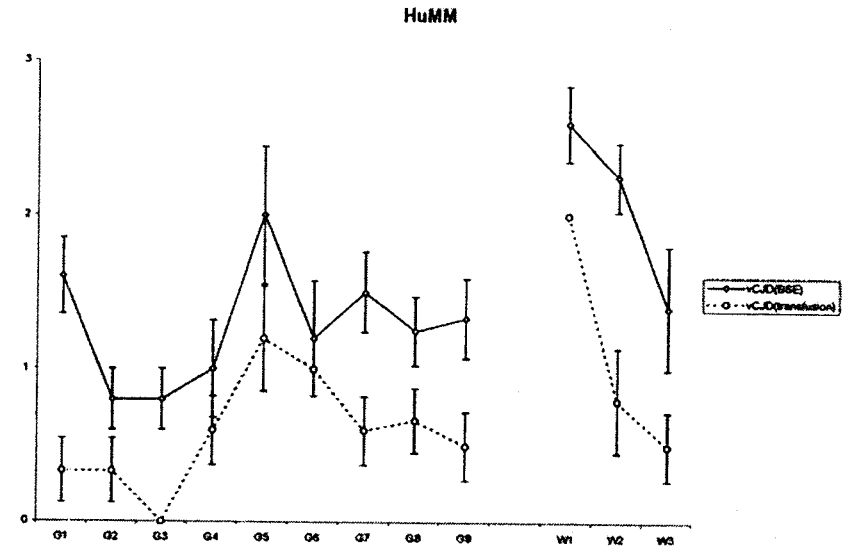
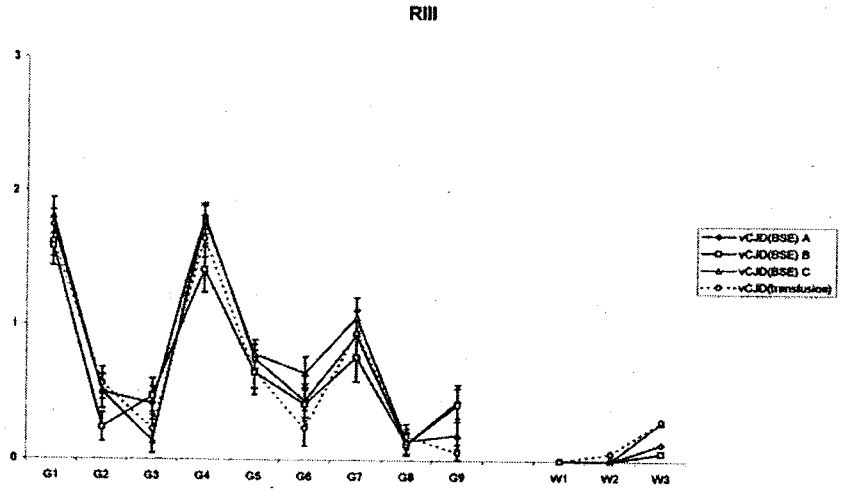

HuMM
vCJD(BSE)
vCJD(transfusion)
G1
G2
G3
G4
G5
G6
G7
G8
G9
W1
W2
W3


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

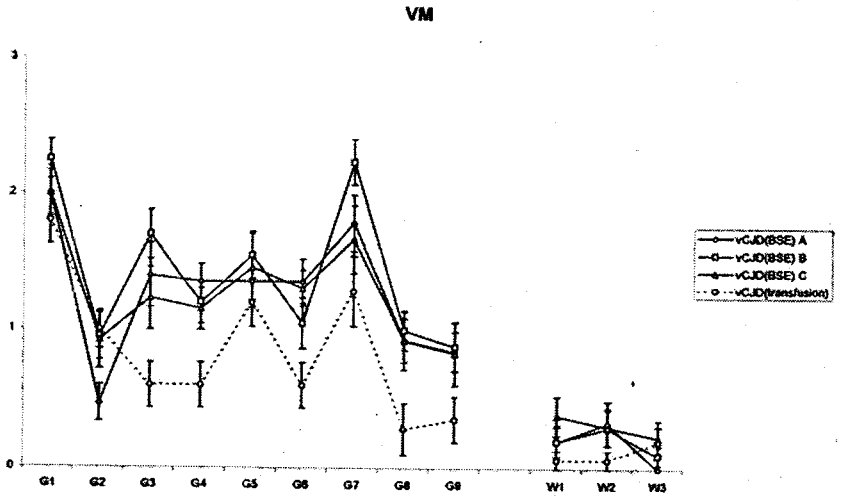

VM
vCJD(BSE) A
vCJD(BSE) B
vCJD(BSE) C
vCJD(transfusion)
G1
G2
G3
G4
G5
G6
G7
G8
G9
W1
W2
W3

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

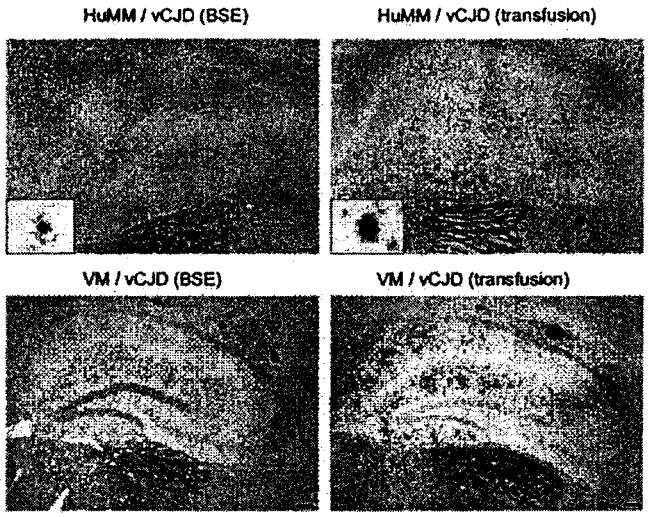


Figure 3. Detection of abnormal PrP in the mouse brain. Immunocytochemical detection of abnormal PrP deposition in hippocampus and thalamus (lateral posterior nucleus) of HuMM transgenic (with additional 40× magnification of florid plaque structure, see box lower left) and VM wild-type mice following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

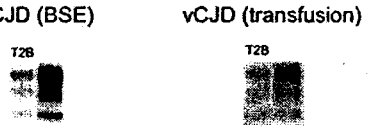


Figure 4. PrP^{Sc} typing by Western blot. Brain homogenates from HuMM mice inoculated with both vCJD (BSE) and vCJD (transfusion) show similar mobility and glycosylation profile (type 2B) as material from vCJD patients. (T2B: control vCJD material; antibody: 6H4)
doi:10.1371/journal.pone.0002878.g004

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 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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We thank Irene McConnell and the Animal Facility staff in the Neuropathogenesis Division, Anne Suttie and the Pathology staff for sectioning the mouse brains and assessing the levels of TSE vacuolation, and Dot Kisielewski for transgenic mouse genotyping.

This study would not be possible without the continued support of the families of those affected by vCJD, and the neurologists and neuropathologists throughout the UK that assist in CJD surveillance.

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。

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。

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。

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PLoS one

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 who lived in the same country 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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Funding: This work has been supported by the Network of Excellence NeuroPrion.

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)
BSE	cattle	100 mg	40
vCJD	human	40 mg	25
vCJD	human	40 mg	32

*Amount of crude brain in 10% brain suspension inoculated intracerebrally. BSE brain had a 10-fold greater concentration of PrP^{res} 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

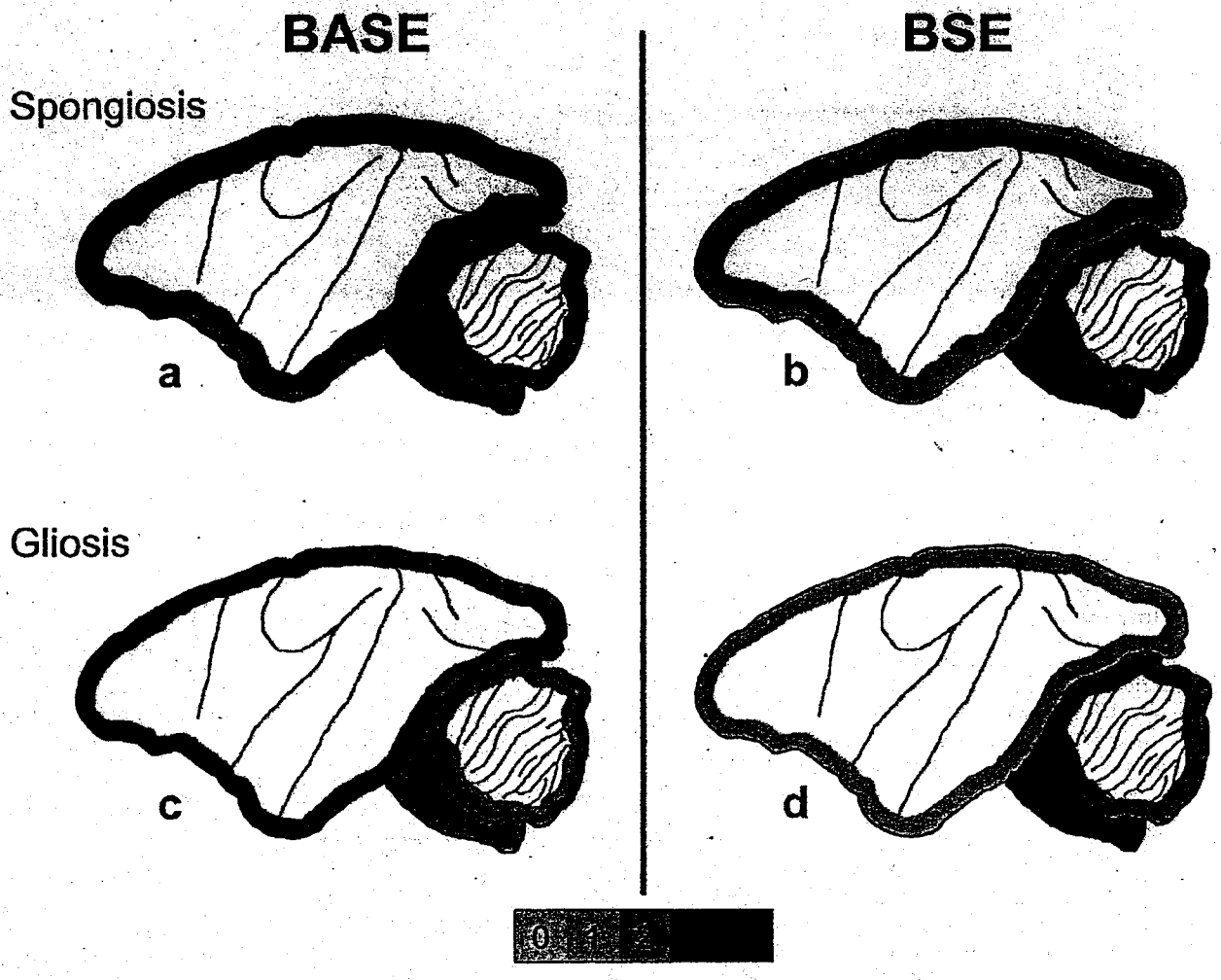

Figure 1. Diagrammatic representation of histologic lesions. Topographic distribution of spongiosis (a and b) or gliosis (c and d) in BASE and cBSE-infected primates. The lesions were scored from 0 to 4 (negative, light, mild, moderate, and severe).

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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

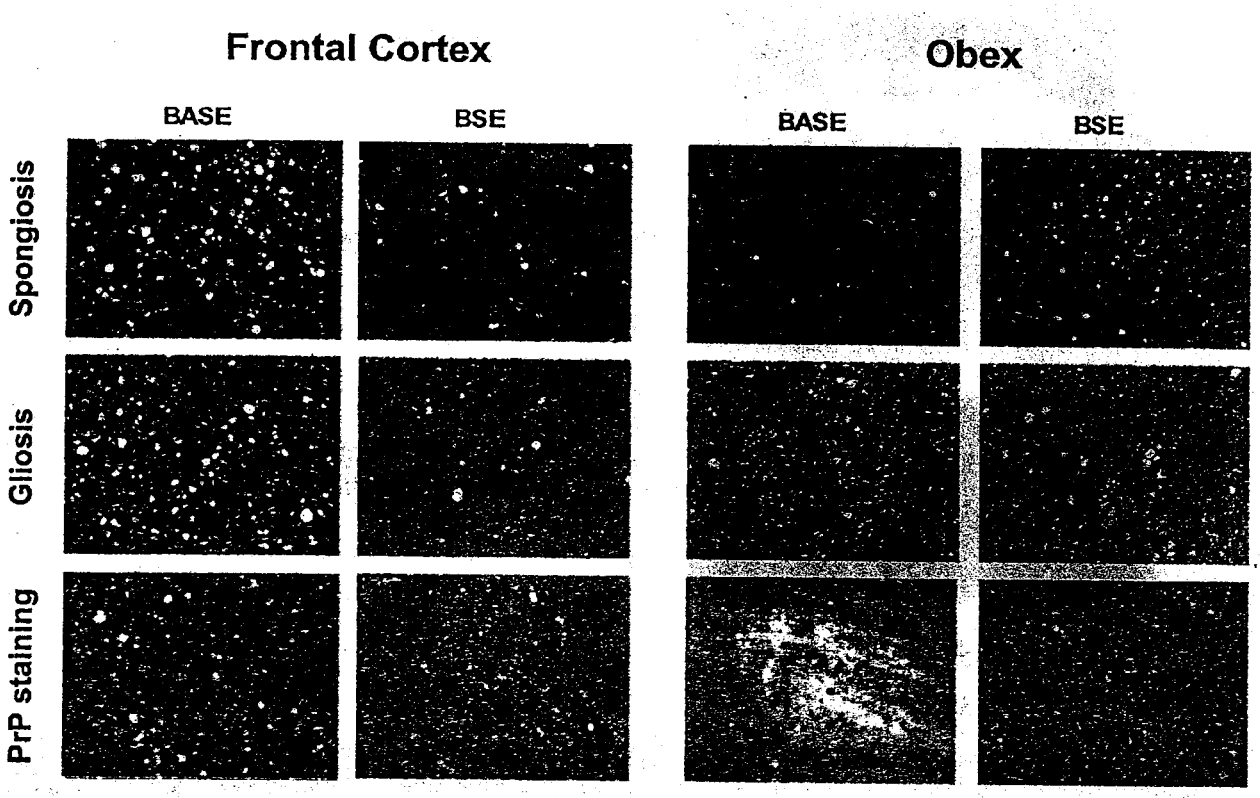

Figure 2. Histopathology and PrPres immunostaining. Spongiosis, gliosis (GFAP staining) and PrPres deposition in frontal cortex and obex in BASE- and cBSE-infected primates (original magnification ×200 for spongiosis and gliosis, ×400 for PrPres staining). Immunostaining of PrPres was performed with 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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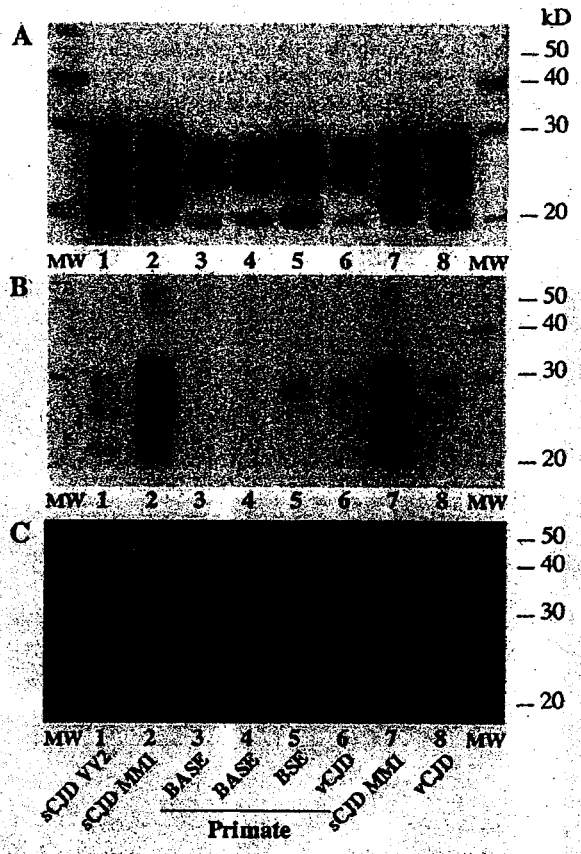
stage of illness at 26 months post inoculation (Table 1). The two cBSE-inoculated animals had longer incubations periods (37.5 months) and survivals (40 months) despite a presumably larger infecting dose (100 mg containing a 10-fold higher PrPres concentration). Moreover, the clinical presentation was 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.



Anti-core Ab
3F4 (109-112)

Anti-octapeptide Ab
(57-88)

Overexposed
Anti-octapeptide Ab
(57-88)

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.

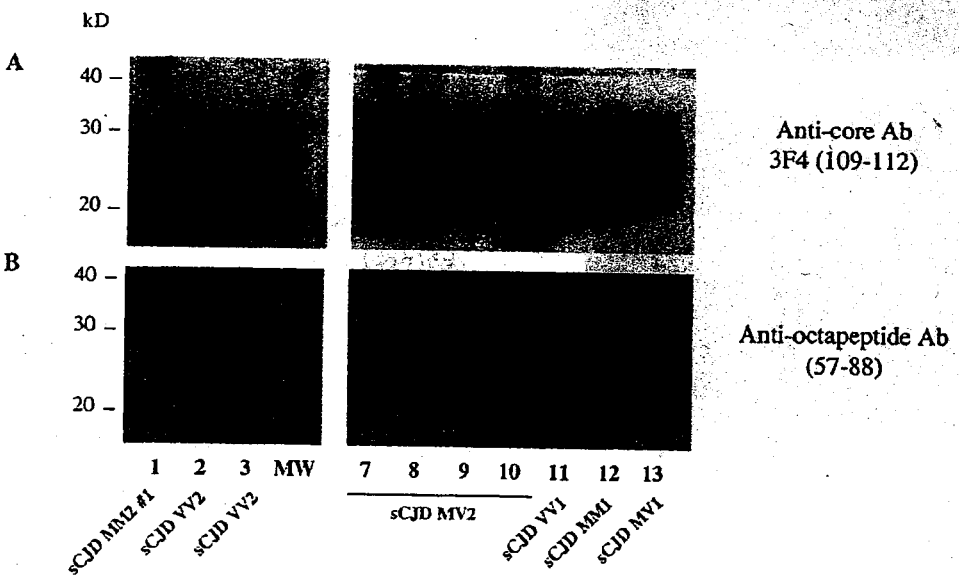

Figure 4. Electrophoretic analysis and differential sensitivity to proteolysis of PrPres in different subtypes of CJD. PrPres from human brain homogenates (MM1, MV1, VV1, MM2, MV2 or VV2 subtypes of sCJD, and vCJD) were purified under high concentrations of proteinase K, and detected with monoclonal 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.
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