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## Articles



## Tissue distribution of bovine spongiform encephalopathy agent in primates after intravenous or oral infection

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### Summary

**Background** The disease-associated form of prion protein (PrP<sup>res</sup>) has been noted in lymphoreticular tissues in patients with variant Creutzfeldt-Jakob disease (vCJD). Thus, the disease could be transmitted iatrogenically by surgery or use of blood products. We aimed to assess transmissibility of the bovine spongiform encephalopathy (BSE) agent to primates by the intravenous route and study its tissue distribution compared with infection by the oral route.

**Methods** Cynomolgus macaques were infected either intravenously or orally with brain homogenates from first-passage animals with BSE. They were clinically monitored for occurrence of neurological signs and killed humanely at the terminal stage of the disease. Brain, lymphoreticular tissues, digestive tract, and peripheral nerves were obtained and analysed by sandwich ELISA and immunohistochemistry for quantitative and qualitative assessment of their PrP<sup>res</sup> content.

**Findings** Incubation periods after intravenous transmission of BSE were much shorter than after oral infection. We noted that PrP<sup>res</sup> was present in lymphoreticular tissues such as spleen and tonsils and in the entire gut from the duodenum to the rectum. In the gut, PrP<sup>res</sup> was present in Peyer's patches and in the enteric nervous system and nerve fibres of intestinal mucosa. Furthermore, PrP<sup>res</sup> was found in locomotor peripheral nerves and the autonomic nervous

system. Amount of PrP<sup>res</sup> ranged from 0.02% to more than 10% of that recorded in brain. Distribution of PrP<sup>res</sup> was similar in animals infected by the intravenous or oral route.

**Interpretation** Our findings suggest that the possible risk of vCJD linked to endoscopic procedures might be currently underestimated. Human iatrogenic vCJD cases infected intravenously raise the same public-health concerns as primary cases and need the same precautionary measures with respect to blood and tissue donations and surgical procedures.

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*See Commentary*

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## Introduction

Tissue distribution of the infectious agent in variant Creutzfeldt-Jakob disease (vCJD)<sup>1</sup>--due to human contamination with the bovine spongiform encephalopathy (BSE) agent<sup>2-4</sup>--has been a public-health issue since the discovery that it differs from that of sporadic CJD. In that disease, infectivity is largely confined to the CNS, although very low amounts of peripheral disease-related prion protein (PrP<sup>res</sup>) or infectivity have been described in some patients.<sup>5,6</sup> By contrast, all vCJD patients to date have harboured PrP<sup>res</sup> and infectivity in tissues of the lymphoreticular system.<sup>7-9</sup> This apparent lymphotropism of the BSE agent in human beings suggests that infectivity might be present in the blood of vCJD patients.<sup>10</sup> Hence, present epidemiological evidence against transmission of sporadic CJD by blood transfusion or injection of blood-derived products cannot be used for vCJD risk assessment.<sup>11</sup> These concerns have led public-health authorities of most countries to gradually implement protective measures to reduce the risk of prion infectivity in labile blood products and blood-derived manufactured compounds. However, Llewelyn and colleagues<sup>12</sup> (see page 417) describe a vCJD patient whose disease is possibly related to the administration, in 1996, of red blood concentrate from an individual who developed vCJD 3 years later. Although the possibility that the disease was acquired through consumption of BSE-contaminated meat cannot be dismissed, this case could be the first of bloodborne vCJD infection in a human being.

Since pathogenesis of transmissible spongiform encephalopathies varies depending on the route of infection,<sup>13,14</sup> and differences have been reported between oral and intravenous routes in BSE-infected sheep,<sup>15</sup> study of the tissue distribution of the molecular marker PrP<sup>res</sup> after intravenous infection in a model close to man in the absence of available human tissues is essential.

We have previously shown that intracerebral inoculation of cynomolgus macaques with the BSE agent reproduces a disease similar to vCJD in human beings.<sup>2,16</sup> We have also shown that secondary passage of vCJD or primate-adapted BSE causes the same disease, showing that the BSE/cynomolgus macaque model can be used to mimick vCJD.<sup>16</sup> We aimed to assess transmissibility of vCJD by the intravenous route with different doses of infectivity and tissue distribution of PrP<sup>res</sup>.

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## Methods

### Animals

We purchased captive-bred cynomolgus macaques (*Macacca fascicularis*) from the Centre de Recherche en Primatologie (Mauritius) and checked them for absence of common primate pathogens. We gave two animals an oral dose of 5 g of macaque-adapted BSE brain homogenate.<sup>16</sup> For intravenous administration, we slowly injected 0.4 mg, 4 mg, and 40 mg of the same brain homogenate--adjusted to a volume of 1 mL with an isotonic glucose solution--into the saphenous vein of animals anaesthetised with ketamine (one animal per dose). We clinically monitored the animals and killed them humanely at an advanced stage of the disease.

### Procedures

We used ELISA for semiquantitative assessment of amounts of PrP<sup>res</sup> in peripheral tissues. We prepared tissue samples by collagenase digestion<sup>17</sup> and homogenised them in a ribolyser (BioRad, Marne La Coquette, France). Tissues were spiked with varying amounts of brain homogenate from a terminally-ill infected animal before collagenase digestion. We purified PrP<sup>res</sup> from tissue homogenates according to a scrapie-associated fibrils protocol.<sup>18</sup> To detect PrP<sup>res</sup> we used a commercial BSE-testing sandwich immunoassay (BioRad).<sup>19</sup> We loaded the equivalent of 9 mg of tissue in every ELISA well and analysed the signal by spectrophotometry.

We determined the exact localisation of PrP<sup>res</sup> immunohistochemically. We fixed small fragments (about 1-2 cm) of peripheral nerves (sciatic nerves and nerve tracts along the carotid artery) and gut by immersion in Carnoy's fluid.<sup>20</sup> We sampled gut fragments at various levels from the duodenum to the rectum, including the ileocaecal junction; these fragments were quickly rinsed in phosphate-buffered saline before being immersed in Carnoy's fluid. After one change of this fixative, tissues were fixed overnight and we transferred them to butanol until paraffin embedding. We cut 4-7 µm-thick sections in an L3 safety laboratory and mounted them on polylysine-coated slides. Adjacent slides were dewaxed and either stained with Masson's trichrome stain or immunolabelled.

For PrP detection we used two monoclonal antibodies: Bar 224, raised against ovine recombinant protein (0.01 mg/L, 2 h room temperature); and biotinylated Saf 32, raised against purified hamster 263K PrP<sup>res</sup> (1 mg/L, 2 h room temperature). The monoclonal antibody 3F4 (1 mg/L, 2 h room temperature; Senetek, St Louis, MO, USA) gave similar results. Briefly, we dewaxed slides and inhibited endogenous peroxidases with 1.5% hydrogen peroxide in methanol. After immersion in 4 mol/L guanidine thiocyanate for 15 min, we rinsed slides in water then incubated them in 0.1% triton X100 in phosphate-buffered saline (PBST). We treated one of two adjacent slides with proteinase K (1 in 10000, 10 min at 37°C; Eurobio, Les Ulis, France). Non-specific labelling was blocked with 20% normal horse serum before incubation with PrP antibodies (diluted in 5% normal horse serum in PBST). After PBST rinses we treated slides with either the peroxidase-coupled polymer Envision (Dako, Trappes, France) for Bar 224 and 3F4 or with streptavidin-peroxidase complex (RTU, Vector, Burlingame, CA, USA) for Saf 32. Peroxidase activity was shown with the chromogen NovaRed (Vector) for 5 min at room temperature, and slides were lightly counterstained with Mayer's haemalum (1 in 10, 2 min), dehydrated, and mounted with Eukitt (Kindler, Freiburg, Germany). We detected enteric nervous structures (plexuses and nerve fibres of the lamina propria) with antihuman neuron-specific enolase and antiGAP43 (1 in 50, 2 h room temperature; Dako).

For fluorescence double-labelling studies we dewaxed paraffin sections and rehydrated them in PBST containing 2% fish gelatine (Sigma, St Quentin Fallavier, France). Slides were then treated with proteinase K (Eurobio) 1 in 10000 for 10 min at 37°C, rinsed in cold water, and treated with 3 mol/L guanidine thiocyanate for 15 min. We applied Bar 224 and polyclonal antiGFAP (Dako) simultaneously at a concentration of 1% each for 2 h. After three 5-min rinses in PBST, we immersed slides in a mix of goat antimouse and goat antirabbit (Alexa 488 and 568; Molecular Probes, Eugene, OR, USA) 1 in 200 for 1 h and rinsed

and mounted them with Vectashield (Vector). We looked at images on an Axiophot microscope (Zeiss, Le Pecq, France), numbered them with a charge-coupled device camera (DP50, Olympus, Hamburg, Germany), and processed them with IM 1000 (Leica, Rueil-Malmaison, France) and Adobe Photoshop 5.5 (Adobe, San Jose, CA, USA).

We did paraffin-embedded tissue blots according to the method developed by Schulz-Schaeffer,<sup>21</sup> which was slightly modified. Briefly, paraffin sections were laid onto nitrocellulose membranes, dewaxed, and treated with 250 mg/L proteinase K (Eurobio) in 50 mmol/L Tris-buffered saline (pH 7.8) containing 0.1% Triton X100 for 2 h at 55°C. The same buffer was used for all consecutive steps. Membranes were then treated with 3 mol/L guanidine thiocyanate for 15 min, rinsed, and blocked with 5% non-fat dried milk. We used Bar 224 at a concentration of 1 in 1000 for 2 h. PrP<sup>res</sup> was then immunodetected and revealed by enhanced chemiluminescence (ECL, Amersham, Orsay, France), as for our classic western-blot procedure.<sup>11</sup> Alternatively, better resolution was obtained with the peroxidase kit mouse Envision (Dako), with diaminobenzidine and nickel as a chromogen. We dried blots and scanned them with a digital camera fitted to a Leica stereomicroscope.

### Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit the report for publication.

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## Results

Animals injected with 40 mg, 4 mg, and 0.4 mg of infectious macaque brain material died at 25, 38, and 33 months, respectively. By contrast, survival times of the cynomolgus macaques orally dosed with 5 g of the same brain material were 47 and 51 months.

Figure 1 shows the signal obtained in 9 mg of liver or ileum spiked with 1.8 µg to 3 mg of positive brain material and 9 mg of pure brain. The retrieval rate was the same in both organs, and level of sensitivity corresponded to a 1.8 µg spike. Hence, we detected PrP<sup>res</sup> in tissues at amounts 5000 times lower than in brain.

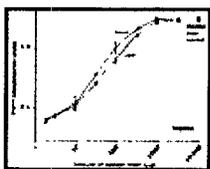


Figure 1: Sensitivity of PrP<sup>res</sup> detection in peripheral organs of primates infected with the BSE agent

Some points on the dilution curve were repeated six times, for which the error bar is shown (SE). Horizontal line shows signal corresponding to the negative control (liver or ileum spiked with normal brain homogenate). Amount of negative brain corresponds to amount of spiked positive brain used.

The highest concentrations of PrP<sup>res</sup> were recorded in the tonsils ( $\geq 10\%$  of that in brain in intravenously infected animals; table). The maximum amount of PrP<sup>res</sup> in spleen tissues was 4% that of brain in intravenously infected animals. In the gut, we detected PrP<sup>res</sup> in intravenously and orally infected animals, albeit at a concentration 1000 times lower than in the brain (table). The liver (negative control) was always negative. However, sciatic nerves were all positive, reaching 0.3% of the PrP<sup>res</sup> concentration in the brain. Comparison of PrP<sup>res</sup> amounts

after either infection route showed that in the tonsil and spleen, PrP<sup>res</sup> accumulated at concentrations up to ten times higher in intravenously versus orally infected animals.

	Liver	Spleen	Tonsil	Intestine	Sciatic nerve
Intravenous	ND	0.4-4%	≥10%	0.02-0.08%	0.03%
Oral	ND	0.1-0.2%	1-10%	0.02-0.08%	0.06-0.3%

ND=not detectable.

#### Amount of PrP<sup>res</sup> detected as a percentage of that present in the brain

In tonsils and spleen, PrP<sup>res</sup> localised to the germinal centres (data not shown), as described in vCJD patients.<sup>8</sup> PrP<sup>res</sup> labelling was positive in the duodenum, jejunum, ileum, caecum, colon, and rectum, and localised to several distinct anatomical compartments. Findings of paraffin-embedded tissue-blot analysis confirmed that these PrP deposits corresponded to proteinase K-resistant PrP<sup>res</sup>. Dense PrP<sup>res</sup> deposits were present in isolated follicles or Peyer's patches follicles, which are found abundantly in the ileocaecal fold (figure 2). Negatively stained follicles were intermingled with positive ones. PrP<sup>res</sup> was also present in the myenteric and submucosal plexuses and in the inner circular smooth-muscle layer (figure 3). Neuron-specific enolase staining of nervous structures showed that the dense PrP<sup>res</sup> deposits in the internal circular layer localised to small sympathetic nerve fibres innervating the intestinal smooth muscle. Furthermore, PrP<sup>res</sup> was also present in nerve fibres of the lamina propria of the intestinal villae (figure 4).



Figure 2: Detection and localisation of PrP<sup>res</sup> in Peyer's patches of the distal ileum

PP=Peyer's patch. PrP<sup>res</sup> labelling in the distal ileum of a cynomolgus macaque infected (A) by the intravenous route and (B) by the oral route. (C) PrP<sup>res</sup> labelling in the distal ileum of an uninfected animal. (D) Paraffin-embedded tissue-blot analysis of a Peyer's patch in a cynomolgus macaque inoculated by the intravenous route. Detection of PrP<sup>res</sup> was done with Bar 224. (A-C) Bars=100  $\mu$ m.



Figure 3: Detection and localisation of PrP<sup>res</sup> in the enteric nervous system and smooth muscle nerve fibres of the small intestine

MP=myenteric plexus, SML=smooth muscle layer (internal circular layer). PrP<sup>res</sup> labelling in the ileum of a cynomolgus macaque infected (A) by the intravenous route and (B) by the oral route. (C) PrP<sup>res</sup> labelling in the ileum of an uninfected animal. (D) Nerve labelling with a neuron-specific enolase antibody in the ileum of a cynomolgus macaque infected orally. Detection of PrP<sup>res</sup> was done with Bar 224. (A-D) Bar=50  $\mu$ m.

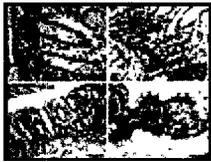


Figure 4: Detection and localisation of PrP<sup>res</sup> in the intestinal mucosa

SMP=submucosal plexa. LP=lamina propria. PrP<sup>res</sup> labelling in the caecum of a cynomolgus macaque infected (A) by the intravenous route and (B) by the oral route. (C) PrP immunohistochemistry in the caecum of an uninfected animal. (D) Labelling of nerve fibres with antiGAP43 in the caecum of a cynomolgus macaque inoculated by the oral route. Detection of PrP<sup>res</sup> was done with Bar 224. (A, B, D) Bar=50  $\mu$ m; (C) Bar=100  $\mu$ m.

We examined the sciatic nerve and sympathetic nerve fibres along the carotid artery. Both nerve types stained heavily for PrP<sup>res</sup> (figure 5). PrP and GFAP double-immunolabelling showed that PrP<sup>res</sup> was localised at the surface of Schwann cells.



Figure 5: Detection and localisation of PrP<sup>res</sup> in peripheral nerves

PrP<sup>res</sup> labelling in the sciatic nerve of a cynomolgus macaque infected (A) by the intravenous route and (B) by the oral route. (Inset) PrP<sup>res</sup> labelling in the sciatic nerve of an uninfected control animal. (C) PrP<sup>res</sup> labelling in a sympathetic nerve fibre along the carotid artery of an animal infected orally with BSE. (D, E) Real colour images of PrP (green) and GFAP (red) labelling in the sciatic nerve of a monkey infected intravenously. (F) PrP and GFAP double-labelling. The same images as in D and F have been acquired in grey scale, pseudocoloured, and superimposed. (A-C) Bar=20  $\mu$ m. (D-F) Bar=10  $\mu$ m.

PrP<sup>res</sup> detection was very weak or negative when formalin-fixed tissues were used.

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## Discussion

Primates infected with infectious brain material had survival times similar to those seen after intracerebral inoculation of the same doses of the BSE agent (20-33 months),<sup>16</sup> which reinforces our finding that the intravenous route of infection is highly efficient for BSE transmission in primates. On the basis of our data, we recommend that for risk-assessment studies the intravenous route should be considered as efficient as the intracerebral route. Amount of infectivity present in the blood of vCJD patients, however, remains to be determined. In rodent models of BSE, the level of infectivity present in blood (buffy coat and plasma) during the clinical phase of the disease has been estimated at 20-30 IU/mL.<sup>22</sup> According to our provisional inoculum titration,<sup>16</sup> the lowest amount of infectivity used for the intravenous infection in this study corresponded to 100 IU or more.

Intravenously and orally infected primates had similar vCJD-like neuropathological lesions (similar to those described previously<sup>2,16</sup>) and western-blot pattern in the brain. PrP<sup>res</sup> was detected in lymphoreticular tissues of the BSE-infected primates, in concentrations similar to those reported in human patients (0.1-15%),<sup>8,23</sup> and our data suggest that tonsils should be judged the

tissue of choice for biopsy screening. No PrP<sup>res</sup> could be detected in the lymphoreticular tissues of cynomolgus macaques infected by either a macaque-adapted sporadic CJD or a Kuru strain (data not shown). Similar observations have been made in human cases of these diseases.

Assessment of the presence of infectivity in the digestive tract or in peripheral nerves was of particular importance in view of the number of medical interventions involving these tissues and the widespread distribution of the peripheral nervous system. In vCJD, one rectal tissue sample has been shown to be PrP<sup>res</sup>-positive, and few peripheral nerves have been looked at.<sup>9,23</sup> Our model obviated the difficulty of restricted access to post-mortem samples, so we could further investigate the PrP<sup>res</sup> content of several portions of the digestive tract and of peripheral nerves.

We could detect widespread PrP<sup>res</sup> distribution in the intestine from the duodenum to the rectum. The extent of PrP<sup>res</sup> contamination of the intestine was similar in primates infected by the intravenous route and in those infected orally. This finding shows that the intestine is a target organ for BSE in primates even if it is not the source of entry of the agent into the organism.

Immunomorphological analysis allowed us to identify two sources of PrP<sup>res</sup> deposition in the gut: gut-associated lymphoid tissue—organised in Peyer's patches all along the small intestine and in the caecum, or present as solitary follicles mainly in the colon and rectum—and the autonomic nervous system. The autonomic nervous system was highly involved, with positive staining noted in both plexuses of the enteric nervous system, in nerve fibres of the intestinal smooth muscle and those lying beyond the submucosal plexus, which defines the boundary of the intestinal mucosa. This finding shows that some prion shedding from the mucosa can happen during endoscopic procedures. However, does the amount of infectivity that can be released this way entail contamination of the instrument that is important enough to convey infection to a subsequent patient?

We investigated the sciatic nerve as an example of a locomotor nerve and nerve fibres from the autonomic nervous system. Both types of nerves were PrP<sup>res</sup>-positive, and findings of immunohistochemistry showed that most axons were surrounded by PrP<sup>res</sup> staining colocalising with Schwann cells. These data should be considered for assessment of the potential risk linked to peripheral nervous system surgery.

In conclusion, the tissue distribution of PrP<sup>res</sup> was closely similar whether the BSE agent entered the body through the gut or directly by the intravenous route, although some quantitative differences were noted, with substantially more PrP<sup>res</sup> detected in the spleen of intravenously infected animals. In addition to lymphoid organs like spleen and tonsils, the digestive tract and the peripheral nervous system are involved tissues. In view of the high efficiency of transmission of the BSE agent to primates by the intravenous route, the latter should be regarded as a likely route of contamination for vCJD patients with a medical history involving a transfusion during the period at risk. To avoid further contamination to human beings from peripheral tissues, the same precautionary measures taken for primary vCJD cases should apply to possible transfusion cases of the disease.

### **Contributors**

C Herzog and N Etchegaray adapted the ELISA method for PrP<sup>res</sup> detection in primates and did biochemical analyses of tissue samples. N Salès developed the highly sensitive immunohistochemistry procedure and, together with A Charbonnier and S Freire, analysed tissue samples. D Dormont was a helpful adviser to the study. J-P Deslys contributed to design and operation of the study. C I Lasmézas coordinated the design and operation of the study and wrote the report.

### **Conflict of interest statement**

None declared.

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