Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples

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Summary

Background Prion diseases are associated with the accumulation of an abnormal isoform of cellular prion protein (PrP^{Sc}), which is the principal constituent of prions. Prions replicate in lymphoreticular tissues before neuroinvasion, suggesting that lymphoreticular biopsy samples may allow early diagnosis by detection of PrP^{Sc}. Variant Creutzfeldt-Jakob disease (variant CJD) is difficult to distinguish from common psychiatric disorders in its early stages and definitive diagnosis has relied on neuropathology. We studied lymphoreticular tissues from a necropsy series and assessed tonsillar biopsy samples as a diagnostic investigation for human prion disease.

Methods Lymphoreticular tissues (68 tonsils, 64 spleens, and 40 lymph nodes) were obtained at necropsy from patients affected by prion disease and from neurological and normal controls. Tonsil biopsy sampling was done on 20 patients with suspected prion disease. Tissues were analysed by western blot to detect and type PrP^{Sc}, by PrP immunohistochemistry, or both.

Findings All lymphoreticular tissues obtained at necropsy from patients with neuropathologically confirmed variant CJD, but not from patients with other prion diseases or controls, were positive for PrP^{Sc}. In addition, PrP^{Sc} typing revealed a consistent pattern (designated type 4t) different from that seen in variant CJD brain (type 4) or in brain from other CJD subtypes (types 1–3). Tonsil biopsy tissue was positive in all eight patients with an adequate biopsy sample and whose subsequent course has confirmed, or is highly consistent with, a diagnosis of variant CJD and negative in all patients subsequently confirmed to have other diagnoses.

Interpretation We found that if, in the appropriate clinical context, a tonsil biopsy sample was positive for PrP^{Sc}, variant CJD could be diagnosed, which obviates the need for a brain biopsy sample to be taken. Our results also show that variant CJD has a different pathogenesis to sporadic CJD.

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Introduction

Around 15% of human prion diseases are inherited and all cases of such diseases have been associated with coding mutations in the prion protein gene (*PRNP*), of which over 20 distinct types have been defined.[1] No such pathogenic mutations in the *PRNP* gene are present in sporadic and acquired prion disease although most cases occur in individuals who are homozygous for a common prion protein (PrP) polymorphism at residue 129.[2,3] Acquired prion diseases, iatrogenic Creutzfeldt-Jakob disease (CJD), and kuru are recognised because of the specific history of exposure to human prions through medical or surgical procedures or participation in cannibalism.

A novel form of human prion disease, variant CJD, was recognised in the UK in 1996.[4] Epidemiological studies argued for a link with bovine spongiform encephalopathy (BSE) and this was strongly supported by molecular strain typing[5] and subsequently by transmission studies into both transgenic and conventional mice,[6,7] confirming that variant CJD and cattle BSE are caused by the same prion strain.

Diagnosis of variant CJD in the living patient presents more difficulties than sporadic CJD. Cerebrospinal fluid 14-3-3 protein, usually positive in sporadic CJD, is presumably a marker of neuronal injury, and appears less useful in the more slowly progressive variant CJD.[8] High T2-weighted signal in the posterior thalamus on magnetic resonance imaging (MRI), seen in several patients with variant CJD, has yet to be formally assessed as a diagnostic tool.[8] Definite diagnosis of variant CJD has therefore remained neuropathological, at necropsy or brain biopsy.

34 cases of variant CJD in the UK and a single case in France have been confirmed by neuropathology. While numbers have risen slowly to date, it remains entirely possible that a substantial epidemic of variant CJD will occur over the coming years. The extremely prolonged incubation periods of these diseases in human beings, allied with the effect of crossing a species barrier (which will further prolong mean incubation periods), means

Diagnosis	Tonsil	Spleen	Lymph node
PrP immunohistochemistry*			
Variant CJD	9/9	10/10	8/8
Iatrogenic CJD	0/1	0/5	0/4
Sporadic CJD	0/16	0/20	0/8
Inherited prion disease	0/1	0/1	0/1
Alzheimer's disease	0/6	0/12	0/8
Other neurological disease†	0/5	..	..
Normal controls	0/13	0/12	0/8
Western blot for PrP*			
Variant CJD	6/6	2/2	..
Iatrogenic CJD	..	0/1	0/1
Sporadic CJD	0/5	0/1	0/1
Inherited prion disease	0/1	0/1	0/1
Normal controls	0/17‡	0/1	..

*Samples were analysed by immunohistochemistry or western blot, or both.
†Motor neurone disease, cerebrovascular disease, global ischaemia, encephalitis.
‡Includes both necropsy and routine tonsillectomy tissue.

Table 1: **Necropsy lymphoreticular tissues**

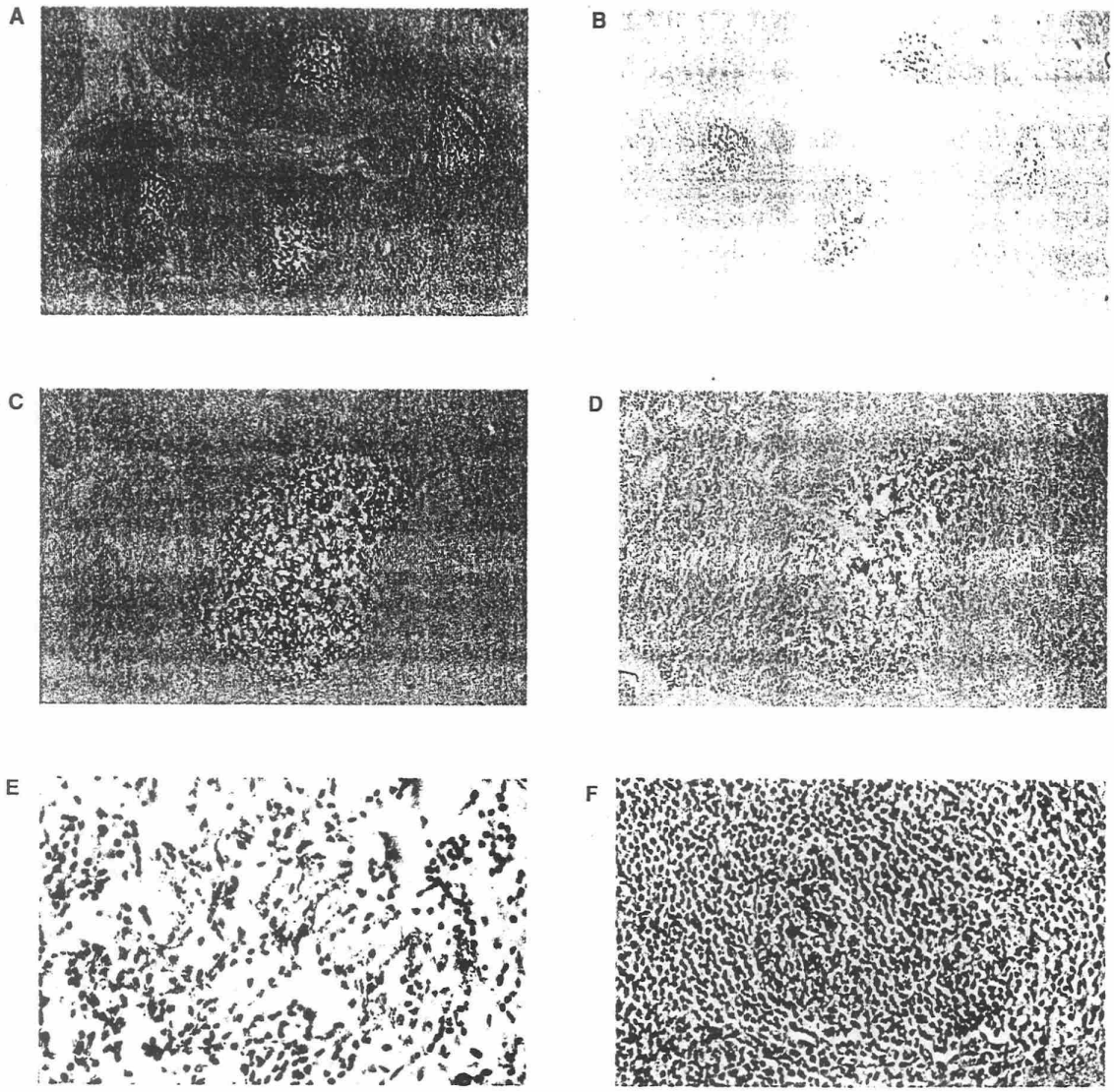

Figure 1: **Immunocytochemistry (A–D) and PrP immunoreactivity (E,F) in tonsil biopsy samples from patients with variant CJD**
(A) Positive staining for PrP and (B) co-localisation of PrP-immunoreactivity with positive immunostaining for CD35 (follicular dendritic cell marker) in adjacent section. T-cell and B-cell areas of tonsil are unstained in both sections. (C) and (D) are single follicle in a tonsil in variant CJD immunostained with antibodies to PrP and CD35, respectively. (E) and (F) are typical PrP immunoreactivity in tonsil biopsy samples from patients with variant CJD.

that it may be some years before the parameters of such an epidemic can be predicted with any degree of confidence. It is possible that significant numbers in the population are incubating this novel disease and that they might pass it on to others via blood transfusion, blood products, tissue and organ transplantation, and contaminated surgical instruments, and other iatrogenic routes.

Prion diseases of human beings and animals are associated with the accumulation in the brain of an abnormal, partly protease-resistant isoform, of cellular prion protein (PrP^{C}), known as PrP^{Sc}. Prion strain-diversity appears to be encoded by differences in PrP conformation[5,9,10] and pattern of glycosylation.[5] Molecular-strain typing has allowed the identification of four main types of CJD: all cases of sporadic and iatrogenic CJD are PrP^{Sc} types 1–3, and cases of variant CJD are all associated with a distinctive type 4 PrP^{Sc} type.[5] The identification of type 4 PrP^{Sc} in human brain tissue by western blotting allows a diagnosis of variant CJD.[5] However, this technique has been limited by the necessity to use brain tissue for PrP^{Sc} typing.

Prion replication occurs in the lymphoreticular system in sheep with scrapie and in rodent models of scrapie,[11] and replication in the spleen and other lymphoreticular tissues may precede detectable neuroinvasion with prions by quite some time. Tonsil biopsy has been shown to be a useful diagnostic test in sheep scrapie—PrP immunohistochemistry is positive in about a third to a half the normal incubation period.[12]

PrP^{Sc} has been found in tonsil tissue in a necropsy sample from a neuropathologically confirmed case of variant CJD,[13] suggesting that tonsil biopsy sampling may be useful for tissue diagnosis of human prion disease. We analysed a series of necropsy samples from patients with sporadic, inherited, and acquired

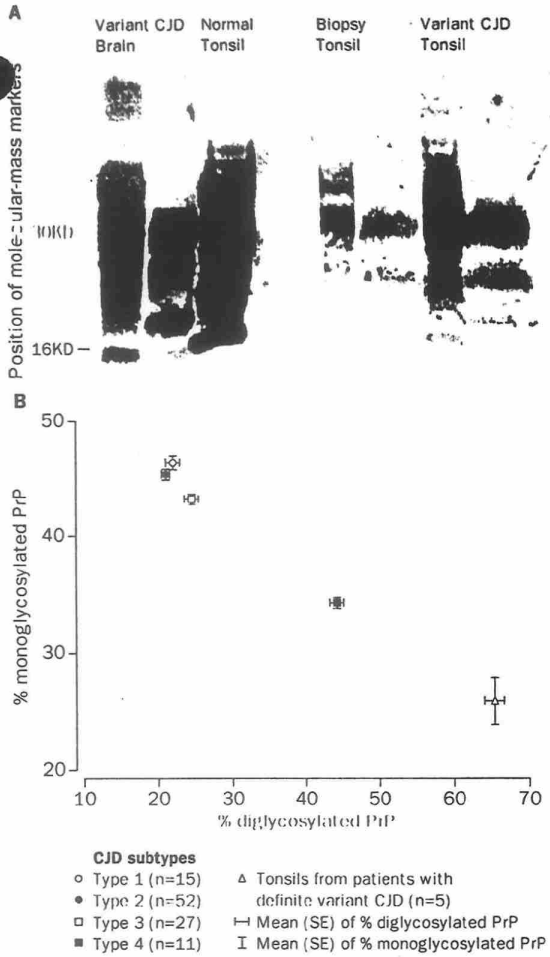

Figure 2: **PrP^{Sc} analysis of tonsil biopsy tissue: western blot (A) and glycoform profiles (B)**

Glycoform profiles are of protease resistant PrP in tonsil of variant CJD compared with those in brain tissue from variant CJD (type 4 PrP^{Sc}) and sporadic and iatrogenic CJD subtypes (types 1–3 PrP^{Sc}).

human prion diseases and tonsil biopsy samples done for the investigation of patients with suspected prion disease.

Methods

Collection of tonsil samples

Tonsil tissue was collected at necropsy from patients with suspected prion disease and normal controls. Where prion disease was suspected, necropsy and tissue handling was done according to established safety guidelines.[14,15]

The use of tonsil biopsy samples for investigation of prion disease was approved by the St Mary's Hospital Ethics Committee. Informed consent for biopsy was obtained from the patients, or a relative, or both. All biopsy samples were taken under general anaesthesia. In all cases, careful microbiological containment precautions were observed in the operating theatre. This included the use of disposable gowns, aprons, drapes, overshoes, and gloves. Gloves were also worn by ancillary theatre staff. Non-essential equipment was removed from the theatre. Both surgeon and scrub nurse wore double-glove protection and disposable safety glasses. Modifications to the anaesthetic procedure included single-use disposable Mackintosh intubating laryngoscope blades (Upsher Laryngoscope Corporation, Foster City, CA, USA). Disposable endotracheal tubes were used. Intersurgical anaesthetic circuits were used with bacterial filters placed at both ends. The anaesthetic circuit, filters, and other disposable materials were destroyed by incineration at the end of the procedure.

The tonsil biopsy sample was taken with a disposable tonsillectomy set (Exmoor Plastics Limited, Taunton, UK). In each case, a wedge of tonsillar tissue was excised from the medial aspect of the middle third of the right tonsil only. Ideally, a 1 cm wedge was taken, but satisfactory analysis could be done with as little as 50 mg tissue. The specimen was sealed in a screw-top sterile plastic container and transferred fresh to a microbiological containment level-three facility for subsequent processing and analysis. In most cases bleeding stopped spontaneously, but if it did not, the cut surfaces were sewn together with 3·0 Vicryl sutures.

Analysis of anonymous tonsil tissue from routine tonsillectomies was approved by the St Mary's Hospital Ethics Committee.

Immunohistochemistry

Paraffin-embedded blocks of formalin-fixed lymphoid tissue from patients with prion disease taken at necropsy and from normal and neurological controls were studied. Paraffin sections were cut every 5 µm and stained with conventional histochemical techniques and by immunohistochemistry for prion protein with monoclonal antibodies 3F4 (Senetek, Maryland Heights, MO, USA) and KG9,[16,17] in a three-step pretreatment with hydrated autoclaving, exposure to formic acid, and guanidine thiocyanate.[18] Lymphoid tissue that contained detectable PrP was then stained with a panel of polyclonal and monoclonal antibodies to characterise the cells in which PrP positivity was detected (S100 protein, CD3, CD20, CD21, CD35, CD45, CD68, and CD79A, Dako). Serial or adjacent sections were compared to identify the subset of cells in which PrP positivity was detected. Double-labelling with antibodies to PrP and lymphoid antigens is unsatisfactory because the pretreatments necessary for PrP detection extensively denature the cell-surface antigens on lymphoid cells. Tonsil biopsy specimens were divided and a portion was fixed in formol saline followed by decontamination in 98% formic acid for 1 h. Immunohistochemistry was then done as above but with PrP monoclonal antibodies 3F4 and 12F10.[16,19]

Western blot analysis of lymphoreticular tissue

All procedures were done in a microbiological containment level three facility. Fresh lymphoreticular tissues were stored within this facility at –80°C until analysed. 20–50 mg tissue was homogenised in lysis buffer (10 mmol/L tris-HCl, pH 7·4, 100 mmol/L sodium chloride, 10 mmol/L edetic acid, 0·5% sodium deoxycholate, 0·5% NP-40) with a Polytron. The homogenate was cleared by centrifugation at 3000 rpm for 5 min. Proteinase K (BDH) was added to a sample of the cleared supernatant at a final concentration of 50 µg/mL and incubated for 1 h at 37°C. The reaction was terminated by the addition of Pefabloc (Boehringer, Mannheim, Germany) to 2 mmol/L. Samples were mixed with an equal volume of sodium dodecyl sulphate loading buffer (125 mmol/L tris-HCl, 4% SDS, 20% glycerol, 0·02% bromophenol blue, pH 6·8) and boiled for 5 min before electrophoresis on 16% tris-glycine gels. The gels were electroblotted onto Immobilon-P (Millipore, Bedford, MA, USA), blocked in 5% Blotto (5% non-fat milk powder in phosphate-buffered saline with 0·05% Tween–20) and then incubated with antibody 6H4 (Prionics) or 3F4 at a 1:5000 dilution overnight. The blots were washed in phosphate-buffered saline with 0·05% Tween–20 and incubated with an alkaline-phosphatase-conjugated antimouse antibody for 1 h at room temperature. The blots were washed and developed with a chemifluorescent substrate (enhanced chemifluorescence; Amersham, Little Chalfont, UK) and visualised on a Storm 840 phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA). PrP glycoforms were quantified with ImageQuaNT software (Molecular Dynamics). All blots included appropriate positive and negative controls.

Age at onset (years)	Clinical duration (months)	Tonsil biopsy at (months)	Presenting features	Other neurological features	MRI brain scan	EEG	CSF 14-3-3 protein	*PRNP* genotype	Neuropathological diagnosis
35	12	11	Depression	Dementia, ataxia myoclonus, chorea to akinetic mutism	Normal	Non-specific: slow waves	N/A	129MM	Definite variant CJD
21	28	9	Depression, ataxia	Dementia, visual hallucinations and persecutory delutions, chorea, dystonia, pyramidal signs to akinetic mutism	Normal	Non-specific: slow waves	Positive	129MM	Definite variant CJD
22	26	16	Social withdrawal, anxiety, dysaesthesiae	Ataxia, dementia, myoclonus, dystonia to akinetic mutism	Normal	Non-specific: slow waves	Positive	129MM	Definite variant CJD
18	>24	10*	Dysaesthesia, panic attacks	Ataxia, dystonia, pyramidal signs to akinetic mutism	Bilateral posterior thalamic high T2 signal	Non-specific: slow waves	Trace	129MM	..
34	>12	8†	Emotional lability, cognitive loss	Ataxia, dysasthesia, myoclonus, pyramidal signs to akinetic mutism	Bilateral posterior thalamic high T2 signal and subcortical white matter lesions	Non-specific: slow waves	Positive	129MM	..
21	5·5	3	Dysaesthesia	Ataxia, pyramidal signs, cognitive loss, athetosis	Normal	Non-specific: widespread theta waves	Trace	129MM	Necropsy not done
29	>14	14	Behavioural disturbance with apathy/withdrawal	Dysaesthesia, cognitive decline, ataxia, chorea, dystonia	Bilateral posterior thalamic high T2 signal	Non-specific: slow waves	Trace	129MM	..
24	>11	11	Dysaesthesia	Ataxia, cognitive decline, pyramidal signs	Bilateral posterior thalamic high T2 signal	Non-specific: slow waves	Positive	129MM	..
28	>12	12	Behavioural disturbance with agitated depression	Ataxia, chorea, cognitive decline	Non-specific white-matter changes	Slow waves, no definite periodic complexes although some suspicious	Positive	129MM	..

MRI=magnetic resonance imaging, EEG=electroencephalogram, CSF=cerebrospinal fluid.
*Inadequate biopsy sample taken but some PrP positivity seen on immunohistochemistry. †Biopsy sample only suitable for PrP immunohistochemistry.

Table 2: **Clinical features and investigations in patients with positive tonsil-biopsy samples**

Results

Analysis of necropsy lymphoreticular tissues

We analysed 68 tonsils, 64 spleens, and 40 lymph nodes obtained at necropsy from patients with prion disease, Alzheimer's disease, and other neurological diseases, and from normal controls. The analysis was done by western blotting for PrP^{Sc} (where fresh tissue was available) by PrP immunohistochemistry, or both (table 1). All lymphoreticular tissues studied from patients with variant CJD were positive. In cases in which both western blotting and immunohistochemistry were used, both methods gave positive results. No PrP^{Sc} on western blot analysis or PrP immunoreactivity on tissue sections was seen in other types of prion disease or in the neurological or other control tissues.

PrP immunohistochemistry

In variant CJD lymphoid tissues, positive staining for PrP was only detected within germinal centres (figure 1A, C). The number of PrP positive cells in the tonsillar germinal centres was higher than in the spleen or lymph nodes; tonsillar tissues generally contained more germinal centres than did other lymphoid tissues in these patients. Comparison with immunohistochemistry for lymphoid cells and macrophages showed co-localisation of PrP positivity with CD35 and CD21 monoclonal antibodies (Ber MAC-DRC, Dako), which label follicular dendritic cells in paraffin sections (figure 1B, 1D).[20,21] Both T-cell and B-cell areas in the tonsil, spleen, and lymph nodes were unstained by the antibodies to PrP. This pattern of immunostaining was similar to that we previously reported in a single patient with variant CJD.[13] While we have not yet been able to do a comprehensive survey of lymphoreticular tissues in variant CJD, all lymph-node regions studied to date have been positive (including cervical, mediastinal, para-aortic, and mesenteric).

Western blot analysis for PrP^{Sc}

Protease-resistant PrP was detected in all lymphoreticular tissues examined from patients with definite variant CJD but was not detectable in tissues from other human prion diseases or in controls. Human prion strains can be distinguished by both molecular mass and relative abundance of the three principal PrP glycoforms detected by western blotting of proteinase-K-digested brain homogenates. Fragment sizes of protease-treated PrP in tonsil tissue were indistinguishable from those seen in brain from patients with variant CJD (figure 2). Figure 2A shows western blot comparison of a tonsil biopsy sample with tonsil and brain tissue from definite variant CJD and normal control tonsil with anti-PrP monoclonal antibody 6H4 (Prionics). After digestion with proteinase K an identical pattern of protease-resistant prion protein fragments was seen in the biopsy sample and in tonsil tissue from a patient with variant CJD confirmed at necropsy. While the glycoform ratios in variant CJD tonsil superficially resembled those seen in variant CJD brain, with a distinctive abundance of diglycosylated PrP, quantitation of these ratios revealed that they were different from those seen in brain ($p<0{\cdot}0001$ with respect to the proportions at each of the three glycoforms [unpaired two-tailed *t*-tests]), but highly consistent between the variant CJD samples studied (figure 2B). The concentrations of PrP^{Sc} detectable in variant CJD tonsil varied and in some cases was about 10% of the concentration seen in variant CJD brain tissue.

Frozen tonsil tissue from five necropsies was provided by the CJD Surveillance Unit and analysed by western blot by technicians unaware of the diagnosis. PrP^{Sc} was detected in two of these necropsy samples. Both of these were from patients with necropsy-confirmed variant CJD; the other tonsils were from patients with sporadic and iatrogenic CJD.

Tonsil biopsy

All patients who had tonsil biopsies made an uneventful recovery. By their first postoperative day, none of the patients required analgesia and all had resumed their

Age at onset (years)	Clinical duration (months)	Tonsil biopsy at (months)	Presenting features	Other neurological features	MRI brain scan	EEG	CSF 14-3-3 protein	*PRNP* genotype	Clinical progress diagnosis	Neuropathological diagnosis
[redacted]	30	14*	Cognitive decline	Parkinsonism, dystonia, myoclonus, pyramidal signs, ataxia	Cerebral atrophy	Focal slow waves and spike and wave activity	Trace	A117V 129MV	Inherited prion disease	Spongiform encephalopathy with PrP plaques, no features of variant CJD
50	3	1·5	Ataxia	Rapid cognitive decline, myoclonus	Normal	Non-specific: slow waves	Positive	E200K 129MM	Inherited prion disease	Typical CJD
79	3·5	2·5*	Disorientation and hypersomnolence	Rapidly progressive dementia, myoclonus to akinetic mutism	Normal	Periodic complexes	Positive	129MM		Sporadic CJD
41	5·5	3	Ataxia, visual impairment unilateral motor, sensory symptoms	Myoclonus, cortical blindness, dystonia to akinetic mutism	Bilateral high-T2 weighted signal in putamen	Periodic complexes	Positive	129MM	Probable sporadic CJD	Necropsy not done
25	>20	14	Social withdrawal, mutism seizures	Cognitive impairment	Normal	Non-specific: slow waves	Negative	129MV	Fluctuating clinical course with recent cognitive improvement	..
18	5	3	Sub-acute confusional state, agitation	Focal seizures, myoclonus	Normal	Non-specific: slow waves	Negative	129MV	Complete recovery after 5 months, undiagnosed encephalopathy	..
34	>13	5	Shaking movements in limbs	Aggression, apathy, reduced verbal and written output	Normal	Non-specific: slow waves	Negative	129MM	Almost complete recovery	..
25	>19	12	Progressive aphasia	Myoclonus, mutism, frontal signs, apraxia	Left-hemisphere atrophy	Non-specific: slow waves	Negative	129VV	Progressive decline, undiagnosed neurodegenerative disorder	..
27	>18	13	Paraesthesia	Pyramidal signs, cognitive deficits, epilepsia partialis continua	Normal	Medication-related fast waves	Negative	129MM	Non-progressive, recent cognitive improvement	..
63	>6	4	Cognitive impairment	Ataxia, visual hallucinations, myoclonus	Changes secondary to childhood neurosurgery for cerebral abcess	Non-specific: slow waves	Negative	129MM	Spontaneous improvement in cognitive skills and balance and cessation of myoclonus	..
56	>33	10	Visual hallucinations, depression	Progressive detioration with cognitive decline, rigidity, tremor, and myoclonus	Normal	Non-specific: slow waves	N/A	129MV	Progressive decline, current clinical diagnosis dementia with Lewy-bodies	..

N/A=not available.
*Inadequate biopsy sample.

Table 3: **Clinical features and investigation of patients with negative tonsil-biopsy samples**

preoperative diet. This compares favourably to the rehabilitation after full bilateral tonsillectomy, where only about one in five patients return to their normal dietary habits before the tenth post-operative day and over two-thirds need analgesics after the first post-operative day.[22]

Diagnostic biopsy samples were taken from 20 patients with suspected prion disease. Biopsy samples were positive by western blot and PrP immunohistochemistry in seven patients. In an eighth patient, the whole biopsy sample was inadvertently placed in fixative, which precluded western blotting, but PrP immunohistochemistry was strongly positive. In a ninth case the tonsils were atrophic and the biopsy sample obtained was largely epithelial tissue such that satisfactory western-blot analysis was not possible, however, fine granular cytoplasmic staining was seen in a small fraction of probable lymphoid cells by immunohistochemistry. The PrP^{Sc} banding pattern that formed after proteinase K digestion in each positive case was indistinguishable from that seen in necropsy-derived tonsil from patients with definite variant CJD (figure 2). PrP immunohistochemistry in these patients also showed a similar pattern of PrP immunoreactivity (figure 1E and 1F) to that of necropsy tonsil from patients with definite variant CJD.

Of these positive tonsil analyses, four patients have subsequently died and necropsies have been done on three. In all three necropsies, a diagnosis of variant CJD was confirmed by neuropathological examination. The other five patients with unequivocally PrP-positive tonsils and the sixth patient with an inadequate biopsy (but some PrP-positive cells) are each thought to have variant CJD and a relentlessly degenerative clinical course has continued, consistent with this diagnosis (table 2). In addition, all these patients are homozygous for methionine at polymorphic PrP residue 129, the *PRNP* genotype seen in all cases of variant CJD recognised to date. Also, all six patients had positive or trace positive 14-3-3 protein in their cerebrospinal fluid and four had high T2-weighted signal in the posterior thalami at MRI, consistent with a diagnosis of variant CJD.

In two of the other 11 biopsy samples, an adequate biopsy sample was not obtained because tonsils were atrophic, or there was insufficient tonsillar tissue because of previous tonsillectomy. Two patients, although without an apparent family history of neurodegenerative disease on referral for biopsy sampling, were diagnosed as having inherited prion disease by *PRNP* analysis. These two patients were confirmed as having the E200K and A117V *PRNP* mutations. A further two patients with negative tonsil biopsy samples have subsequently died. In one, a diagnosis of sporadic CJD was confirmed

on neuropathological examination, and the second was diagnosed as sporadic CJD with a typical electroencephalogram, but necropsy was not done. Of the seven living patients, five have made a part or complete recovery, inconsistent with a diagnosis of prion disease. The remaining two patients were not homozygous for *PRNP* codon 129 methionine. One has been affected for over 33 months and is thought to have dementia with Lewy bodies. The second patient, who presented with a most unusual clinical picture of very young onset progressive aphasia, has continued to deteriorate—no diagnosis having been reached. Brief case summaries of these biopsy-negative patients are given in table 3.

Discussion

The numbers of patients studied are still relatively small, but all definite or clinically highly probable cases of variant CJD studied had PrP^{Sc} detectable in tonsil tissue, or had abnormal PrP immunoreactivity, or both, and no case of other forms of prion disease were positive. These findings suggest that tonsil biopsy, at least in advanced disease, is both an extremely specific and sensitive diagnostic test. Our results, allied with previous studies that failed to detect PrP^{Sc} in lymphoreticular tissues of patients with sporadic CJD,[20] show a clear distinction between the degree of lymphoreticular involvement in variant and sporadic CJD. This could be due to a prion-strain effect, with the BSE-like (bovine-spongiform-encephalopathy) strain that causes variant CJD being highly lymphotrophic in human beings, or could relate to a first passage or species-barrier effect. The degree of involvement of the lymphoreticular system in prion pathogenesis is known to vary between prion strains in the same host and also between hosts infected with the same prion strain.[14] It has also been suggested that prion replication is required in the lymphoreticular system on first passage of a prion strain from a different species to the inoculated animal.[23] The route of exposure of the patients developing variant CJD may be crucial. We were unable to detect PrP^{Sc} in lymphoreticular tissues from patients with iatrogenic CJD. It is possible that the oral route of exposure, presumed to be the route of BSE infection of patients with variant CJD, results in a more pronounced lymphoreticular phase. In this regard, it will be of interest to study lymphoreticular tissues from patients with kuru.

The stage at which PrP^{Sc} is detectable in human tonsil in patients incubating variant CJD is unknown. In sheep scrapie and in animal models of prion disease, prion replication is typically detected first in the spleen and rises to a plateau before detectable neuroinvasion. Mice with severe combined immunodeficiency are highly resistant to prion infection if inoculated peripherally.[24,25] The precise cell type or types involved in lymphoreticular prion replication and transport to the central nervous system remains controversial although several lines of evidence argue for a key role for follicular dendritic cells in peripheral replication.[26,27] Extrapolation from these studies would argue for an early pre-clinical involvement of the lymphoreticular system in variant CJD. This is supported by the finding of abnormal PrP immunoreactivity in an appendix removed 8 months before the onset of neurological symptoms in a patient subsequently confirmed, at necropsy, as having definite variant CJD.[28] It is of interest that in cattle experimentally infected with BSE, infectivity (by mouse bioassay) is detectable only in the distal ileum at 6 months post-exposure, suggesting early involvement of Peyer's patches and other gut lymphoreticular tissue.[29] These findings suggest that human tonsil biopsy samples may allow presymptomatic diagnosis of variant CJD. Indeed, it is possible, assuming sufficiently sensitive tests were available, that PrP^{Sc} will be detectable in human tonsil within months of exposure to BSE. It is possible, although speculative, that all human beings incubating BSE could currently be detected from tonsil biopsy samples. It may therefore be possible to obtain useful prevalence information about preclinical variant CJD by anonymous screening of routine tonsillectomy samples, appendicectomy tissues and archival material. The methods described could, in principle, be used to screen cadaveric or live organs, or tissue donors, including bone-marrow donors, if the prevalence of preclinical variant CJD were of a sufficient level to justify this. It is possible however, for the reasons outlined above, that secondary variant CJD cases, arising from iatrogenic exposure to variant CJD prions, may not have positive tonsil biopsy samples, and may be undetectable by these methods. It is also possible that the current patients affected by variant CJD will prove to be atypical in the degree of their lymphoreticular involvement. There is, at present, no epidemiological evidence to suggest an unusual dietary or occupational exposure to BSE amongst the patients currently recognised as having variant CJD, and therefore it is possible that they represent a particularly susceptible subgroup with unusual lymphoreticular sensitivity to BSE prions resulting in short incubation periods.

Experimental evidence to suggest infectivity of human blood in sporadic CJD has been questioned,[30] and epidemiological studies provide no evidence that blood transfusion or blood products are a risk factor for the development of CJD.[31] However, our finding of significant concentrations of PrP^{Sc} in lymphoreticular tissues of variant CJD patients, but not in classical CJD patients, together with evidence for a key role for mature B lymphocytes in prion pathogenesis,[32] emphasised concerns that blood and blood products derived from patients incubating variant CJD may represent a greater risk than with material from classical CJD. In this regard, it will be important to identify the cell type or types involved in propagating and carrying prion infectivity, or both, in peripheral tissues. Our studies are consistent with a role for follicular dendritic cells and it is possible that the involvement of B lymphocytes is simply to allow maturation of follicular dendritic cells.[33,34] The intimate association of B cells and follicular dendritic cells in lymphoid follicles, with interdigitation of their cellular membranes, complicates dissection of their individual roles because glycosylphosphatidylinositol anchored cell-surface proteins such as PrP can transfer between cells.[35] If prion propagation were to be restricted to non-circulating cell types such as follicular dendritic cells, this would not necessarily exclude transport of PrP^{Sc} by circulating cells such as B lymphocytes.

It is known that different tissues, and different cell types within tissues, glycosylate proteins differently. It is therefore unsurprising that the ratios of protease-resistant PrP glycoforms seen in the tonsil in variant CJD differs to some extent from those seen in brain tissue. However, diglycosylated PrP represents the predominant

glycoform as in variant CJD brain, in clear distinction to the pattern seen in the brain in the various types of sporadic CJD, where monoglycosylated PrP predominates. Presumably, the glycoform ratios seen in variant CJD tonsil represent a superimposition of prion strain and tissue-specific effects. This tonsillar PrP^{Sc} type in variant CJD is designated type 4t to distinguish it from the type 4 pattern seen in the brains of all patients with variant CJD studied to date. Clearly, the PrP glycosylation patterns in tonsil of other human prion diseases cannot be compared because, if PrP^{Sc} is present in tonsil tissue in these patients, it is undetectable by current methods.

Contributors

A F Hill, S Joiner, and G Jackson developed tonsillar PrP^{Sc} analysis, analysed all the frozen material, and contributed to drafting the manuscript. R J Butterworth, M N Rossor, and D J Thomas assessed patients, and contributed to the design of the study and drafting the manuscript. A Frosh and N Tolley developed tonsil biopsy methods and contributed to the design of the study and drafting the manuscript. J E Bell, M Spencer, A King, S Al-Sarraj, J Ironside, and P L Lantos developed tonsil PrP immunohistochemical methods, analysed all the fixed material, and contributed to drafting the manuscript. J Collinge co-ordinated the design and operation of the study, assessed the patients, and drafted the manuscript.

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