Time-course studies of 14-3-3 protein isoforms in cerebrospinal fluid and brain of primates after oral or intracerebral infection with bovine spongiform encephalopathy agent

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Experimental transmission of bovine spongiform encephalopathy (BSE) to cynomolgus monkeys (Macaca fascicularis) is an animal model for variant Creutzfeldt–Jakob disease (vCJD). The presence of 14-3-3 proteins in cerebrospinal fluid (CSF) samples indicates neuronal destruction and is therefore used as a clinical biomarker. However, time-course studies using 14-3-3 proteins have not been performed until now in simian vCJD. The main goals of this study were to determine isoform patterns, to examine kinetics and to correlate the clinical course with the occurrence of this biomarker in simian vCJD. In monkeys dosed intracerebrally with BSE, the earliest clinical sign of illness was a drop in body weight that was detected months before the onset of mild neurological signs. Macaques dosed orally or intracerebrally with BSE developed neurological signs 4.3 (3.7–4.6) and 4.8 (2.9–6.0) years post-infection, respectively. 14-3-3b and -c-positive CSF samples were found around the time of onset of mild neurological signs, but not earlier. In contrast, 14-3-3e and -g isoforms were not detectable. 14-3-3 levels increased with time and were positively correlated with the degree of neurological symptoms. Post-mortem examination of brain samples revealed a positive correlation between PrP res and 14-3-3e levels. Interestingly, florid plaques characteristic of human vCJD could not be detected in diseased monkeys. It was concluded that analysis of 14-3-3 proteins in CSF is a reliable tool to characterize the time course of brain degeneration in simian vCJD. However, there are differences in the clinical course between orally and intracerebrally infected animals that may influence the detection of other biomarkers.

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs) affect a wide range of mammals (reviewed by Lasmézas, 2003). Cattle are affected by bovine spongiform encephalopathy (BSE) and small ruminants by scrapie. Human prion diseases include sporadic Creutzfeldt–Jakob disease (sCJD) and its variant form, vCJD (Ironside, 1998). In humans, it is likely that vCJD was caused by the consumption of BSE-contaminated food (Ironside, 1998). The causative agent of TSEs is regarded as a conformational variant of the ubiquitous host prion protein (PrPc) (Weissmann, 2005), generally termed PrPSc, as the oldest known TSE is scrapie. PrPSc is defined as an aggregated form of PrPc that is largely resistant to proteinase K (PK) digestion (Weissmann, 2005). Human prion diseases can be transmitted experimentally to non-human primates (NHPs) (Gajdusek et al., 1966; Gajdusek & Gibbs, 1967, 1971, 1975; Gibbs et al., 1968, 1980; Cathala et al., 1975; Masters et al., 1976; Baker et al., 1993; Bons et al., 1999; Williams et al., 2007). BSE transmitted to cynomolgus monkeys (Macaca fascicularis) causes a simian form of vCJD after an incubation period of 3–5 years (Lasmézas et al., 1996, 2001, 2005; Herzog et al., 2004).

A number of isoforms, named β, γ, ε, τ, ζ, σ and η, comprise the family of mammalian 14-3-3 proteins. 14-3-3 proteins are crucial for various physiological cellular processes, such as signalling, cell growth, division, adhesion, differentiation, apoptosis and regulation of ion channels. These intracytoplasmic molecules are expressed across a broad range of neuronal and non-neuronal

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cell types. However, the highest tissue concentration is found in the central nervous system (CNS), where it is present in the cytoplasmic compartment (Berg et al., 2003).

Extensive destruction of the brain leads to leakage of 14-3-3 proteins into the cerebrospinal fluid (CSF), which can be detected by ELISA (Kenney et al., 2000; Green et al., 2002) or Western blotting (Hsich et al., 1996; Takahashi et al., 1999). Elevated 14-3-3 levels are found in the CSF of patients with dementia (Burkhard et al., 2001) or suffering from prion diseases (Hsich et al., 1996; Will et al., 1996; Collins et al., 2000; Zerr et al., 2000; Giraud et al., 2002; Geschwind et al., 2003). In this regard, detection of 14-3-3 proteins in CSF is not disease-specific, but rather reflects the process of damaged neurons spilling their proteins into CSF (Pocchiari et al., 2004; Cuadrado-Corrales et al., 2006).

CSF 14-3-3 immunoassays have been used as ante-mortem tests for both sCJD and vCJD (Hsich et al., 1996; Will et al., 2000; Zerr et al., 2000; Cuadrado-Corrales et al., 2006; Peo’ch et al., 2006; Satoh et al., 2006). However, detection of 14-3-3 proteins in the CSF from vCJD patients has a lower specificity and sensitivity compared with sCJD (Van Everbroeck et al., 2005). Epidemiological studies have shown that 14-3-3-negative CSF test results are significantly correlated with relatively prolonged survival in sCJD (Pocchiari et al., 2004). The total amount of 14-3-3 proteins in the CSF during neurodegeneration is in the range of ng ml⁻¹ (Kenney et al., 2000; Aksamit et al., 2001). In sCJD, the lowest 14-3-3 levels are found at the onset and during the end stage of the disease (Green et al., 2001; Van Everbroeck et al., 2003, 2005; Sanchez-Valle et al., 2004; Shiga et al., 2006). In CJD patients, β, γ, α and η isotypes can be detected in CSF samples (Wiltfang et al., 1999). 14-3-3 protein isoforms have been found in the endoplasmic reticulum (Shikano et al., 2006) and in the Golgi apparatus (Pietromonaco et al., 1996). Subcellularly, the γ, ε, η, β and ζ isotypes have been found in synaptic vesicle membranes, and there are hints that some isoatypes (γ, ε and η) might be located at the synaptic junction and bind to the synaptic membrane (Martin et al., 1994; Jones et al., 1995; Baxter et al., 2002).

Interestingly, the cellular prion protein (PrP⁰) is also localized at the synaptic plasma membrane (Moya et al., 2000). 14-3-3 proteins also play a role in the conformational stabilization of other proteins and could therefore be involved in the axonal transport of prion proteins and their delivery through secretory pathways to the cell surface (Wiltfang et al., 1999; Satoh et al., 2005).

Here, we present data from a longitudinal study of BSE-infected cynomolgus monkeys. Our main goals were to determine the isoform pattern, to examine 14-3-3 kinetics and to correlate the clinical course with the occurrence of CSF 14-3-3 proteins in this NHP animal model. For the latter, we used two clinical parameters: neurological signs and body weight.

**METHODS**

**Animals and clinical check-up.** Data shown in this study were obtained from 20 cynomolgus monkeys (M. fascicularis). These animals belonged to a group comprising more than 100 macaques that were purchased as intact female 1-year-old animals from the Centre de Recherche en Primatologie, Mauritius, and used in an EU-supported study of BSE in primates (http://cordis.europa.eu/data/PROJ_FPS/ACTIONeqDnSSESSIONeq112482005919ndDOCeq322ndTBLeqEN_PROJ.htm). Monkeys were chosen randomly to serve as either BSE-infected animals, mock controls or uninfected controls and were housed at five different European collaborating centres.

The 20 animals were housed in a microbiological containment level 3 facility of the Paul-Ehrlich-Institut’s primate centre and were maintained in social groups of up to six or seven monkeys comprising stable prion and stable subordinates. Behavioural disorders could thus be detected easily and early under these housing conditions by changed social behaviour.

The animals tested negative for common primate pathogens. The prion protein gene (PRNP) was sequenced and showed no polymorphism, and all animals were homozygous for methionine at codon 129.

The study was approved by the Hessian Animal Protection Committee before experiments started (local authority permit no. V54–19c 20/15–F107/63). All animal experiments were supervised by local authorities (Regierungspräsidium Darmstadt) and were performed in accordance with section 8 of the German Animal Protection Law in compliance with EC Directive 86/609.

All animals were observed daily for any abnormalities in movement or general behaviour by an animal caretaker and weekly by a veterinarian. EDTA-anticoagulated whole blood samples for complete haematology and CSF samples were collected at regular intervals from anaesthetized monkeys (2.5 mg xylazine/HCl kg⁻¹ and 6 mg ketamine/HCl kg⁻¹ administered intramuscularly). Clinical check-ups included measurements of body weight and temperature.

**BSE inoculum.** The inoculum was a pool of homogenized bovine brain stems from 11 naturally infected, histopathologically and immunohistochemically confirmed cases of BSE. The BSE stock tested positive for PK-resistant prion protein (PrP⁰) by Western immunoblotting. The material was generously provided by the Veterinary Laboratories Agency, Weybridge, UK, and processed at the Institute for Reference Material and Measurements, Geel, Belgium. Briefly, a 90 % (w/v) brain homogenate was prepared in sucrose solution (90 % brain, 10 % sucrose solution) for oral infection, and a 10 % (w/v) brain homogenate was prepared for intracerebral infection.

**Experimental BSE infection.** Six monkeys, each 3 years of age, were injected intracerebrally (right frontal lobe) with 0.25 ml 10 % BSE brain homogenate mixture corresponding to 5 mg BSE brain. All animals recovered well from the inoculation procedure. For the dietary exposure study, seven trained monkeys, each 4 years of age, were fed with muesli balls containing 5 g BSE brain homogenate. The monkeys were closely observed during food uptake (within 10 min) to confirm that each consumed the total amount of brain homogenate. Seven animals remained non-inoculated, serving as age- and sex-matched controls.

**CSF specimens.** CSF was obtained from the cerebellomedullary cistern (cisterna magna) by suboccipital puncture (Spinocan disposable needle, 0.70 × 40 mm/22 G × 1.5; B. Braun Melsungen AG) at regular intervals during the asymptomatic phase (two samples per year). Samples were collected monthly as soon as body weights dropped to significantly lower levels, or when changes in social behaviour were observed. Freshly obtained CSF samples were kept at
4 °C immediately after puncture, carefully tested for cell contamination, centrifuged and aliquotted (50 μl) within 2 h of collection. One aliquot was tested on the day of collection to exclude effects caused by a freeze–thaw cycle and the remaining aliquots were stored at –80 °C within 2 h of collection. Aliquots from each time point were tested five to ten times by Western immunoblotting using a panel of different antisera and monoclonal antibodies against different 14-3-3 protein isoforms to determine the overall reproducibility. Two positive controls (simian brain homogenate and recombinant human 14-3-3 γ protein) and one negative control (incubation of secondary antibody without primary antibody) were included.

**Post-mortem examination.** As a rule, diseased monkeys were sacrificed ~28 days after the onset of gait ataxia. Two orally dosed monkeys showing no neurological symptoms were sacrificed at 365 days and one orally dosed monkey at 1460 days after infection. Two uninfected control animals were sacrificed at the ages of 3 and 5 years, respectively, serving as age-/sex-matched controls. At necropsy, the brain was taken and the left hemisphere was fixed in phosphate-buffered paraformaldehyde solution or in Carnoy’s fixative, as described previously (Giaccone et al., 2000). Paraaffin-embedded tissues were cut into 5 μm sections and stained with haematoxylon and eosin (H&E). Lesion profiles were determined in H&E-stained serial sections by routine histopathology. The right hemisphere was cut into ten coronary sections (6–8 mm), which were rapidly frozen on dry ice and stored at –80 °C. Tissue samples from defined areas (cortex cerebri, nucleus caudatus, thalamus, hypothalamus, pons, medulla oblongata and deep cerebellar nuclei) were taken from coronal sections of the right hemisphere and tested for the presence of PrP^res and 14-3-3 proteins by Western blotting, as described below.

**Reagents.** Monoclonal antibodies (mAbs) and polyclonal antisera used for the detection of 14-3-3 proteins are shown in Table 1. A recombinant human 14-3-3γ protein containing an N-terminal glutathione S-transferase tag with a total molecular mass of 60 kDa (Biomol) and homogenized simian brain were used as positive controls and to determine the detection limit of our immunoassay.

PrP^res was detected in PK-treated brain homogenate samples with mAb 6H4 (Prionics AG). Brain homogenates were co-stained for PrP and actin (mouse polyclonal antisera; Chemicon Int.). Primary antibodies were detected with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody.

**Tissue homogenization.** Defined brain areas as indicated above were taken from frozen coronary brain sections, cut into pieces and homogenized with commercially available kits according to the manufacturers’ guidelines (beads from Lysing Matrix D, MP Biomedicals; homogenizing buffer from BetaPrion BSE Purification kit, Roboscreen; FastPrep homogenizer from MP Biomedicals). Homogenized tissue was cleared by two centrifugation steps. Supernatant was recovered after the last centrifugation step and protein concentrations were determined (BCA protein assay kit; Pierce). Homogenates were adjusted to a final protein concentration of 10 mg ml⁻¹, aliquotted (50 μl) and stored at –80 °C.

**PrP^res extraction from homogenized brain and Western blotting.** Lyophilized PK (recombinant PK; Roche Diagnostics) was diluted in a commercially available PK buffer (BetaPrion BSE purification kit; Roboscreen). The sample was added to the PK/buffer solution to obtain a final concentration of 20 μg PK ml⁻¹ and incubated at 37 °C for 20 min. The sample was then processed using a commercially available purification kit (BetaPrion BSE purification kit) according to the manufacturer’s guidelines, loaded onto a 12 % Tris/glycine polyacrylamide gel (NuPAGE Novex 12 %; Invitrogen) and run at 200 V for 50 min [XCell SureLock Minicell (Bio-Rad) with NuPAGE MOPS SDS running buffer (Invitrogen)]. Gels were electroblotted onto a nitrocellulose membrane (semi-dry blot apparatus at 25 V for 30 min, XCell SureLock MiniCell), which was subsequently blocked with horse serum (10 %, v/v, in Tris-buffered saline containing 0.1 %, v/v, Tween 20; Gibco) and co-incubated with a 1:10 000 dilution of mAb 6H4 in blocking buffer together with a 1:100 000 dilution of anti-actin antiserum for 60 min at room temperature. Blots were incubated with a 1:10 000 dilution of goat anti-mouse IgG–HRP (Dianova) in blocking buffer for 45 min at room temperature, developed in chemiluminescent substrate (Supersignal, West Pico, Pierce; or ECL kit, Amersham) and visualized using ECL Hyperfilm (Amersham).

**Detection and quantification of 14-3-3 proteins by Western blotting.** Ten microlitres of CSF was treated with an equal volume of 2 × Laemmlı buffer (Bio-Rad) at 100 °C for 5 min, loaded onto a 12 % Tris/glycine polyacrylamide gel and blotted as described above with the following modifications. Gels were electroblotted in a wet-blot apparatus (Invitrogen) and blots were developed in ECL Plus chemiluminescent substrate (Amersham) and visualized with ECL Hyperfilm (Amersham). Western blots were evaluated by visual inspection and by densitometric analyses using UN-SCAN-IT gel software (version 6.1; Silk Scientific). Values were expressed as either the percentage of a loading control (2 ng recombinant 14-3-3γ) or as a 14-3-3 : actin ratio.

### Table 1. Antibodies and antisera used for the detection of simian 14-3-3 isoforms

<table>
<thead>
<tr>
<th>Name</th>
<th>Specificities</th>
<th>Secondary antibody (working dilution and source)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-8 (mAb)*</td>
<td>β, γ, ε, ζ, σ, η</td>
<td>Goat anti-mouse IgG–HRP, 1:10000; Dianova</td>
</tr>
<tr>
<td>C23-1 (mAb)†</td>
<td>β, γ, ε, ζ, σ, η</td>
<td>Goat anti-mouse IgG–HRP, 1:10000; Dianova</td>
</tr>
<tr>
<td>C-20 (antiserum)*</td>
<td>β (γ, ε, ζ, σ, η)</td>
<td>Donkey anti-rabbit IgG–HRP, 1:5000; Amersham</td>
</tr>
<tr>
<td>C-16 (antiserum)*</td>
<td>γ, η</td>
<td>Donkey anti-rabbit IgG–HRP, 1:5000; Amersham</td>
</tr>
<tr>
<td>E-12 (antiserum)*</td>
<td>η</td>
<td>Donkey anti-rabbit IgG–HRP, 1:2000; Santa Cruz</td>
</tr>
<tr>
<td>FL-246 (antiserum)*</td>
<td>β, γ, ε, ζ, σ, η</td>
<td>Donkey anti-rabbit IgG–HRP, 1:5000; Amersham</td>
</tr>
<tr>
<td>T-16 (antiserum)*</td>
<td>ε</td>
<td>Donkey anti-rabbit IgG–HRP, 1:5000; Amersham</td>
</tr>
</tbody>
</table>

*1: 200 working dilution; Santa Cruz.  
†1: 200 working dilution; Becton & Dickinson.
RESULTS

Incubation time, clinical findings and confirmation of simian vCJD

The incubation period, defined as the period from infection until the first neurological signs, varied from 1066 to 2208 days in intracerebrally infected animals (cases #1 to #6) and from 1372 to 1700 days in orally dosed macaques (cases #7 to #10), as shown in Table 2. Three other primates dosed orally with BSE were sacrificed during the incubation period on day 365 (n=2, cases #11 and #12) and day 1460 (n=1, case #13) post-infection (p.i.) to examine the PrP<sup>res</sup> tissue distribution in asymptomatic primates (see below).

Six out of ten BSE-inoculated monkeys that ultimately developed simian vCJD showed a progressive loss in body weight, or at least no further increase, compared with the control animals: these comprised 5/6 intracerebrally inoculated and 1/4 orally inoculated monkeys (Table 2). Time courses are shown in Fig. 1(a) (cases 1, 2, 3 and 8). In five cases (1, 2, 5, 6 and 8), this altered growth preceded the onset of neurological signs (143–246 days earlier, mean 196 days). In two cases, body weight loss was seen in combination with a voracious appetite. A ruffled coat was observed in 10/10 cases long before the onset of neurological symptoms.

The main neurological signs were as follows: gait ataxia (10/10), disturbed social behaviour or loss of social status ranking (8/10), panic-stricken responses provoked by optical stimuli (7/10), reduced escape reactions in the presence of humans (7/10), abnormal shrieking (7/10), optical stimuli (7/10), decreased competitiveness (5/10), restlessness (4/10), aggressiveness (3/10), a mild tremor (3/10) and reduced field of vision/visual disturbances (2/10). The latter sign was found only in primates dosed intracerebrally, whereas the other described symptoms did not correlate with inoculation route. Neurological signs became progressively worse in 9/10 cases, whereas one case seemed to recover in between but ultimately also developed severe neurological signs. In addition, animals rapidly became tired after being made agitated (6/10). Weeks to months after the onset of these neurological signs, ataxia worsened (10/10). Table 2 summarizes the clinical data of 10 BSE-inoculated monkeys (cases #1 to #10) that developed vCJD. Another three orally dosed monkeys (cases #11 to #13) were sacrificed during the clinically asymptomatic incubation period (not shown).

The symptomatic phase of simian vCJD could thus be characterized by five different stages based on the predominant clinical symptoms: stage 1 was characterized by a disturbance of body growth, followed by stage 2 dominated by behavioural disorders, combined with a lack of coordination in stage 3. All clinical symptoms worsened in stage 4, and truncal ataxia was the main symptom during this stage. However, appetite remained good in 10/10 diseased monkeys until this stage. One monkey (case #1) rapidly progressed to stage 5 (dementia). The median duration from stage 2 until the time point of necropsy was 99 days and 121 days in intracerebrally and orally dosed animals, respectively (Table 2).

In each of the ten diseased monkeys, spongiform encephalopathy and PrP<sup>res</sup> deposits were found by histological examination of the brain (data not shown) and Western blotting of brain homogenate samples, respectively (Fig. 1b). However, florid plaques were not seen.

<table>
<thead>
<tr>
<th>vCJD case no.</th>
<th>Onset of altered body weight (days p.i.)</th>
<th>Onset of CNS symptoms (days p.i.)</th>
<th>Last time point of 14-3-3-negative CSF (days p.i.)</th>
<th>First time point of 14-3-3-positive CSF (days p.i.)</th>
<th>Death by euthanasia (days p.i.)</th>
<th>Body weight at necropsy (kg) [mean of eight age/sex-matched controls, min.–max.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracerebral</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>&gt;820</td>
<td>1066</td>
<td>865</td>
<td>1040</td>
<td>1159</td>
<td>3.0 [4.8, 3.7–6.0]</td>
</tr>
<tr>
<td>2</td>
<td>&gt;1400</td>
<td>1610</td>
<td>1235</td>
<td>1607</td>
<td>1677</td>
<td>3.4 [5.6, 4.3–6.8]</td>
</tr>
<tr>
<td>3</td>
<td>&gt;1760</td>
<td>1749</td>
<td>1607</td>
<td>1825</td>
<td>1917</td>
<td>5.1 [6.3, 5.1–7.4]</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>1908</td>
<td>1784</td>
<td>1965</td>
<td>1993</td>
<td>6.0 [6.1, 4.9–6.9]</td>
</tr>
<tr>
<td>5</td>
<td>&gt;1825</td>
<td>2037</td>
<td>1978</td>
<td>2041</td>
<td>2189</td>
<td>5.8 [6.3, 5.6–7.6]</td>
</tr>
<tr>
<td>6</td>
<td>&gt;2040</td>
<td>2208</td>
<td>2159</td>
<td>2223</td>
<td>2237</td>
<td>3.7 [6.2, 4.9–7.7]</td>
</tr>
<tr>
<td>Oral</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>7</td>
<td>NA</td>
<td>1372</td>
<td>1345</td>
<td>1442</td>
<td>1555</td>
<td>7.2 [6.3, 5.5–7.6]</td>
</tr>
<tr>
<td>8</td>
<td>&gt;1420</td>
<td>1563</td>
<td>1380</td>
<td>1568</td>
<td>1590</td>
<td>3.8 [6.1, 4.8–6.8]</td>
</tr>
<tr>
<td>9</td>
<td>NA</td>
<td>1688</td>
<td>1380</td>
<td>1638</td>
<td>1701</td>
<td>7.0 [6.3, 5.3–7.4]</td>
</tr>
<tr>
<td>10</td>
<td>NA</td>
<td>1700</td>
<td>1638</td>
<td>1749</td>
<td>1848</td>
<td>6.2 [6.2, 4.9–7.7]</td>
</tr>
</tbody>
</table>

NA, Measured body weight was not altered during the entire observation period.
Evaluation of an immunoassay to detect simian 14-3-3 proteins

To establish a simian 14-3-3 protein immunoassay, we electrophoresed simian brain homogenates and blotted samples onto a nitrocellulose membrane, as described in Methods. A number of commercially available mAbs and polyclonal antisera directed against human 14-3-3 proteins (Table 1) were then tested for cross-reactivity. As shown in Fig. 2(a), antisera against the \( \gamma \) (C-16, lane 2), \( \beta \) (FL-246, lane 3), \( \varepsilon \) (T-16, lane 4) and \( \eta \) (E-12, not shown) isoforms cross-reacted with simian 14-3-3 proteins. Two mAbs recognizing all human isoforms (mAb H-8, lane 8; mAb C23-1, lane 9) also cross-reacted with simian 14-3-3 proteins. Antibody C23-1 could not recognize the simian \( \epsilon \) isoform. However, there was a marked variability in titres among different batches of the above-mentioned polyclonal antisera (data not shown).

To determine the detection limit of our immunoassay, serial dilutions of simian brain homogenate or recombinant human 14-3-3 protein were electrophoresed, blotted, incubated with antisera C-16 or H-8 and analysed. Thirty-two nanograms of total protein had to be loaded to detect 14-3-3 proteins in simian whole brain homogenate of an uninfected monkey and \( \geq 0.2 \) ng recombinant 14-3-3 protein was detected in a volume of 10 \( \mu l \) (data not shown).

Detection of 14-3-3 proteins in CSF samples from diseased animals

mAb H-8 and polyclonal antisera C-16, C-20, E-12 and T-16 were used as standard antibodies to detect 14-3-3 protein isoforms in CSF samples. First, we analysed CSF samples obtained from BSE-infected animals showing mild neurological signs (Fig. 2b) and from uninfected age- and sex-matched controls. We readily detected 30 kDa bands in animals showing ataxia using C-16 (Figs 2b and 3a), C-20 and H-8 (Fig. 3). The intensity of the 30 kDa band differed among samples collected at different stages of the clinical phase (Fig. 2b, lanes 3 and 4; Fig. 3). However, the \( \eta \) and \( \varepsilon \) isoforms could not be detected in any examined CSF samples (data not shown). Faint 30 kDa bands were sometimes visible in CSF samples collected from uninfected controls in specimens free from blood contamination using antisera C-16 (Fig. 2b, lane 5) or C-20.

Time-course studies of 14-3-3 proteins in CSF samples

In a number of cases (#3, #4, #6, #7 and #10), positive CSF samples were found several weeks after the onset of mild neurological signs (Table 2, columns 3 and 5), whereas in others the onset of clinical symptoms could be...
confirmed by 14-3-3 testing procedures within days (cases #2, #5 and #8). However, in 2/10 cases (#1 and #9), a positive CSF result was obtained distinctly earlier than clinical symptoms. Unfortunately, there was a diagnostic 14-3-3 gap (Table 2, columns 4 and 5) that ranged from 63 days in case #5 (the last time point of a 14-3-3 negative CSF was at 1978 days p.i. and the first time point of a 14-3-3-positive test was 2041 days p.i.) to 372 days in case #2. This was often due to CSF samples that were collected in between being contaminated with blood and unable to be used for 14-3-3 analyses. Nevertheless, two different isoforms could be detected by Western blotting in CSF samples collected over time in diseased primates: β and γ isoforms (Figs 2b and 3a). We found a progressive increase in these two isoforms during the symptomatic phase, as detected by an increase in the optical density of Western blot bands (Fig. 3b, c). This increase was seen in 10/10 monkeys (Fig. 4).

Interestingly, in a number of cases, altered body weight was seen long before 14-3-3-positive CSF samples were detectable or neurological signs could be observed (Fig. 1a).

**14-3-3 expression patterns in brain samples**

A number of different brain localizations (medulla oblongata, pons, deep cerebellar nuclei, hypothalamus, thalamus, nucleus caudatus and cortex cerebri) were examined for the presence of PrPres and 14-3-3 protein isoforms. PrPres patterns were semi-quantitatively grouped into three classes: negative (−), weakly positive (+) and strongly positive (++). A positive correlation was found between the amount of PrPres and the 14-3-3-γ (Fig. 5) and -η isoforms (not shown) that was not seen for β and (not shown).

**DISCUSSION**

The cynomolgus monkey PrPC has a high degree of homology with that of man (Schätzl et al., 1995) and has been established as an animal model to study the pathogenesis of vCJD (Herzog et al., 2004, 2005; LasmeÌÁzas et al., 2005). However, there are only a few studies reporting the detection of 14-3-3 proteins in TSE-infected primates (Hsich et al., 1996). Furthermore, the timing of appearance of 14-3-3 proteins in CSF in simian vCJD was unknown previous to this study. Therefore, we initiated a 14-3-3 protein time-course study in BSE-infected cynomolgus monkeys. As early clinical symptoms are sometimes difficult to detect in an NHP model, detection of 14-3-3 proteins in CSF may be more suitable for diagnosis of the disease during the early stages. Finally, detection of 14-3-3 proteins in CSF was evaluated as a humane end point.

There was variation in the incubation periods of intracerebrally dosed primates. In intracerebrally infected animals, this was mostly due to one rapid progressor, whereas the 25th to 75th percentile range was lower: 1338–2123 days p.i. Exogenous factors such as stress may have been responsible for the early onset of clinical signs in this single case. Moreover, the variation in incubation time was much lower in orally dosed primates compared with intracerebrally dosed animals. This was mostly due to the different doses that were given to the monkeys. Orally dosed primates in this study received a high dose, which contributed to a low variability in incubation time, whereas the 5 mg intracerebral dose did not represent a high dose, thereby leading to a prolonged incubation period compared with animals infected with 50 mg each (data not shown).

The symptomatic phase of simian vCJD was characterized by five different stages based on the predominant clinical
symptoms. However, stage 1 (disturbed body growth, no neurological signs, CSF-negative for 14-3-3 proteins) was seen in 5/6 intracerebrally inoculated monkeys, but only in 1/4 orally dosed monkeys, indicating that the clinical course may be influenced by the inoculation route. The efficacy of intracerebral versus oral inoculation will be described elsewhere, involving a larger number of infected cynomolgus monkeys.

The underlying mechanisms for the disturbed body growth are unknown. Body weight loss has been described in clinically symptomatic vCJD patients (Zeidler et al., 1997) and in intracerebrally infected primates (Williams et al., 2007), but to our knowledge there has been no description so far of its onset occurring several months before the onset of early neurological signs. Body weight in cynomolgus monkeys is influenced by social ranking when animals are kept together in groups: dominants always have higher body weight than subordinates, explaining the wide range in body weights of the described simian vCJD cases. For example, in one infected subordinate, body weight dropped from 5 to 3 kg, whereas body weight decreased from 8 to 6 kg in an infected dominant (Table 2). We can exclude that the observed changes were caused by alterations in the social ranking of our animal groups. It is tempting to speculate that this phenomenon was caused by disturbed hypothalamic regulation, as food intake is regulated by hypothalamic nuclei (Cota et al., 2006; Morton et al., 2006). We therefore examined the hypothalamus from a monkey that was sacrificed during this stage (case #13), but found no evidence of PrP\textsuperscript{res}. Moreover, CSF samples were negative for 14-3-3 proteins throughout stage 1 in 6/6

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{(a–b) Representative 14-3-3 isoform patterns in CSF samples of cynomolgus monkeys dosed intracerebrally with BSE as detected by Western blot analysis. Two representative examples are shown: case #5 (a) and case #3 (b). (c) Densitometric analyses of 14-3-3 bands (case #3) revealed a progressive increase during the symptomatic phase using antiserum C-16.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{14-3-3; CSF kinetics in monkeys dosed intracerebrally or orally with BSE. Results are shown as a time course of 14-3-3; detection in CSF, expressed as a percentage of the density of the loading control (2 ng rec14-3-3\textsuperscript{3\gamma}). 14-3-3 levels progressively increased in all ten simian vCJD cases (case #10 is not shown).}
\end{figure}
simian vCJD cases with altered body weight, indicating that PrPres-induced brain degeneration did not cause this phenomenon. Alternatively, very low amounts of PrPres might cause neuronal defects, leading to disturbed hypothalamic regulation. However, the fact that food intake was not disturbed and appetite remained good in all monkeys during stage 1 also did not support this explanation. Thus, the underlying mechanism remains to be elucidated.

Post-mortem examination of the brain revealed large vacuoles in neurons from different areas of the brain. However, florid plaques typical of human vCJD (Ironside, 1998), which were described in cynomolgus monkeys intracerebrally infected with BSE (Lasmézas et al., 1996), were not found in this study either in intracerebrally or in orally dosed cynomolgus monkeys. Interestingly, Williams et al. (2007) were also unable to detect amyloid plaques in squirrel monkeys that were intracerebrally infected with vCJD brain homogenate when they were adults. They hypothesized that the absence of florid plaques in their animal model was due to the host species, an explanation that cannot account for the absence in our primate model.

Another characteristic was non-glycosylated double bands in brain homogenates digested with PK by Western blotting (Fig. 1b, case #6). These double bands were found in the thalamus of all cases when 4–12 % gels were used (data not shown). We are currently examining different brain areas for the presence of this double band using modified laboratory protocols to explain this phenomenon.

14-3-3 proteins form homo- and heterodimers: the ε isoform monomer has a molecular mass of 32 kDa (Takahashi et al., 1999) and all other isoform monomers have a molecular mass of 28–30 kDa (Martin et al., 1993). At the beginning of the present study, we used a Western blot assay to detect simian 14-3-3 protein isoforms. For this purpose, a number of commercially available antibodies and antisera were tested for cross-reactivity using simian brain homogenate as antigen. However, there were a number of samples without any red blood cell contaminations from both infected and uninfected monkeys in which sequential testing gave rise to contradictory results: in one instance negative and in the other weakly positive, especially when antiserum C-20 was used. This faint 30 kDa band was regarded as a false-positive, as it also occurred in CSF samples from healthy control animals. Finally, there was great variability in titres among different batches of the above-mentioned commercially available polyclonal antiserum. The use of standardized positive controls is therefore highly recommended to exclude false-negative test results (Aksamit, 2003). False-positive results were observed in samples contaminated with red blood cells. These samples were excluded from further analyses.

All CSF samples collected during stage 1 were negative for all examined 14-3-3 protein isoforms, but CSF samples were positive for β and γ isoforms in animals showing neurological signs (stages 2–5). 14-3-3 ε and -η isoforms could not be detected in any of the ten simian vCJD cases, most probably because concentrations were below the detection limit of our immunoassay or due to their complete absence in the NHP animal model. Concentrations of 14-3-3 proteins progressively increased over time in BSE-infected monkeys. This is not in line with what is known from 14-3-3 kinetics in sCJD patients (Green et al., 2001; Van Everbroeck et al., 2003, 2005; Shiga et al., 2006) and may suggest a difference between sCJD and vCJD.

Neurons constitutively express the β, γ, ζ and η isoforms, but do not constantly express the ε or σ isoforms (Satoh et al., 2004). 14-3-3 proteins are also expressed by astrocytes, microglia and oligodendrocytes (Satoh et al., 2004). A changed 14-3-3 expression pattern has been described under pathological conditions. The ε isoform is upregulated in reactive human astrocytes (Satoh et al., 2004) and is detectable in CSF samples from sCJD patients (Takahashi et al., 1999). In murine scrapie, 14-3-3 isoform expression is decreased in some neuroanatomical areas and increased in others (Baxter et al., 2002). Indeed, in this study, we found higher 14-3-3 ε and -η levels (data not shown) in brain areas with large amounts of PrPres deposits. It is tempting to speculate that the large amounts of the ε isoform was due to reactive astrocytes, but this assumption can only be proved by immunohistochemical methods, which we have recently initiated. However, no

**Fig. 5.** Dot plots showing densitometric analyses of 14-3-3: isoform expression in brain samples of BSE-inoculated monkeys as detected by Western blotting. Brain localizations were chosen on the basis of the amount of PrPres and were semi-quantitatively grouped into three classes: negative (open triangles), weak positive (shaded squares) and strong positive (filled squares) for PrPres. Values are expressed as the 14-3-3:ε:actin ratio.
differences could be detected when we examined the levels of the 14-3-3β and -γ isofoms.

We conclude that the detection of 14-3-3 proteins in CSF is a reliable and excellent tool to characterize the course of brain degeneration and to standardize a humane end point in simian vCJD. 14-3-3 levels progressively increased in simian vCJD. However, there were differences in the clinical course between orally and intracerebrally infected animals that may influence the detection of other biomarkers. In addition, the brain pathology of simian vCJD was characterized by the absence of florid plaques.

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REFERENCES


