブリノゲン及びトロンビンは，ともに1バイアル当たりの病原菌が伝播する残遺リスクが極めて低く，A型，B型及びC型肝炎及びHIVについては10^{15}分の1未満と算出された。B19Vについては，トロンビンが1000万分の1未満，フィブリノゲンが50万分の1未満と算出された。同様に，vCJDの病原体伝播リスクも非常に低いと推定された。新型ウイルス（西ナイルウイルス，H5N1型インフルエンザウイルス，重症急性呼吸器症候群（SARS）ウイルス又はチクングニヤウイルスーいずれもエンベロープを持つ）の脅威に関しては，現在の製造工程が完全な不活化をもたらすことがわかっている。したがって，血漿製造業界は，血液製剤の安全性を増強するために過去10～20年に実施された多くの改善点により，優良な安全性プロファイルをもつ製品の製造し，提供していると著者らは結論付けた。	BYL-2008-0323

報告企業の意見	今後の対応
著名な2研究機関が発表した本研究では，新たな脅威として現れたエンベロープウイルスに対する完全不活化法や非エンベロープウイルスやプリオンに対する実質的除去法など，病原体不活化法が常に改良され続けてきたことを確認している。したがって，実質的リスクが存在する血液製剤と違い，血漿分画製剤における病原体伝播リスクは極めて低いと考えられる。	現時点で新たな安全対策上の措置を講じる必要はないと考える。

Estimating the pathogen safety of manufactured human plasma products: application to fibrin sealants and to thrombin

Bernard Horowitz and Michael Busch

BACKGROUND: Plasma fractionators have implemented many improvements over the past decade directed toward reducing the likelihood of pathogen transmission by purified blood products, yet little has been published attempting to assess the overall impact of these improvements on the probability of safety of the final product.

STUDY DESIGN AND METHODS: Safety margins for human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), hepatitis A virus (HAV), parvovirus B19, and variant form of Creutzfeldt-Jakob disease (vCJD) were calculated for the two fibrin sealants licensed in the United States and for thrombin. These products were selected because their use in a clinical setting is, in most cases, optional, and both were relatively recently approved for marketing by the US Food and Drug Administration (FDA). Moreover, thrombin and fibrinogen both undergo two dedicated virus inactivation steps and/or removal steps in accord with the recommendations of regulatory agencies worldwide. Safety margins were determined by comparing the potential maximum viral loads in contaminated units to viral clearance factors, ultimately leading to the calculation of the residual risk per vial.

RESULTS: The residual risk of pathogen transmission per vial was calculated to be less than 1 in 10^{-15} for HIV, HCV, HBV, and HAV for both fibrinogen and thrombin. Owing to the greater quantities that can be present and its greater thermal stability, the calculated risk for parvovirus transmission was 1 in 500,000 vials for fibrinogen and less than 1 in 10^{7} per vial for thrombin. Assuming that vCJD is found to be present in plasma donations, its risk of transmission by these purified and processed plasma derivatives would appear to be very low.

CONCLUSIONS: The pathogen safety initiatives implemented by plasma fractionators over the past 10 to 20 years have resulted in products with excellent pathogen safety profiles. Of the agents examined, parvovirus continues to have the lowest calculated margin of safety. Despite this, parvovirus transmissions should be rare. Manufacturers are encouraged to continue exploring processes to further enlarge parvovirus safety margins and to continue exploring ways of eliminating prions.

Products derived from human plasma have important therapeutic uses, including substitution therapy for hemophilia and primary immune deficiency disorders, plasma expanders after trauma and surgery, and as hemostatic agents.[1-3] Plasma proteins and their functions are so diverse that new applications for currently licensed plasma protein products continue to be investigated[4] and novel plasma protein products continue to be developed.[5-7] Consequently, there has been an increase in the quantity of plasma processed worldwide, and significant improvements have been made in manufacturing procedures and in plant design and operation. Many of these improvements were implemented with the goal of assuring safety of plasma derivatives from transfusion-transmissible pathogens. These include 1) improved selection of donors, 2) use of plasma only from "qualified" donors who repeatedly pass viral screening procedures, 3) use of nucleic acid amplification testing (NAT) methods to detect and eliminate virus before the pooling of donor units, 4) inventory hold policies that allow interdiction of "window-phase"

ABBREVIATIONS: BVDV = bovine viral diarrhea virus; HAV = hepatitis A virus; ID(s) = infectious dose(s); PRV = pseudorabies virus; SARS = severe acute respiratory syndrome; S/D = solvent/detergent (method of virus inactivation); TNBP = tri-(*n*-butyl)phosphate (the solvent in S/D treatment); vCJD = variant form of Creutzfeldt-Jakob disease that infects man and presumptively has arisen from the epidemic of bovine spongiform encephalopathy in cattle; WNV = West Nile virus.

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TRANSFUSION **;**:**-**.

units before pooling, 5) employment of purification procedures shown to remove virus or prions should they be present, 6) the use of two complementary or "orthogonal" methods of virus inactivation, and 7) the engineering and design of facilities so as to prevent contamination of downstream process streams with upstream fractions.

Products that promote hemostasis and tissue sealing following trauma and surgery are among the more recently licensed human plasma products in the United States. Two fibrin sealants, one from Omrix (New York, NY) and one from Baxter (Deerfield, IL), are licensed in the United States by the Food and Drug Administration (FDA), and Omrix also recently received approval of a topically applied thrombin. While they cannot be used in all surgical settings, such as to control high-pressure (arterial) bleeds, these products have been shown to improve surgical outcomes, reduce the time to hemostasis, reduce blood loss, and reduce surgical complications.[8] Substitutes for these human plasma-derived hemostatic agents have also been developed, including bovine thrombin and recombinant-derived human thrombin. Bovine thrombin is antigenically distinct from human thrombin and has been shown to elicit antibodies when used in man.[9] These antibodies, as well as antibodies elicited to bovine impurities in the product, especially antibodies to coagulation factor (F)V, have resulted in severe bleeding complications due to cross-reaction with their human counterparts.[10-13] Higher purification has reduced this complication, although a recent report[14] indicates that antibody formation still occurs. Products made by recombinant technology have their own, somewhat unique, issues. Depending on the gene construct used and the cell line chosen, the amino acid sequence may differ from that which occurs naturally, and differences in posttranscriptional processing often result in altered patterns of glycosylation or other molecular changes.[15-19] Consequently, immunogenicity is a potential problem that needs to be continually assessed. Also, depending on specific production details, manufacturing procedures must employ steps designed to inactivate and/or remove viral contaminants (and other potential pathogens) known to be present in the cell line and/or in the culture medium employed.[20] Additionally, in many circumstances, the higher cost associated with recombinant proteins limits their use.

In the past decade, many estimates of the viral safety of transfused whole blood and its components (i.e., red blood cells, platelet concentrates, and fresh-frozen plasma) have been published, with each passing year showing improved viral and bacterial safety.[21,22] In the same time frame, aside from monitoring clinical outcomes and despite the aforementioned improvements, little has been published to assess the parallel increase in safety of manufactured plasma products. A recent publication by Janssen and colleagues[23] used a probabilistic, Monte Carlo model to estimate the risk of human immunodeficiency virus (HIV) and hepatitis B virus (HBV) in a hypothetical plasma derivative subjected to what appears to be a single method of viral inactivation. Based on their assumptions, they calculated that the risk per vial approximated 1 in 1 million. Given these improvements, the recent licensure of human plasma-derived topical thrombin, and the frequent surgical use of fibrin sealants, consisting of fibrinogen in addition to thrombin, it is timely to estimate their pathogen safety. These estimates are especially useful since the fibrinogen component of fibrin sealants is among the least processed blood derivatives, while the manufacturing procedures for thrombin, whether part of a fibrin sealant kit or used by itself, are typical of those employed with most newer plasma derivatives. Thrombin and fibrinogen both undergo two dedicated virus inactivation steps and/or removal steps. Fibrinogen and thrombin from each company are each treated by solvent/detergent (S/D). Additionally, Omrix pasteurizes its fibrinogen and nanofilters its thrombin, while Baxter vapor heats both components following lyophilization. The fibrinogen preparation cannot be nanofiltered without suffering large losses in fibrinogen and fibronectin due to their large size. The presence of fibronectin may be important since it contributes to cell adhesion.[24] For HIV, hepatitis C virus (HCV), and HBV, this report updates estimates made by one of us (BH) in 1990[25] using better information on viral loads than was available then and enlarges the pathogen list to include hepatitis A virus (HAV) and parvovirus, both of which are nonenveloped viruses, and the prion that causes variant form of Creutzfeldt-Jakob disease (vCJD). It is anticipated that the method of approach reported here can be applied to other existing or experimental blood protein products.

MATERIALS AND METHODS

Two distinct methods can be used to calculate pathogen safety. The first is to calculate safety margins by comparing the number of infectious units or doses of pathogen in the starting material to the clearance capacity of the manufacturing process. The second is to measure clinical outcomes, comparing the incidence of transmission to the quantity of product infused. Transmissions in a clinical setting should be considered the gold standard since they involve actually measuring what we want to know, and with this information, one can back-calculate clearance capacities of processes for known pathogen burdens. On the other hand, clinical studies of the type required can be extremely lengthy and expensive and the results possibly misleading. The former method has the advantage that estimates of safety can be made in advance of clinical testing. Moreover, the safety margins calculated for a wide range of viruses likely will also be applicable to unstudied and newly emerging viruses.

Product safety margins can be calculated by comparing potential viral loads with the viral clearance capacity by the formulas

$$VL = N \times C,$$

where VL is viral load, N is the number of units in a plasma pool containing infectious virus, and C is the concentration of virus in those units, and

$$\text{Safety Margin} = CC/VL,$$

where CC is the clearance capacity or the ability of the process to remove or inactivate the infectious agent being studied.

Viral load

The pathogens of interest for manufactured plasma products are largely viruses that are present in blood predominantly as cell-free virions (e.g., HBV, HCV, HIV, HAV, and parvovirus B19). Other examples include West Nile virus (WNV) and dengue viruses. The newly described vCJD agent, presumably a prion, is also a potential concern despite the absence of evidence that it is transmitted by purified plasma protein products.[26,27] Cell-associated viruses like cytomegalovirus, Epstein-Barr virus, and human herpes virus 8 are not a concern since infected cells are removed by the apheresis and filtration procedures in common use. Bacteria and fungi are also effectively removed by the terminal sterile filtrations applied to all biologic products, including plasma products, recombinant products, monoclonal antibodies, and so forth, and therefore will not be addressed here.

For the major transfusion-transmitted viral pathogens, the viral loads are typically measured as genome-equivalents (geq) per mL of plasma based on results of quantitative NAT. These loads vary dramatically during the progressive stages of infection with the highest viral loads seen transiently during the acute preseroconversion (i.e., so-called window period) stage of infection; moreover, infectiousness is also highest during this same period.[28-30] Subsequent to antibody seroconversion (and coincident with innate and adaptive cellular immune responses to infection), the agents are 1) eliminated from the body (e.g., eradication of infection, as occurs with WNV, HAV, and dengue); 2) cleared from plasma but with persistence of cell-associated virus in tissues (e.g., latent infections such as herpes viruses, parvovirus B19, and "occult" HBV infections); or 3) persistent at reduced concentrations in plasma (i.e., so-called set-point viremia after establishment of chronic HBV, HCV, and HIV infections). In addition to variations in viral load measured by NAT, as infections evolve the infectivity of viruses change profoundly.[31-39] For HIV (and its model agent simian immunodeficiency virus), HBV, HCV, and WNV, it is now well established that during the acute preseroconversion phase of infection (pre–ramp-up and ramp-up stages), virion particles in plasma are highly infectious, with 10 or fewer geq in the entire volume of plasma sufficient to transmit infection following parenteral injection. In contrast, viral particles present in plasma from the same infected individuals have significantly (10- to 1000-fold) reduced infectious potential weeks to years after seroconversion.[29-35,40-43] The reduced infectivity of plasma virus from postseroconversion phases of chronic infection is attributable to a combination of factors, including presence of endogenous neutralizing antibodies, generation of defective virions (i.e., lacking full genomes or other required infectivity factors), and immune selection of virions with reduced fitness. Hence, viral load distributions observed during acute versus chronic stages of infection need to be adjusted by a factor to account for the relative infectivity of virion particles to derive estimates for the functional viral load during each stage.

All donated blood in the United States, whether for the preparation of components or for use in manufactured plasma products, is screened by serologic assays for HIV-1 and -2, HBV, HCV, and human lymphotropic virus-1 and -2 and by NAT for HIV and HCV. Donors are also excluded if they have certain risk factors that make their exposure to viruses or prions more likely. Additionally, plasma manufacturers screen donated plasma in a minipool format for HBV, HAV, and parvovirus by NAT. The use of NAT greatly reduces viral loads since positive units missed by serologic screening procedures typically have the highest concentrations of virus, which is also highly infectious. Consequently, with very rare exceptions of concordant testing errors in serology and NAT screening, only units that test both serologically negative (i.e., window-phase units) and that have relatively low titers of infectious virus (<500-5000 infectious doses [IDs]/mL) are pooled. Furthermore, manufacturing pools are retested by NAT before fractionation to assure that high-titer viremic units were not missed as a result of erroneous testing. As a result, the probability that a fractionation pool contains a significant level of virus is extraordinarily remote.

Pathogen infectious load estimates are given in Table 1. To estimate the number of positive units missed by the screening procedures employed, currently observed NAT yields, expressed as number of positive samples per million donations, were adjusted to account for the amount the window period is believed to be closed through the use of NAT. From this, we conclude that few plasma pools will contain HIV, HCV, or HAV while contamination by HBV and parvovirus B19 will be considerably more frequent. Based on the analytical sensitivity of the NAT assays, the dilution factors during assay, and the volume of an individual donor unit, we calculated the

TABLE 1. Viral load estimates

Virus	NAT yield (number/million donations)* (A)	Percent NAT closes window period† (B)	Number of positive units missed by NAT/million donations (C) = (A/B – A)	NAT analytic sensitivity‡ (geq/ml)	NAT operational sensitivity‡ (geq/mL in the donor unit)	Maximum genomic load (log geq/ fractionation pool)§	Ratio of IDs to geq¶	Maximum viral load (log ID/pool)
HIV	0.58	48% (11/23)	0.63	1.40	717	5.7	1:1	5.7
HCV	4.08	88% (50/57)	0.56	3.10	1,587	6.0	1:1	6.0
HBV	13	23% (10/43)	44	0.66	338	5.4	1:10	4.4
HAV	0.30	71% (5/7)	0.12	2.0	1,024	5.9	1:1,000	2.9
Parvovirus B19	50.5	71% (5/7)	20.6	22.6	5,120,000	9.6	1:1,000	6.6
vCJD						4.3	1:1	4.3

* For HIV, HCV, and HBV, a NAT yield unit is defined as an antibody- or hepatitis B surface antigen (HBsAg)-negative donation detected by RNA and/or DNA screening using pooled NAT systems. Rates presented are published rates from United States and European whole-blood donor screening programs.[76-78] Although NAT yield rates for these viruses among source plasma donors are higher, this is offset by source plasma policies that stipulate that only plasma from "qualified donors" be released for fractionation and that frozen units be held in inventory enabling interdiction of quarantined potential window-phase units when donors later test reactive for infectious disease markers or are deferred for other reasons.[79] For HAV and parvovirus B19, we use the rate of detection of high titer viremic donations by low sensitivity NAT screening of whole-blood and plasma donors, irrespective of serostatus of viremic units.[80,81]

† The percentage is determined by dividing the number of days NAT detects positive samples by the number of days from when a donor becomes infectious until there is sufficient antibody to be detected serologically (HIV, HCV, HBV) or there is sufficient antibody to render the donation noninfectious (HAV, parvovirus; see Busch et al.[82] for conceptual basis for this approach and Kleinman and Busch[83] for application of this approach to HBV). The residual infectious window periods are defined as the number of days from viremia reaching the minimal infectious threshold (set as 1 copy per 20 mL of plasma; Busch et al.[84]) to the level of viremia detected by pooled-sample NAT, using the viral doubling-times during the acute ramp-up phases established for each agent (20.5 hr for HIV, 10.8 hr for HCV, 2.6 days for HBV, and approx. 1 day for HAV and parvovirus B19). This yielded pre-NAT infectious window periods of 12 days for HIV, 7 days for HCV, 33 days for HBV, and 2 days for HAV and parvovirus B19. The NAT detection windows are based on time from reaching the 50 percent sensitivity of the NAT screening assays to the point of seroconversion for HIV (11 days), HCV (50 days), and HBV (10 days) or the duration of the estimated NAT yield window period for HAV (5 days) and parvovirus B19 (5 days).

‡ We assumed that the 50 percent sensitivity levels for assays used by source plasma donors are in the same range as those reported by the National Genetics Institute (NGI). NGI NAT assays are used by approximately 60 percent of the source plasma sector for all five viruses, as well as by the American Red Cross for HAV and parvovirus B19. For HIV-1, HBV, and HCV, the analytic sensitivity quoted is that of the assay itself without taking sample dilutions or pooling into account (Schreiber et al.[79]). Operational sensitivity takes these dilutions into account and refers to the maximum quantity that could be present in the contaminated donor unit. For parvovirus, Omrix's acceptance requirement for a pool of 512 units is less than 10,000 geq per mL, and thus for operational sensitivity we used $10,000 \times 512$ (the number of units in the minipool).

§ NAT operational sensitivity was multiplied by 700, the assumed volume of the donation. Based on the number of units missed by NAT per million donations and a pool size of 6000 L, we assume that only one positive unit will enter a fractionation pool. For vCJD, we assumed 30 ID per mL in a contaminated unit.

¶ For HIV and HCV, the infectious load is considered to be equivalent to the viral load expressed in geq or copies, given that we are restricting consideration to the acute preseroconversion viremic phase, which is known to be highly infectious (see text), and that seropositive units from other donors, which might contain neutralizing antibodies, have been detected by serologic screening and excluded from the manufacturing pools. For HBV, we similarly assume high-level infectivity of window-phase donations[35,85] but reduce this to a ratio of 1 in 10, in part, because of the likely presence of anti-HBsAg in the plasma pool. We used a ratio of 1:1000 for HAV and parvovirus. We believe this to be justified since the neutralization capacity of anti-HAV is well established. While the ratio of infectious units to geq for parvovirus is unknown, results from tissue culture infectivity studies indicate that the ratio is 1:5000 for genotype 1 and 1:260,000 for genotype 2[86] for products devoid of parvovirus antibody, the lowest ID that has been reported on infusion into a seronegative recipient is 2×10^4 geq,[70] and the infectivity of products containing parvovirus antibody has been shown to be reduced considerably.[83,87,88]

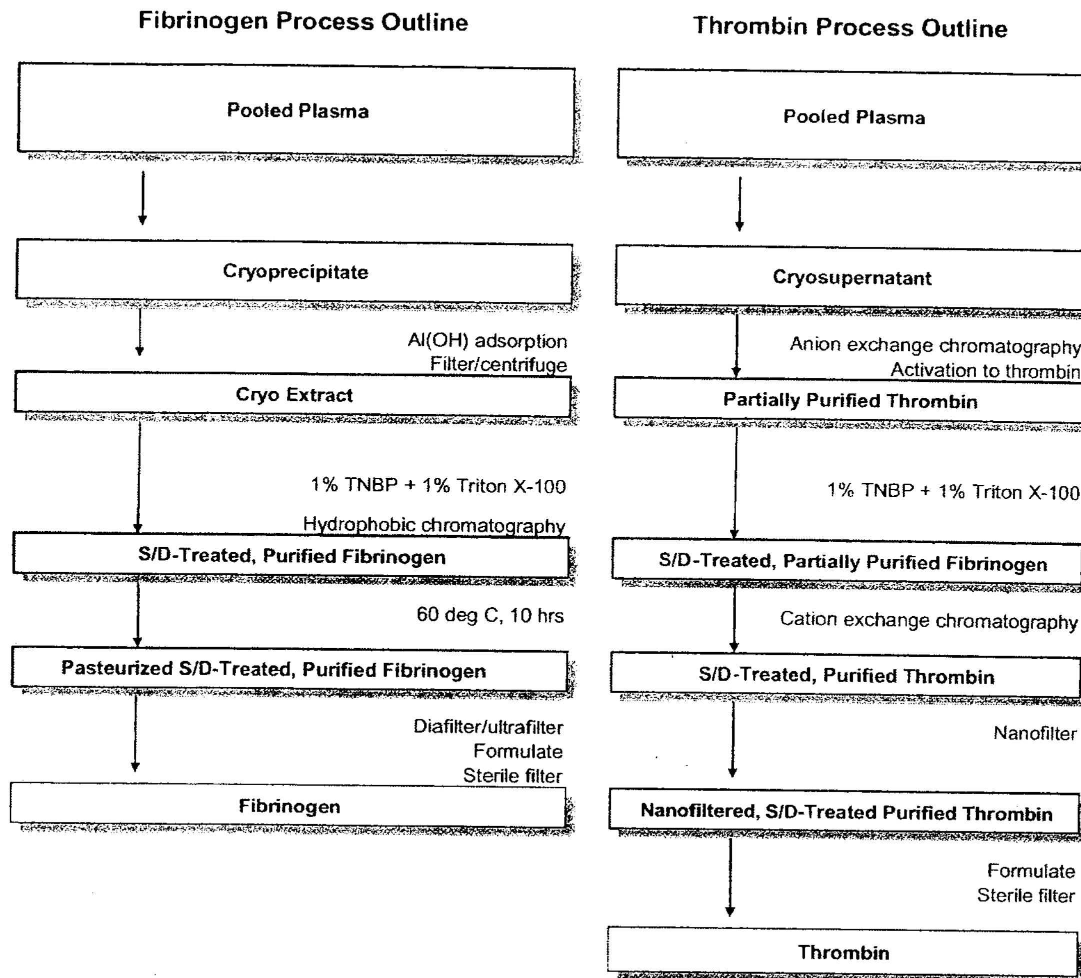

Fig. 1. Process outlines for fibrinogen and thrombin.

maximum genomic load likely to be present in a fractionation pool (Table 1, Column 7). This was adjusted downward for HBV, HAV, and parvovirus B19 to account for the reduced infectivity of virus that occurs as a result of neutralizing antibodies derived from other donors in the pool, since antibody screening is not performed for these prevalent agents. No adjustment was applied to HIV or HCV since later-stage infections with potential neutralizing antibodies are interdicted by the currently deployed serologic tests. Although attempts to transmit vCJD by human plasma have failed,[44] epidemiologic evidence supports its transmission by whole blood and blood components.[45-47] Based on animal models, its concentration is likely to be quite low, estimated at 20 to 30 IDs per mL.[48] With the use of this estimate, the maximum concentration in the plasma pool approximates 0.003 IDs per mL (3 IDs/L), and the total maximum load in a 6000-L plasma pool will approximate $10^{4.3}$ IDs (Table 1).

Clearance capacity

The clearance capacity for pathogens is a function of the extent to which pathogen is removed during steps designed to purify the protein of interest, the inclusion within the manufacturing process of dedicated viral inactivation and removal steps, the presence of neutralizing antibody in final product, and serendipitous inactivation that occurs. The process steps for fibrinogen and thrombin used by Omrix are outlined in Fig. 1. As is typical of modern plasma protein products, each process includes two dedicated, viral elimination steps: fibrinogen is treated with S/D and is pasteurized, and thrombin is treated with S/D and is passed through a purposefully designed, virus removal filter (so-called nanofiltration). Additionally, each chromatographic step and filtration in the presence of filter aid can contribute to pathogen removal. Baxter's Tisseel is processed similarly except, when first introduced in 1998, it utilized vapor heating as

TABLE 2. Validated viral elimination when processing fibrinogen: Omrix process

		Log kill or removal				
	Virus:	HIV-1	BVDV	PRV	HAV	CPV
	Model for:	HIV	HCV	HBV	HAV	Parvovirus B19
Step	Enveloped virus?:	Yes	Yes	Yes	No	No
Cryoprecipitation + Al(OH) treatment		ND	ND	ND	1.5	1.5
S/D treatment		>4.4*	>4.4†	>4.4†	ND	0
Pasteurization		>4.4‡	>5.5‡	ND	>5.8‡	1.3
	Sum:	>8.8	>9.9	>4.0	>7.3	2.8

* No infectivity after 5 minutes. Treatment is for 4 hours.
† No infectivity after 10 minutes, the first time point taken.
‡ 9 to 10 hours were required to achieve reported kills. Treatment is for 10 hours.
Al(OH) = aluminum hydroxide; CPV = canine parvovirus; ND = not done.

TABLE 3. Validated viral elimination when processing thrombin: Omrix process

		Log kill or removal						
	Virus:	HIV-1	BVDV	Sindbis	PRV	EMCV	CPV	MMV
	Model for:	HIV	HCV	HCV	HBV	HAV	Parvovirus B19	Parvovirus B19
Step	Enveloped virus?:	Yes	Yes	Yes	Yes	No	No	No
Cryo removal		ND	ND	ND	ND	ND	ND	ND
Anion-exchange chromatography		ND	ND	ND	ND	ND	ND	ND
S/D treatment		>5.8*	>4.7†	>5.3‡	>4.3†	ND	0	ND
Cation-exchange chromatography		ND	ND	ND	ND	ND	ND	ND
Nanofiltration		>4.4	ND	>5.3	>5.5	7.0	5.9	5.8
	Sum:	>10.2	>4.7	>10.6	>9.8	7.0	5.9	5.8

* No infectivity after 5 minutes. Treatment is for 6 hours for thrombin.
† No infectivity after 10 minutes, the first time point taken.
‡ No infectivity after 15 minutes, the first time point taken.
EMCV = encephalomyocarditis virus; MMV = mouse minute virus; ND = not done.

its sole, dedicated virus inactivation step; S/D treatment has been added recently. Another difference is that its thrombin component is isolated starting with Baxter's activated prothrombin factor complex.

The FDA and other applicable regulatory authorities demand that formal viral inactivation and/or removal studies be performed and that these adhere to international standards as they relate to the selection of viruses to be used, the conduct of these studies under Good Laboratory Practice guidelines and the calculations provided. We need not reiterate those guidelines here, except to say that the model viruses selected were chosen to represent multiple viral types and, in particular, the viruses of concern for products derived from human blood. Thus, viral elimination studies typically use HIV, bovine viral diarrhea virus (BVDV; model for HCV), pseudorabies virus (PRV; model for HBV), HAV or another picornovirus such as encephalomyocarditis virus, and canine parvovirus (or another model for human parvovirus B19).

The results from these formal studies for the fibrinogen and thrombin components of Omrix's and Baxter's fibrin sealant products are given in Tables 2 and 3 and Table 4, respectively (see product package inserts, with updates from manufacturers; see Acknowledgments). The clearance factors for enveloped viruses and the models for HIV, HCV, and HBV exceed the challenge dose for each of the dedicated viral elimination steps (i.e., S/D, pasteurization, nanofiltration, and vapor heating). Consequently, when the same virus has been studied in each of the two dedicated steps, the validated clearance factors exceed 9 log, and where higher doses of virus have been used or more steps validated, clearance factors as large as 18 log have been reported. The validated clearance of nonenveloped viruses is significantly less than for enveloped viruses since only one of the two dedicated viral elimination methods is effective against these viruses. Parvoviruses are a special case since they are especially heat-stable, and only 1 to 2 log of animal parvoviruses are inactivated by either pasteurization or vapor heating. It should be noted, however, that human parvovirus B19 may be more heat-sensitive than the models used here.[49] Nanofiltration is significantly more effective, and Omrix has shown for its thrombin preparation that nanofiltration removes approximately 6 log of parvoviruses.

A more complete estimate of safety margin needs to take into account the contribution of the other steps in the process that contribute to safety despite not being formally validated. It is commonly accepted that immune neutralization contributes to HAV and parvovirus B19 safety and that the neutralization capacity of antibodies to

TABLE 4. Validated viral elimination when processing fibrinogen and thrombin: Baxter process

	Log kill or removal				
Virus:	HIV-1	BVDV or TBEV	PRV	HAV or ERV	MMV
Model for:	HIV	HCV	HBV	HAV	Parvovirus B19
Process and step / Enveloped virus?:	Yes	Yes	Yes	No	No
Fibrinogen					
Cryoprecipitation + wash	2.6	1.3	1.5	1.8	ND
Lyophilization + vapor heating	>6.2	>6.8	>7.1	>6.5	1.5
S/D treatment	>6.6	>6.5	>6.7	NA	NA
Sum:	>15.4	>14.6	>15.3	>8.3	>1.5
Thrombin					
Cryoprecipitation + wash	1.4	ND	1.1	ND	ND
Anion-exchange chromatography	2	ND	3.1	ND	ND
Lyophilization + vapor heating	>5.3	>5.9	>7.0	>4.7	1.0
S/D treatment	>5.2	>6.0	>6.9	NA	NA
Sum:	>13.9	>11.9	>18.1	>4.7	1.0

MMV = mouse minute virus; NA = not applicable; ND = not done.

TABLE 5. Assignment of additional virucidal activity based on reserve capacity

Time required for complete kill (% of total)	Estimated minimal additional cidal power (log)
>100	0
76-100	1
51-75	2
26-50	3
≤25	4

these viruses is at least 3 to 4 log.[50] Since fibrinogen is purified by simple precipitations, it, like intermediate-purity FVIII preparations, likely benefits from the copresence of antibody in the final preparation.[51] Ion-exchange chromatography typically removes 2 to 3 log of virus.[52-55] Finally, some contribution to the calculation of safety margins should be ascribed to the "reserve capacity" of the viral inactivation method(s) employed, defined as the ability to achieve complete virus kill in a fraction of the treatment time allotted. While numerous publications make clear that linear extrapolation of virus inactivation curves overstates inactivation potential,[56,57] assigning no benefit to reserve capacity when calculating safety margins clearly underestimates inactivation capacity. Unless data indicate otherwise, we propose adopting the scheme described in Table 5. Although seemingly arbitrary, this scheme has the value of simplicity. Its use is supported by the dozens if not hundreds of times results with S/D and heat treatment methods have been reproduced, thereby increasing the quantity of virus subjected to challenge. Also, for S/D treatment methods, viral kill has been shown to be complete even when using tri-(*n*-butyl)phosphate (TNBP) together with sodium cholate, a combination that provides far slower kill kinetics than the more frequently employed TNBP-Tween 80 or TNBP-Triton X-100 combinations (Fig. 2), and the fact that reagent concentration can be halved without affecting viral kill (data not shown). We have not made reserve capacity estimates for vapor heating since much of the loss in viral infectivity occurs before initiation of the heat cycle (Fig. 3).

Taking these factors into account, along with published information on the inactivation of HIV, HCV, and HBV,[58] for fibrinogen, we estimate that 15 to 17 log of enveloped viruses, 10 to 11.5 log of HAV or most other nonenveloped viruses, and 7 log of parvovirus are eliminated. For thrombin, we estimate that 17 to 22 log of enveloped viruses, 11 to 13 log of HAV, and 7 to 10 log of parvoviruses are eliminated (Table 6).

Calculation of safety margin

The calculated margins of safety are given in Table 7. For enveloped viruses, safety margins are exceedingly large, estimated at about 100 billion-fold for fibrinogen and 1 trillion-fold for thrombin. Although lower, the safety margins for HAV for both fibrinogen and thrombin exceed 1 million-fold. Owing to the potentially significantly higher content of parvovirus and its greater resistance to inactivation, fibrinogen enjoys only a small safety margin while that for thrombin is approximately 2000-fold. Even still, when expressed as risk of transmission per vial, a calculation typically required by regulatory authorities, the risk with fibrinogen is calculated at 1 in 500,000 vials and that with thrombin is approximately 1 in 100 million vials or less. Thus, parvovirus transmission should still be an infrequent event.

Clinical experience

Tisseel has been marketed in the United States since 1998, and in Europe it was introduced clinically more than a decade earlier. Evicel and, except for a formulation change, its identical predecessor product Crosseal have been marketed in the United States since 2003 and were available in Europe several years earlier. Throughout this use, there have been no known cases of hepatitis or HIV

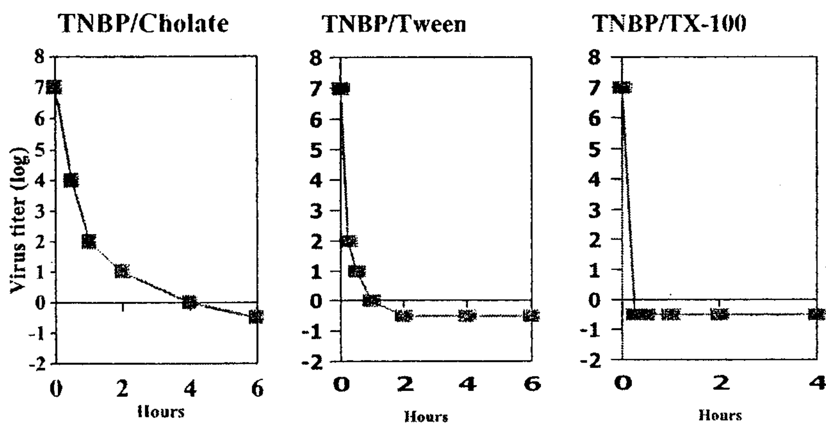

Fig. 2. S/D inactivation of vesicular stomatitis virus added to an antihemophilic factor concentrate.

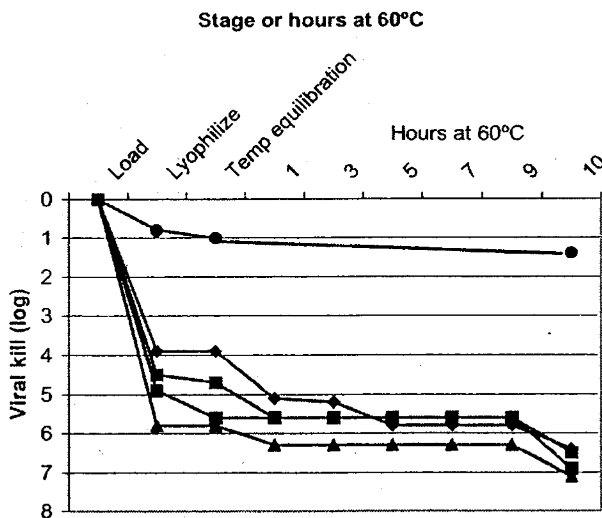

Fig. 3. Validated viral kill on vapor heating of fibrinogen (currently used Baxter process). BVDV (■) and PRV (▲) were undetectable upon reaching 60°C, and HIV (◆) and HAV (■) were undetectable after 5 and 1 hour, respectively, at 60°C. (●) Mouse minute virus.

transmission associated with commercial fibrin sealants.[59] This is notable since, for much of this time, the manufacturing process for Tisseel utilized only one dedicated viral elimination step while modern processes utilize two. On the other hand, epidemiologic evidence suggests that among patients who do not have parvovirus antibody at the time of fibrin sealant application, approximately one-fifth have reduced reticulocyte counts 12 to 20 days after surgery and develop parvovirus antibodies 12 to 48 weeks after surgery.[60] This finding is consistent with the calculations presented above since the study was performed using fibrin sealant prepared from plasma pools that were not screened by NAT for parvovirus, thereby potentially starting with 10,000-fold higher parvovirus loads. Experience with S/D-plasma indicates that reducing the quantity of parvovirus DNA to no more than 10^4 geq per mL (10^6 geq/patient exposure) eliminates parvovirus transmission as measured by DNA replication or seroconversion when the patient also receives product containing parvovirus antibody.[61] Additional clinical studies are needed to show whether the fibrin sealants manufactured today can still transmit parvovirus B19 or one of the newly described, human blood-borne parvoviruses.[62]

New viral threats

In the past 5 to 8 years, three new pathogens, WNV, Chikungunya virus, and vCJD, have emerged as potential threats to the blood supply. Other infectious agents, like H5N1 influenza virus, the strain of corona virus that causes severe acute respiratory syndrome (SARS); dengue virus; and vaccinia virus are potentially transmissible by blood and blood products. With the exception of vCJD, all are enveloped viruses and would be expected to be completely cleared by the processes now in place for manufactured blood products like fibrin sealant or thrombin. As shown in Table 8, WNV, H5N1 influenza virus, SARS-associated corona virus, and Chikungunya virus were all inactivated completely to the extent of challenge by the methods of viral inactivation discussed above.

The infectious agent of vCJD is believed to be a protein that resists most methods of inactivation including all that are applied to manufactured plasma products. Although there is evidence that it can be found in blood (see above), despite years of surveillance there have been no reported transmissions by manufactured plasma products. Model studies indicate that significant quantities would be removed by the purification processes now in use, including cryoprecipitation, depth filtration with filter aids, nanofiltration, and ion-exchange or affinity chromatography.[25,63-69] Based mostly on published findings, compared with a total maximum load of about 4 log of vCJD per plasma pool, the fibrinogen and thrombin processes should remove greater than 7.6 and greater than 13 log, respectively (Table 9), providing large safety margins.

DISCUSSION

The safety of modern plasma-derived products with respect to HBV, HCV, and HIV has been proven clinically over the past decade or more, mostly using manufacturing procedures employing only one dedicated method of

TABLE 6. Estimated viral elimination when processing fibrinogen and thrombin: based on both Omrix's and Baxter's processes

Step		Log reduction				
		HIV	HCV	HBV	HAV	Parvovirus B19
Fibrinogen						
Cryoprecipitation + Al(OH) or wash		1.5	1.5	1.5	1.5	1.5
Immune neutralization					3*	3*
S/D treatment		>6	>6	>6	0	0
Heat treatment		>6	>6	>6	>5.8 to >6.5†	1.3
Greater heat sensitivity of B19 than CPV						1
Reserve capacity of virucidal methods‡		4	4	2	0	0
	Sum:	17.5	17.5	15.5	10.3-11.5	6.8
Thrombin						
Cryo removal		1	1	1	1	1
Immune neutralization		0	0	0	0	0
Initial fractionation (Baxter)§		2	2	2	2	2
Chromatographic purification		3	3	3	3	3
S/D treatment		>6	>6	>6	0	0
S/D reserve capacity		4	4	2	NA	NA
Heat treatment (Baxter)		>6	>6	>6	6.9	1.3
Nanofiltration (Omrix)		>4.4	>5.3	>5.5	7	5.9
	Sum:	18-22	19-22	17.5-20	11-13	7.3-9.9¶

* Virus neutralization is predicated on the fibrinogen containing antibody.
† The lower number applies to Omrix's fibrinogen and the higher number applies to Baxter's fibrin.
‡ The reserve capacity of vapor treating is estimated at zero since most of the reported viral kill takes place prior to initiating the heat cycle. The estimate of HBV reserve capacity with S/D treatment comes from studies with duck HBV added to whole plasma.
§ A mean of 2 log removal during cold alcohol fractionation is assumed.
¶ The lower number applies to Baxter's thrombin and the higher number applies to Omrix's thrombin.
NA = not applicable.

TABLE 7. Calculation of viral safety margins

	HIV	HCV	HBV	HAV	Parvovirus
Viral load (log; from Table 1)	5.7	6.0	4.4	2.9	6.6
Fibrinogen					
Viral clearance capacity (log)	17.5	17.5	15.5	10.3	6.8
Safety margin (fold)	6.3×10^{11}	3.2×10^{11}	1.3×10^{11}	2.5×10^{7}	1.6
Risk/vial (with virus at maximum load)*	3×10^{-16}	5×10^{-16}	1×10^{-15}	7×10^{-12}	1×10^{-4}
Adjusted risk/vial (all lots)†	1×10^{-19}	3×10^{-19}	5×10^{-17}	2×10^{-16}	2×10^{-5}
Thrombin					
Viral clearance capacity (log)	18	19	17	11	7.3-9.9
Safety margin (fold)	2×10^{12}	1×10^{13}	4×10^{12}	1×10^{8}	2×10^{3}
Risk/vial (with virus at maximum load)‡	8×10^{-18}	2×10^{-18}	4×10^{-18}	1×10^{-13}	3×10^{-6}-8×10^{-9}§
Adjusted risk/vial (all lots)†	4×10^{-21}	8×10^{-22}	2×10^{-19}	3×10^{-18}	7×10^{-8}-2×10^{-10} §

* Assumes 1 vial per L of plasma.
† The risk was adjusted to include lots without virus (calculated from Table 1, Column 4) and further assumes that the average viral load of contaminated lots is 1 log lower than the maximum load.
‡ Assumes 10 vials per L of plasma.
§ The larger number applies to Baxter's thrombin and the smaller number applies to Omrix's thrombin.

virus inactivation.[70] The safety record of fibrin sealant products, composed of both fibrinogen and thrombin, matches the safety record of other manufactured plasma products.[57] This suggests that for these and other enveloped viruses, the safety margins of fibrin sealant or stand-alone thrombin should be much higher than required since both employ an additional, dedicated method of viral elimination and, indeed, our calculated safety margins for enveloped viruses are extremely high. As a consequence, when new threats from enveloped viruses (e.g., WNV, pandemic influenza, and dengue viruses) are identified, the procedures in place for manufactured blood products are sufficient to ensure safety. This contrasts with the record of so-called labile blood components that have been shown to transmit, for example, WNV, at least until new screening tests are developed and deployed. From a patient safety perspective, it is also important to note that the safety margins for fibrin sealant and thrombin exceed those for labile blood components by many orders of magnitude. This is a direct consequence of the multiple improvements adopted by manufacturers of purified blood products over the past 20 years including deploying robust methods of virus inactivation.

TABLE 8. Inactivation of new viral threats

Virus	Preparation	Treatment	Log kill	First time point where infectious virus was not detected	Reference
WNV	α1-proteinase inhibitor	Pasteurization at 60°C for 10 hr	≥6.5	5 hr	Remington et al.[89]
	Antihemophilic factor concentrate	S/D (0.3% TNBP/1.0% Tween 80) at 28°C for 6 hr	≥5.9	1 hr	
WNV	FEIBA	Vapor heating (60°C for 10 hr and 80°C for 1 hr)	>7.6	6 hr	Kreil et al.[90]
	Antihemophilic factor concentrate	S/D (0.3% TNBP and 1% Triton X-100) at 20°C for 60 min	>6.0	<1 min	
H5N1 influenza virus	FEIBA	Vapor heating (60°C for 10 hr and 80°C for 1 hr)	>5.3	10 hr	Kreil et al.[91]
	IVIG	S/D (0.3% TNBP, 1% Triton X-100 and 0.3% Tween 80) at 18°C for 60 min	>4.7	<2 min	
SARS-associated corona virus	Haptoglobin, AT III, or IVIG	Pasteurization at 60°C for 10 hr	>3.3 to >6.5	1 hr	Yunoki et al.[92]
Chikungunya virus	IVIG	Pasteurization at 60°C for 10 hr	>5.2	1 hr	Uemura et al.[93]

AT III = antithrombin III; FEIBA = factor VIII bypassing activity produced by CSL Behring; H5N1 = the strain of influenza virus that causes SARS; IVIG = intravenous immune globulin.

Calculated safety margins for nonenveloped viruses are smaller since the manufacturing procedures for many plasma proteins, including both fibrinogen and thrombin, typically employ only one dedicated viral inactivation and/or removal method effective against these viruses, and parvoviruses are especially stable to thermal inactivation. There are no reports of HAV transmission by fibrin sealants even before adoption of NAT screening procedures. This is in accord with the finding that coagulation FVIII preparations did not transmit HAV provided they were either heat-treated or affinity-purified.[71] Additionally, given the modest processing fibrinogen undergoes and the known presence of immunoglobulin G in cryoprecipitate, it is reasonable to assume that fibrinogen is further protected by anti-HAV, present as a "contaminant."

Numerous reports describe the transmission of parvovirus B19 by coagulation factor concentrates[72] and its transmission by fibrin sealant has also been reported.[58] Frequent transmission results from the high concentration of virus that can be present in plasma pools containing units from donors with acute-phase viremia[73] and because parvovirus is not inactivated by S/D and is relatively stable to heat treatment methods. Beginning around 2001, commercial manufacturers of plasma products began employing NAT to screen incoming plasma units in a minipool format to limit viral loads. Originally, testing was performed to ensure that titers did not exceed 10^5 geq per mL; for Omrix's and Baxter's fibrin sealant products, a standard of not more than 10^4 geq per mL has been adopted for the minipool being tested. A recent article by Geng et al.[51] confirms the benefits of screening incoming plasma for parvovirus B19 by NAT. Despite this improvement, the maximum load of infectious virus that might be present remains considerable, and it would appear that the fibrinogen component might still transmit parvovirus B19, albeit at very low frequency. Clinical studies will be required to confirm this since the antibody content of fibrinogen might provide adequate protection, or parvovirus B19 might be more sensitive to heat treatment than the animal parvovirus models used, as has been suggested.[74] The greater safety margin calculated for the thrombin component arises from the use of more vigorous purification procedures and the overall effectiveness of nanofilters in removing parvovirus. Because of its size and shape, fibrinogen cannot be nanofiltered successfully.

All evidence to date indicates that vCJD is not transmitted by manufactured plasma products. Safety may result from the geographic restrictions that have been instituted to eliminate individuals who are at high risk of exposure, the very low levels in blood, and its removal by steps in common use including precipitations, filtrations, and column chromatography. If a vCJD contaminated unit was included in the plasma pool, the calculated safety margin for fibrinogen and thrombin is very high. Unlike viruses used in spiking studies, however, the structure of

TABLE 9. Clearance of prions: Omrix process

	Prion load (log; from Table 1): 4.3 Log removal	Reference*
Fibrinogen		
Cryoprecipitation	1.6 mean (0.6-2.6)	Foster, 2000[56] and 2006[69]
Al(OH) extraction and filtration	>2	Omrix validated study
Oil extraction of S/D reagents	2	Omrix preliminary study
Hydrophobic chromatography	2	Foster, 1999[63]
Clearance capacity (log)	>7.6	
Safety margin (fold)	1995	
Adjusted risk/vial†	7×10^{-10}	
Thrombin		
Cryo removal	1	Foster, 2000[56] and 2006[69]
Filtrations	2	Foster, 2000[56] and 2006[69]
Anion-exchange chromatography	3	Foster, 2000[56] and 2006[69]
Cation-exchange chromatography	3	Foster, 2000[56] and 2006[69]
Nanofiltration	4.4 mean (1.6 to >5.9)	Foster, 2000[56] and 2006[69]
Clearance capacity (log 10)	13.4	
Safety margin (fold)	1.3×10^9	
Adjusted risk/vial†	1×10^{-16}	

* See also additional Foster references.[61,64,65,67] It should be noted that the form that the infectious vCJD agent takes in plasma is unknown and that, should it be present in plasma pools, its behavior may differ from the materials used.
† We employed the same assumptions as used in Table 7 plus assumed risk of vCJD presence was the same as for CJD, i.e., 1 per 1 million donations, and mean load was same as maximum load.

the causative agent of vCJD is unknown and may differ significantly from the models in use. Because of this uncertainty and the devastating nature of the disease, the authorities in the United Kingdom have concluded that recipients of plasma-derived FVIII, F IX, and antithrombin prepared using donations from individuals who subsequently developed vCJD should be told that they may be at increased risk for developing the disease.[75] This emphasizes the importance of donor exclusion criteria implemented for all products licensed in the United States or Europe. With these exclusions taken into account, the risk for vialed product should remain well less than 1 in 1 million for fibrinogen and less than 1 in 1 trillion for thrombin.

Finally, we should comment about the methods employed in making these calculations. In addition to the validated studies presented to the FDA and other regulatory agencies, we examined other steps in the manufacturing process that were likely to contribute to safety together with published information from other related processes. We also have taken into account the benefit of using viral inactivation methods that have a large reserve capacity. Our intent in employing this approach was not to replace the stricter approach taken by regulatory authorities, but simply to more completely assess safety. Nonetheless, we acknowledge that our calculations are estimates and actual findings may differ. Acknowledging these shortcomings but based on our calculations, we encourage manufacturers and other interested parties to continue seeking ways to enlarge the safety margin, especially for nonenveloped viruses and to address the theoretical risk posed by vCJD

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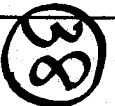
研究報告の概要	使用上の注意記載状況・その他参考事項等
○輸血用血液製剤の病原体不活化についての欧州の見解 血液、血液成分および血漿由来製剤の安全性および有効性の向上は、現在も重要な問題である。医療技術の進歩と寿命の延長に伴い、血液需要は増加し続けている。現在、供血者の選別によって、輸血用血液製剤の安全性が確保されているが、ウイルス、細菌、寄生虫感染の残存リスクに加えて、新たなウイルスによる感染の危険が存在する。化学的、光化学的なゲノム修飾による病原体不活化は、広範囲の病原体に対応する予防的アプローチである。多くの不活化技術が利用され、血漿由来製剤の不活化に成功した。有機溶媒・界面活性剤とメチレンブルーを用いた方法はヨーロッパの多くの国で利用され、副作用の発生もなく製剤の安全性を向上させているが、赤血球と血小板製剤(PC)には適用できない。アモトサレン(Intercept)、リボフラビン(Mirasol)を用いた新しい方法は、CEマークを受けヨーロッパで導入されている。MirasolはPCおよび血漿用に開発されており、赤血球にも適用される可能性がある。血小板減少症患者を対象としたMirasol PCの第III相臨床試験の結果は、近いうちに報告される予定である。Intercept PCは、ヨーロッパの複数施設で既に15,000単位以上が輸血されている。フランスでは、レユニオン島でチクングニヤウイルスの流行時(2006年)に、またマルティニークとグアドループ-ギアナではデングおよびシャーガス病対策として導入された。アルザス(不活化のパイロット実施地域)では、Intercept PCが2006年5月に、Intercept血漿が2007年7月に導入された。2008年1月時点で、Intercept PCは22,000本以上、Intercept血漿は8,000本が輸血された。Intercept PCの臨床的止血効果は従来の未処理PCと同等(血小板量同等)であり、輸血副作用は約50%減少した。Intercept血漿は、貯留保管を経た血漿の現行適応症に対して使用され、有効性は同等であった。2007年のトロントのコンセンサス会議では、すべての輸血用血液製剤の不活化完全実施を求める声明が発表された。将来、全ての輸血用血液製剤の不活化を可能とするべきである。	合成血-LR「日赤」 照射合成血-LR「日赤」 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

報告企業の意見	今後の対応
輸血用血液製剤の病原体不活化について、ヨーロッパでは有機溶媒・界面活性剤、メチレンブルー、アモトサレン、リボフラビンなどを用いた方法が開発され、フランスのアルザス地方ではこれを導入し、3万本の使用実績が報告された。	日本赤十字社では8項目の安全対策の一つとして、不活化技術の導入について、各不活化技術の効果、血液成分への影響、製造作業への影響などについて評価検討を行っている。厚生労働省の薬事・食品衛生審議会へ不活化技術導入にかかる基本的考え方を報告したところである。



MedDRA/J Ver.11.0J

JRC2008T-037

Monday: Parallel Sessions S1 - Pathogen Reduction/ Inactivation

2A-S01-01

PATHOGEN REDUCTION: AN AMERICAN VIEW

Klein H
NIH, Bethesda, MD, USA

Blood transfusion is extremely safe in the United States. The risks of known viral infections are now so low that they must be calculated from donor data rather than measured directly. Nevertheless, measures for interdicting bacterial contamination remain imperfect, a variety of known pathogens, including viruses and parasites, are not screened out of the blood supply, and the risk of emerging infections transmitted by blood remains a concern of the public, the regulatory agencies, and the medical establishment. Following the HIV epidemic of the early 1980's, the plasma fractionation industry adopted pathogen reduction technology and has improved the process continuously; no transmission of major pathogens has since been reported when proper validated plasma fraction production has been performed, and transmission of some newly recognized agents, such as West Nile virus has been prevented. The blood collection services and the regulatory agencies have remained wedded to the reactive strategy of surveillance, screening, and testing as an approach to new infectious threats. The result has been an accepted disease burden prior to introduction of screening methods and a continued loss of blood donors. Barriers to adopting pathogen reduction technology include concerns about product safety, reduced therapeutic dose, absence of a single technique to treat all blood components, recognition that no technology inactivates all pathogens, and the added cost and complexity of the inactivation process. In January 2008, the Advisory Committee on Blood Safety and Availability recommended to the US Secretary of Health and Human Services that the potential benefits of pathogen reduction warrant a commitment and concerted effort to add this technology as a broadly applicable safeguard to the nation's blood supply. Pathogen reduction was seen as a pro-active and pre-emptive strategy to address the residual risk of known agents and to prevent emerging agents from becoming transfusion risks. The Committee recognized that to achieve this goal, government, industry, blood organizations, and public stakeholders must work in concert to commit the required financial and technical resources.

2A-S01-02

EUROPEAN VIEW ABOUT PATHOGEN INACTIVATION IN LABILE BLOOD PRODUCTS

Cazenave JP
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Increased safety and efficiency of blood, blood components and drugs derived from plasma remain a major concern. Blood transfusion in Europe is tightly regulated. The demand for blood has continually increased as health care and life expectancy have increased. The safety of labile blood products [red blood cell concentrates (RBCC), platelet concentrates (PC) and plasma] is currently ensured by medical and biological donor selection measures. Nonetheless, in addition to the residual risk of viral, bacterial and parasitic infection, there is the emerging danger associated with new viruses. PI based on chemical or photochemical genomic modifications is a broad-spectrum and pro-active approach. A number of PI techniques have been use with success to inactivate plasma derived products. The solvent-detergent (SD) and the methylene blue (MB) methods are used in many countries in Europe, increasing the safety of the products and without side effects. Unfortunately, SD and PI technologies cannot be applied to RBCC and PC. New PI methods, amotosalen (Intercept, Cerus) and riboflavin (Mirasol, Gambro) have received CE marking and are being implemented in Europe. A PI process (Mirasol PRT, Gambro) is being developed for PC, plasma and possibly RBCC, using riboflavin, UV and visible light. The procedure inactivates a wide range of pathogens. Toxicity is reduced. A phase III clinical study to evaluate the efficacy and safety of Mirasol PC in thrombocytopenic patients is to be reported. Amotosalen hydrochloride and UVA (Intercept, Cerus) inactivate a broad spectrum of pathogens in PC and plasma. Intercept PC (both apheresis and buffy-coat derived) have been implemented in several centres in Europe (more than 15, 000 units transfused). In France Intercept PC have been implemented during an epidemic of Chikungunya virus in the Ile de la Réunion in 2006 and in EFS-Martinique and EFS-Guadeloupe-Guyane in 2007 (dengue and Chagas disease). EFS-Alsace, a pilot region, has introduced Intercept PI for PC (40% apheresis and 60% buffy coat derived PC, about 15 000 units/year) in May 2006 and Intercept PI for plasma (about 15 000 units/year) in July 2007. The distribution of both products is universal to patients. As of January 2008 more than 22 000 Intercept PC and 8,000 Intercept plasma have been transfused. For all patients, clinical haemostasis provided by Intercept PC is equivalent (same platelet dose) to conventional non treated PC and transfusion adverse reactions are reduced by about 50%. Intercept plasma has been used for current indications with equivalent effects as quarantine plasma. Inactivation of RBCC is a major undertaking. The use of FRALE S-303 (Cerus) is in the more advanced stage of development. In 2007, the Consensus Conference of Toronto concluded with statements that will guide the ultimate implementation of PI for all labile blood products: (1) active surveillance cannot account for the risk of an emerging transfusion-transmitted pathogen; (2) such risks require a proactive approach; (3) PI should be implemented when feasible and safe methods are available; and (4) costs and benefits should be assessed. Universal inactivation of all labile blood products should be possible in future.

2A-S01-03

A RANDOMIZED, CONTROLLED, 2-PERIOD CROSSOVER STUDY OF RECOVERY AND LIFESPAN OF RADIOLABELED AUTOLOGOUS 35-DAY-OLD RED BLOOD CELLS PREPARED WITH A MODIFIED S-303 TREATMENT FOR PATHOGEN INACTIVATION

Cancelas JA[1], Dumont L[2], Herschel L[2], Roger J[2], Rugg N[1], Garratty G[3], Arndt P[3], Propst M[4], Laurence L[4], Sundin D[4], AuBuchon J[2]
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Background: The S-303 Treatment System for Red Blood Cell concentrates (RBC) developed by Cerus Corporation uses S-303, a frangible anchor-linker-effector compound, to irreversibly inactivate contaminating bacteria, viruses, protozoa, and leukocytes. Following observations of antibodies specific for S-303 treated RBCs in a Phase three trial the treatment process was modified to reduce S 303 binding to treated RBCs.
Aims: The present study was conducted to evaluate recovery/lifespan of 35-day old autologous RBCs prepared with the modified S-303 process.
Study Design: This was a proof-of-concept, radiolabeled, crossover Phase I study conducted in 28 healthy subjects (10 male, 18 female). The study was divided into three periods: Screening and enrollment, Treatment Period 1, and Treatment Period 2. In each treatment period, subjects underwent autologous blood donation on Day 0 and infusion of double-label (51Cr/99mTc) autologous RBCs on Day 35. All whole blood units were processed into AS-3 solution, and leukocyte reduced. In random sequence, one unit (Test) from each subject was treated with the modified pathogen inactivation process (0.2 mM S-303 and 20 mM GSH) and stored at 4°C for 35 day. The other unit (Control) was prepared as conventional RBC and stored at 4°C for 35 day. Following infusion, blood samples were obtained over a 24 hour period (for single and double radio-isotope determinations of post-transfusion recovery). Additional samples were collected for 35 day post-infusion to determine lifespan. Biochemical assessments of study units (e.g. ATP, 2,3-DPG, PCV) were performed on days 0 and 35 of storage. Crossmatch reactivity to S303 treated RBC was conducted during the study using conventional gel cards.

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医薬品
医薬部外品　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2008 年 8 月 27 日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①乾燥抗 HBs 人免疫グロブリン ②ポリエチレングリコール処理抗 HBs 人免疫グロブリン	研究報告の公表状況	ProMED/20080826.2660	公表国 中国		
販売名（企業名）	①ヘブスブリン（ベネシス） ②静注用ヘブスブリンーIH（ベネシス）					

研究報告の概要

中国の新しい研究が、狂犬病感染が劇的に急増していることを報告した。この報告によると、中国のいくつかの省においてヒトの狂犬病の症例数が、2000 年以降急に跳ね上がったという。
報告者らが調査したのは、中国保健省のサーベイランス・データベースから得た、1990 年 1 月から 2007 年 7 月までの 22,527 のヒト狂犬病症例のデータである。報告者らは、ヒトの狂犬病は 1990-1996 年に下火になり、このときはわずか 159 の症例が報告されただけであったが、この数字は、2006 年に 3,279 症例に跳ね上がったことを見出した。
さらに、狂犬病に遭遇する頻度が多いのは、中国の南西部および南部の省、特に人口密度の高い地域であることを見出した。
報告者の 1 人は、「狂犬病流行のこの 4 つの省では、イヌの狂犬病を排除する厳しい強制的措置が欠けているか、またはヒトへ投与する最新技術による細胞培養の狂犬病ワクチンがないのです」と述べた。報告者らによると、最も影響が大きかった広東省では、患者の 62.5%が、受けた傷への適切な治療を受けておらず、92.5%が曝露後に十分なワクチン接種を受けていなかったという。また 91.25%が抗狂犬病免疫グロブリンの投与を受けなかった。
この報告者らは、現在の狂犬病の管理プログラムを、監督を強化することによって改善し、これによって地方と政府との人的交流を改善し、狂犬病への意識を高め、都市の計画立案と開発を変更してヒトと動物とのふれ合いのバランスを図るべきであると勧告している。
（本研究は in press であり、'Rabies trend in China (1990-2007) and post-exposure prophylaxis in the Guangdong province' と題され、BMC Infectious Diseases に掲載される予定である。）

使用上の注意記載状況・その他参考事項等

代表として静注用ヘブスブリンーIH の記載を示す。
2. 重要な基本的注意
(1) 本剤の原材料となる血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体陰性で、かつALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗HBs抗体を含有する血漿を原料として、Cohnの低温エタノール分画で得た画分からポリエチレングリコール4000処理、DEAEセファデックス処理等により抗HBs人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びろ過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
中国における狂犬病が 2006 年に急増したとの報告である。 血漿分画製剤からの狂犬病ウイルス伝播の事例は報告されていない。また、万一原料血漿に狂犬病ウイルスが混入したとしても、BVDをモデルウイルスとしたウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

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ヘブスブリン

Archive Number 20080826.2660
Published Date 26-AUG-2008
Subject PRO/AH/EDR> Rabies - China: increased incidence

RABIES - CHINA: INCREASED INCIDENCE
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A ProMED-mail post
<http://www.promedmail.org>
ProMED-mail is a program of the
International Society for Infectious Diseases
<http://www.isid.org>

Date: Fri 22 Aug 2008
Source: Science Daily [edited]
<http://www.sciencedaily.com/releases/2008/08/080820194839.htm>

A new Chinese study has reported a dramatic spike in rabies infections. The research shows that in some provinces of China the number of human rabies cases has jumped since the new millennium.

Jia-Hai Lu, from the School of Public Health at Sun Yat-Sen University, China, led a team of researchers who studied the rabies trend in China between 1990 and 2007. Lu describes how things have changed in the last 8 years: "In China, human rabies was largely under control during the years 1990-1996, via nation-wide rabies vaccination programmes. Since the end of the century, however, cases of human rabies have jumped high enough to trigger a warning sign for control and prevention."

Rabies, an infection of the nervous system transmitted by animal bites, causes over 50 000 deaths each year around the world. During recent years, most of the research on control of rabies has concentrated on the development of post-exposure prophylaxis (preventative treatment — in this case, preventing the worsening of an infection). According to the researchers, "The use of human and equine rabies immunoglobulins (HRIG/ERIG) has saved the lives of countless patients who would have died if treated with vaccine alone. However, both products are often in short supply worldwide and are virtually unaffordable in developing countries." [See ProMED post 20080826.2659 Announcements (03): Rabies vaccine supply limited - USA (CDC)].

Data from 22 527 human rabies cases from January 1990 to July 2007 were obtained from a surveillance database from the Ministry of Health of China. The authors found that human rabies was under control from 1990 to 1996, when only 159 cases of rabies were reported, but this figure had leapt to 3279 cases in 2006.

The authors found that rabies was most frequently encountered in the southwestern and southern territories of China, especially in highly populated areas. Lu said, "The 4 rabies-endemic provinces lacked strictly enforced measures to eliminate dog rabies or an ample supply

of modern cell culture rabies vaccines for humans." Most of the patients were children or teenagers, and most contracted the disease after being bitten by a dog, usually on the head and neck. According to the authors, "In the worst-affected province, Guangdong, 62.5 percent of patients did not receive proper treatment on their wounds, 92.5 percent did not receive adequate post-exposure vaccination, and 91.25 percent did not receive any anti-rabies immunoglobulin."

The authors recommend that the current rabies control programme be improved by increasing supervision, improving the interaction between local and national authorities, increasing rabies awareness, and altering urban planning and development to balance the interaction between humans and animals.

Reference

Han Si, Zhong-Min Guo, Yuan-Tao Hao, Yu-Ge Liu, Ding-Mei Zhang, Shao-Qi Rao, and Jia-Hai Lu: Rabies trend in China (1990-2007) and post-exposure prophylaxis in the Guangdong province. BMC Infectious Diseases, (in press) [available at <http://www.biomedcentral.com/content/pdf/1471-2334-8-113.pdf>].

Adapted from materials provided by BMC Infectious Diseases (<http://www.biomedcentral.com/bmcinfectdis/>) via EurekAlert!, a service of AAAS (<http://www.eurekalert.org>).

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[It is useful to read the full article, not so much for the summary of incidence trends or methods but to fully appreciate the application of potential control mechanisms. The authors emphasize the need for improved availability and timely application of anti-rabies biologicals and the undertaking of dog vaccination programs for the control of rabies in dogs as critical elements for success in reducing the rate of occurrence of rabies in China. Such strategies have worked in other countries around the world and have even previously worked in China in the 1990s. The failure of effective dog vaccination programs in China is a step back.

CDC's (US Centers for Disease Control and Prevention) Advisory Council on the subject agrees with the importance of vaccination in dogs in the following introduction:

"As a result of improved canine vaccination programs and stray animal control, a marked decrease in domestic animal rabies cases in the United States occurred after World War II. This decline led to a substantial decrease in indigenously acquired rabies among humans. In 1946, a total of 8384 indigenous rabies cases were reported among dogs and 33 cases in humans. In 2006, a total of 79 cases of rabies were reported in domestic dogs, none of which was attributed to enzootic dog-to-dog transmission, and 3 cases were reported in

humans. The infectious sources of the 79 cases in dogs were wildlife reservoirs or dogs that were translocated from localities where canine rabies virus variants still circulate. None of the 2006 human rabies cases was acquired from indigenous domestic animals. Thus, the likelihood of human exposure to a rabid domestic animal in the United States has decreased substantially."

See "Human Rabies Prevention - United States, 2008, Recommendations of the Advisory Committee on Immunization Practices" at <http://www.cdc.gov/mmwr/pdf/rr/rr57e507.pdf>.

WHO's introduction to their section on rabies from the "WHO recommended standards and strategies for surveillance, prevention, and control of communicable diseases" includes 3 main control strategies: post-exposure prophylaxis, pre-exposure immunization in high risk groups, and control of the disease in dogs.

WHO provides further information in the introduction as follows: "Rabies is a vaccine-preventable disease, and it is still a significant public health problem in many countries of Asia and Africa, even though safe, effective vaccines for both human and veterinary use exist. Most of the 55 000 deaths from rabies reported annually around the world occur in Asia and Africa, and most of the victims are children: 30-50 percent of the reported cases of rabies -- and therefore deaths -- occur in children under 15 years of age. The main route of transmission is the bite of rabid dogs. Most of the children who die from rabies were not treated or did not receive adequate post-exposure treatment. Although the efficacy and safety of modern cell culture vaccines have been recognized, some Asian countries still produce and use nervous tissue vaccines, which are less effective, require repeated visits to the hospital, and often have severe side-effects. Moreover, these patients do not receive the necessary rabies immunoglobulin, because of a perennial global shortage and because of its high price, so that it is unaffordable in countries where canine rabies is endemic.

"Due to complete absence of any successful medical treatment for clinical rabies and the horrific nature of the disease, most rabies victims die at home rather than being admitted to a hospital in abysmal conditions. These circumstances add to the notorious lack of surveillance data. Underestimating the health implications of rabies leads many high ranking decision-makers in public health and animal health to perceive rabies as a rare disease of humans resulting from a bite of an economically unimportant animal (the dog). Therefore, rabies usually falls between 2 stools and is not dealt with appropriately either by the Ministry of Health or the Ministry of Agriculture."
See "Human and Animal Rabies" at <http://www.who.int/rabies/en/>. - Mod.PC]

[see also:
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......................................pc/mj/jw

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2008. 7. 3	該当なし	

一般的名称	(製造販売承認書に記載なし)	研究報告の公表状況	Dujardin JC, Campino L, Cañavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M. Emerg Infect Dis. 2008 Jul;14(7):1013-8.	公表国
販売名(企業名)	合成血-LR「日赤」(日本赤十字社) 照射合成血-LR「日赤」(日本赤十字社)			ヨーロッパ

研究報告の概要

○ヨーロッパにおける生物媒介性疾患の拡大とリーシュマニア症に対する軽視(ネグレクト)
リーシュマニア症は南ヨーロッパ各国に定着しており、毎年700例近く、トルコを含めると3,950例の地域内感染症例が報告される。
ヒトでのリーシュマニア症の発現率は100,000人当り0.02～0.49(トルコを含むと8.53/100,000)である。無症候症例は、臨床症例1例に対して30～100例程度発生すると見られている。これは、血液事業に重大な影響を及ぼす可能性がある。南フランスとギリシャの流行地域に住む供血者由来の血液の血清陽性率は、それぞれ3.4%、15%であった。スペインの高流行地域の供血者の22.1%は、PCR法でリーシュマニア症陽性であった。また、無症候感染は、エイズ患者などの免疫不全者で重度の臨床型に進行する場合がある。飼い犬のリーシュマニア症血清陽性率は最高25%と推定されている。薬剤耐性*L. infantum*は、イヌを介してヨーロッパ国外に輸出されるかもしれない。
薬剤耐性の出現などの問題があるにもかかわらず、全ヨーロッパレベルでの協調的な疾患調査は行われていない。リーシュマニア症は、睡眠病やシャーガス病などと同様に、発展途上国で最も軽視された疾患の1つであり、有効で安価で使用簡便な薬剤の開発、調査や対策は行われてこなかった。この主な理由の1つには、リーシュマニア症が発展途上国の貧しい者の疾患であるということがある。
2001年以降、複数の研究チームが欧州一地中海諸国から科学者を集め、リーシュマニア症研究者のネットワークが形成された。今後研究者は、基礎研究を進めると共に、結果を発表することで政策決定に影響を与え、生物媒介性疾患の1つとして対策が行われるよう働きかけなくてはならない。

使用上の注意記載状況・その他参考事項等

合成血-LR「日赤」
照射合成血-LR「日赤」

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

報告企業の意見	今後の対応
リーシュマニア症は南ヨーロッパ各国に定着しており、毎年700例近くの症例が報告されているが、全ヨーロッパレベルでの調査や対策が行われていないとの報告である。リーシュマニア症をはじめとするダニ媒介性疾患の対策は難しく、流行状況に注意が必要である。	日本赤十字社では、輸血感染症対策として問診時に海外渡航歴の有無を確認し、帰国(入国)後4週間は献血不適としている。今後も引き続き、新興・再興感染症の発生状況等に関する情報の収集に努めるとともに、ヨーロッパにおける輸血感染症の動向等に注意する。

Spread of Vector-borne Diseases and Neglect of Leishmaniasis, Europe

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The risk for reintroduction of some exotic vector-borne diseases in Europe has become a hot topic, while the reality of others is neglected at the public health policy level. Leishmaniasis is endemic in all southern countries of Europe, with ≈700 autochthonous human cases reported each year (3,950 if Turkey is included). Asymptomatic cases have been estimated at 30–100/1 symptomatic case, and leishmaniasis has up to 25% seroprevalence in domestic dogs. Even though leishmaniasis is essentially associated with *Leishmania infantum* and visceral leishmaniasis, new species, such as *L. donovani* and *L. tropica*, might colonize European sand fly vectors. Drug-resistant *L. infantum* strains might be exported outside Europe through dogs. Despite this possibility, no coordinated surveillance of the disease exists at the European level. In this review of leishmaniasis importance in Europe, we would like to bridge the gap between research and surveillance and control.

In August through September of 2007, a chikungunya outbreak occurred in the province of Ravenna, Italy (*1*). The risk for reintroduction of vector-borne diseases in Europe as a consequence of global warming was highlighted, although long-distance tourism, travel, and trade could also play major roles in the transcontinental transport of microorganisms (*2*). The European Centre for Disease Control is currently assessing the magnitude and importance of vector-borne diseases in Europe, focusing on Lyme disease, tick-borne encephalitis, leptospirosis, malaria, plague, tularemia, viral hemorrhagic fevers, hantavirus, and West Nile fever. Concern about the impact of global warming and the spread of arthropod-borne diseases and other infectious agents in Europe is justifiable. However, existing autochthonous vector-borne infections should not be forgotten or ignored, which may be the case, as illustrated here for leishmaniasis.

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Leishmaniasis in Europe

Leishmaniasis is a major vector-borne disease, which is endemic to 88 countries and is the only tropical vector-borne disease that has been endemic to southern Europe for decades. In southern Europe, most of the reported cases are due to zoonotic visceral leishmaniasis (VL), which is the most dangerous form and is lethal when untreated. Cutaneous leishmaniasis (CL), which is more benign than VL, is also present. Incidence of leishmaniasis in humans is relatively low, ranging from 0.02/100,000 to 0.49/100,000 (8.53/100,000 including Turkey). We estimate that this corresponds to a total of ≈700 reported new cases per year for southern European countries (3,950 if Turkey is included; Table and Figure). However, autochthonous leishmaniasis appears not to be limited to the Mediterranean region anymore. It has spread northward, as shown by the recent reports of indigenous VL cases in northern Italy and southern Germany (*8,9*).

However, these numbers are misleading for several reasons. First, data from patients infected in southern Europe, but diagnosed elsewhere, are not taken into consideration. For instance, a leishmaniasis reference center established on a voluntary basis in Germany identified within 2 years 70 cases of leishmaniasis. Of the 27 VL case-patients, most

Table. Leishmaniasis situation in 7 disease-endemic countries of Europe (including Turkey)*

Country	Human leishmaniasis: Notification status	Current information from reference centers (2000–2006)	VL + CL incidence x 100,000†	Imported cases (VL + CL)	Canine leishmaniasis
Portugal‡	Compulsory for VL	≈22 VL cases/y recorded at IHMT	0.07–0.17	≈2 cases/y recorded at IHMT	Average 20% seroprevalence in disease-endemic areas (3)
Spain§	Compulsory in 12/17 autonomous communities; 20%–45% underreporting for VL, ≈100% for CL (4)	≈100 VL cases/y recorded by National Epidemiologic Surveillance Network, RENAVE	0.18–0.29	≈5 cases/y recorded at ISCIII	Average 8.5% seroprevalence (5)
France¶	Not compulsory, but spontaneous reports at UMON	≈24 VL + CL cases/y reported at UMON	0.02–0.19	≈65 cases/y recorded at UMON	Seroprevalence in disease-endemic areas of southern France 4%–20%#
Italy**	Compulsory for both VL and CL, but CL underreported	≈200 VL cases/y recorded at ISS; ≈300 CL cases/y estimated by ISS	0.15–0.38	≈8 cases/y recorded at ISS	Average 15% seroprevalence in peninsular Italy; average 2% seroprevalence in continental Italy (6)
Greece††	Compulsory for both VL and CL, but underreported	≈21 VL cases/y notified	0.06–0.49	Unknown	Average seroprevalence 25% in disease-endemic areas (7)
Cyprus‡‡	Compulsory for both VL and CL, but underreported	5 VL + CL cases recorded in 2006	0.25–0.47	Unknown	Average seroprevalence 20% in disease-endemic areas
Turkey§§	Compulsory for both VL and CL	≈37 VL cases/y and ≈2,300 CL cases/y notified	1.6–8.53	Unknown	Average 15.7% seroprevalence

*Authors' institutions are national reference laboratories for leishmaniasis diagnosis and surveillance and rely on consolidated countrywide networks of collaborating clinical health centers. Diagnosis records are cross-checked with case notifications to provide more realistic figures and estimates. VL, visceral leishmaniasis; CL, cutaneous Leishmaniasis; WHO, World Health Organization.
†WHO-EURO, WHO Europe, 1996–2005; http://data.euro.who.int/CISID.
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§Instituto de Salud Carlos III (ISCIII), Madrid, Spain.
¶Université de Montpellier (UMON), data from Centre National de Référence des Leishmania, Montpellier, France.
#Source: retrospective canine leishmaniasis database, Centre National de Référence des Leishmania.
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(17) had been infected within European Union boundaries: Spain, Portugal, Greece, or France (*10*). Five cases were in children. Similarly, a retrospective study in the Hospital for Tropical Diseases in London showed that most of the imported VL case-patients in the United Kingdom were adult men touring the Mediterranean (*11*). Second, in the absence of public health surveillance at the European level, underreporting is common (see the Leishmaniasis and the Globalization of Neglect section). Third, asymptomatic infections may be common in some regions: for 1 clinical case of VL, there may be 30–100 subclinical infections (*12*). This underreporting can have major consequences for blood banks: blood from donors living in areas of endemicity in southern France and Greece had 3.4% and 15% seropositivity, respectively (*13,14*). In addition, 22.1% of blood donors in a highly disease-endemic area from Spain were PCR positive for leishmaniasis (*15*). Furthermore, asymptomatic infections may progress to severe clinical forms in immunocompromised persons, for example, in AIDS patients (*16*). Fourth, the etiologic agent of southern European VL, *Leishmania infantum*, is also infecting dogs (with a seroprevalence of up to 34% in areas of Spain where the disease is highly endemic) (Table). Dogs with leishmaniasis infections are generally very sick, causing a major problem in southern Europe (e.g., ≈5,000 clinical cases occur each year in France) (Table). However, sick as well as asymptomatic dogs also represent a risk for humans, as they constitute the major reservoir of the parasite on which sand fly vectors may feed and transmit the infection.

Import–Export Balance of European Leishmaniasis

In addition to the reality of autochthonous leishmaniasis in Europe, the risk for introduction of new species through travelers or immigrants from countries where

non-European species are endemic should also be considered. However, the probability that these species could enter in a transmission cycle is relatively low. The probability depends on contact between infected persons and sand flies, the capacity of the infected person to act as reservoir, and the susceptibility of European sand flies to the different *Leishmania* species. For most species, humans are generally a transmission dead-end. However, for 2 species, the risk might theoretically be higher: *L. tropica*, which is causing CL in Africa, the Middle East, and Southwest Asia, and *L. donovani*, the etiologic agent of VL in East Africa and the Indian subcontinent. These 2 species are indeed associated with an anthroponotic transmission cycle. On one hand, *L. donovani*, which is transmitted by a different species of sand fly outside Europe, might be hosted by most European sand flies, except *Phlebotomus papatasi* and *P. sergenti* (*17*). On the other hand, *L. tropica*, which has more stringent requirements in terms of vector, would need *P. sergenti*, which was reported in several places in southern Europe, from Portugal (*18*) to Cyprus (*19*). *L. tropica* was indeed encountered in Greece (*20*), and according to a very recent report, the first autochthonous cases of *L. donovani* in Europe have been detected in Cyprus (*21*). The clinical phenotype associated with both species needs also to be considered for an exhaustive risk evaluation. *L. tropica* causes lesions that are generally more difficult to treat with antimonial drugs (*22*), whereas *L. donovani* is considered to be more aggressive than *L. infantum* and often does not respond to treatment with first-line drugs (*23*).

In addition to being concerned about importation and spread of exotic *Leishmania* species in Europe, exportation should also be considered. The best known historical example of the spread of leishmaniasis is the migration of *L. infantum* from Europe to Latin America, where it colonized in *Lutzomyia longipalpis* and is now causing a serious public health problem (>3,500 cases of VL per year in Brazil) (*24*). This spread is thought to have been caused by conquistadores' dogs (*25*). Another and current example concerns the *L. major/L. infantum* hybrids recently described in HIV-positive VL patients from Portugal (*26*). Indeed, these hybrids were shown to be able to develop in *P. papatasi* (*27*), a vector that is widespread in Europe, Africa, and Asia. Considering the reservoir role of HIV–co-infected patients and the peridomestic and anthropophilic nature of *P. papatasi*, these hybrid strains might circulate by using this sand fly vector, thereby increasing the risk of their spreading into new foci throughout the broad range of *P. papatasi* distribution (*27*). Finally, the way Europe deals with its leishmaniasis public and animal health problem can still have major consequences for the rest of the world. Miltefosine, one of the few available antileishmania drugs, has been recently launched in the market for canine leishmaniasis treatment in Portugal, Spain, Italy, Greece, and Cyprus. Because dogs are never cured parasitologically and given the long half-life of the drug, the lack of European policy might contribute to the emergence of parasites resistant to miltefosine. This resistance could be a problem for European human patients, as miltefosine is being used on a compassionate basis in several European AIDS co-infected patients unresponsive to amphotericin B or pentavalent antimonials (*28,29*). Furthermore, if dogs infected with miltefosine-resistant strains were to migrate to Latin America, where several countries have registered the drug for human use (currently Colombia, Guatemala, Argentina, Venezuela, Paraguay, Ecuador, and Honduras; *30*), the impact might be greater.

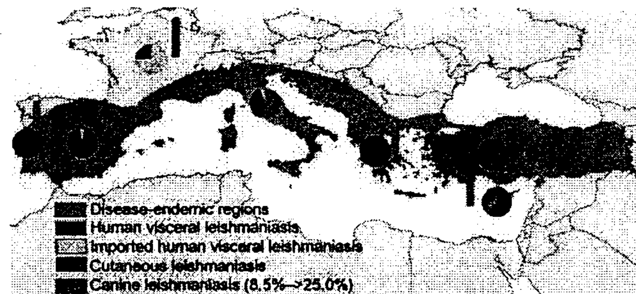

Figure. Leishmaniasis in southern Europe. Distribution of the endemic disease; relative proportion of autochthonous (visceral, cutaneous) and imported human cases and seroprevalence in dogs (from data reported in Table).

Leishmaniasis and the Globalization of Neglect

Twelve million persons have leishmaniasis, and 500,000 new cases of VL occur each year. More than 50,000 die of this disease each year. The disease is spreading because of several risk factors, climate being only one. Humanmade changes to the environment and population movements (for economic or political reasons) may lead to alterations in the range and densities of the vectors and reservoirs, increasing human exposure to infected sand flies. Urbanization of leishmaniasis becomes more common and in conjunction with the ruralization of HIV/AIDS, it contributes to increase the problem of co-infections in contexts where access to highly active antiretroviral therapy is not the same as in industrialized countries. *Leishmania* spp. have already become resistant to antimonial drugs (the first-line drug in many developing countries) in some regions and may soon become resistant to miltefosine (*23*). Despite this increasing resistance, leishmaniasis is one of the most neglected diseases in developing countries, along with others like sleeping sickness or Chagas disease. Leishmaniasis is a disease for which we lack effective, affordable, and easy to use drugs, and the pharmaceutical industry has had few incentives to engage