LETTERS

Irregular presence of abnormal prion protein in appendix in variant Creutzfeldt-Jakob disease

We have investigated the presence of disease related prion protein (PrP^{Sc}) in appendix samples obtained at necropsy from four neuropathologically confirmed cases of variant Creutzfeldt-Jakob disease (vCJD). PrP^{Sc} was detected in only one vCJD appendix, at a level lower than found in a diagnostic tonsil biopsy sample obtained from the same patient. The single PrP^{Sc} positive appendix, but not the other samples, also showed abnormal prion protein immunohistochemistry. The finding that appendix samples from three of four cases of vCJD are devoid of detectable PrP^{Sc} questions the utility of screening archival appendicectomy tissues to estimate the prevalence of pre-clinical vCJD infection within the UK population.

The appearance of a novel human prion disease, variant Creutzfeldt-Jakob disease (vCJD), in the United Kingdom from 1995 onwards, and the experimental confirmation that this is caused by the same prion strain as that causing BSE in cattle, has raised the possibility that a major epidemic of vCJD will occur in the United Kingdom and other countries as a result of dietary or other exposure to BSE prions.[1] The pathogenesis of vCJD differs significantly from that of other forms of CJD. Disease associated prion protein (PrP^{Sc}) is readily detectable in lymphoreticular tissues in vCJD and not in classic CJD.[1–3] High levels of PrP^{Sc} are uniformly found in the central nervous system and lymphoreticular system of vCJD patients.[2 3] The highest levels of PrP^{Sc} seen outside the central nervous system in vCJD are in tonsil (about 10% of that found in brain)[2 3] and tonsil biopsy is used for antemortem diagnosis of vCJD.[1–3] To date, positive prion protein immunohistochemistry has been reported in only a single appendix sample, although, importantly, this was removed from the patient before the onset of overt features of vCJD.[4] While the stage at which lymphoreticular infection occurs in vCJD is unknown, PrP^{Sc} accumulation is detectable in the lymphoreticular system in natural sheep scrapie and in experimental rodent models of scrapie at a very early stage of the incubation period, long before the clinical phase of the disease. Based upon these data it has been suggested that large scale screening of surgical tonsillectomy and appendicectomy tissues for PrP^{Sc} could provide early warning of a high level of vCJD prion infection and several such studies are in progress.[2 5]

Recently we reported our concern after finding that PrP^{Sc} was undetectable in appendix samples obtained at necropsy from two neuropathologically confirmed vCJD cases.[3] While we were not able to examine these samples using immunohistochemical methods, we have now had the opportunity to investigate appendixes from two further vCJD cases by both high sensitivity western blotting and immunohistochemistry.

Methods

Tissue samples

Tissues were collected at necropsy with consent of relatives from two patients with clinical presentations consistent with vCJD. Definite diagnoses of vCJD were confirmed by neuropathological examination and the demonstration of type 4t PrP^{Sc} in tonsil.[2 3] Appendix samples from these vCJD cases, and appendixes from single neuropathologically confirmed cases of either sporadic CJD or inherited prion disease (144 base-pair insertion), were divided and prepared as either 10% homogenates in phosphate buffered saline (PBS) or fixed in 10% formal saline.

Immunohistochemistry

Tonsil tissue was fixed in 10% buffered formal saline and inactivation of prion infectivity was accomplished by incubation in 98% formic acid for one hour. After further washing for 24 hours in 10% buffered formal saline, tissue samples were processed and paraffin wax embedded. Sections were cut at a nominal thickness of 4 µm, treated with 98% formic acid for five minutes and then boiled in EDTA-TRIS-citrate buffer pH 7.8 for 20 minutes. Immunohistochemical staining was performed with anti-PrP monoclonal antibody 12F10 on a Ventana automated immunohistochemical staining machine using a basic diaminobenzidine detection system according to the manufacturers instructions (Ventana Medical Systems, Tucson, Arizona).

Detection of PrP^{Sc}

Sodium phosphotungstic acid precipitation of PrP^{Sc} from 0.5 ml 10% tissue homogenates and western blotting using high sensitivity enhanced chemiluminescence was performed as described previously.[3]

Results

Recently we reported that appendix samples obtained at necropsy from two neuropathologically confirmed vCJD cases contained undetectable levels of PrP^{Sc}.[3] We have now examined appendix samples from two further neuropathologically confirmed vCJD cases and have detected PrP^{Sc} in only one vCJD appendix (fig 1A). The level of PrP^{Sc} present in this appendix was compared directly with the level of PrP^{Sc} present in a diagnostic tonsil biopsy sample obtained from the same patient. After proteinase K treatment of equivalent aliquots (20 µl) of 10% appendix homogenate or 10% tonsil biopsy homogenate, we observed clear detection of PrP^{Sc} in biopsy tonsil homogenate, but not in appendix homogenate (fig 1B). Similarly, PrP^{Sc} was readily detectable in necropsy tonsil obtained from the vCJD patient with PrP^{Sc} negative appendix (fig 1C). The background single immunoreactive band seen in appendix is also

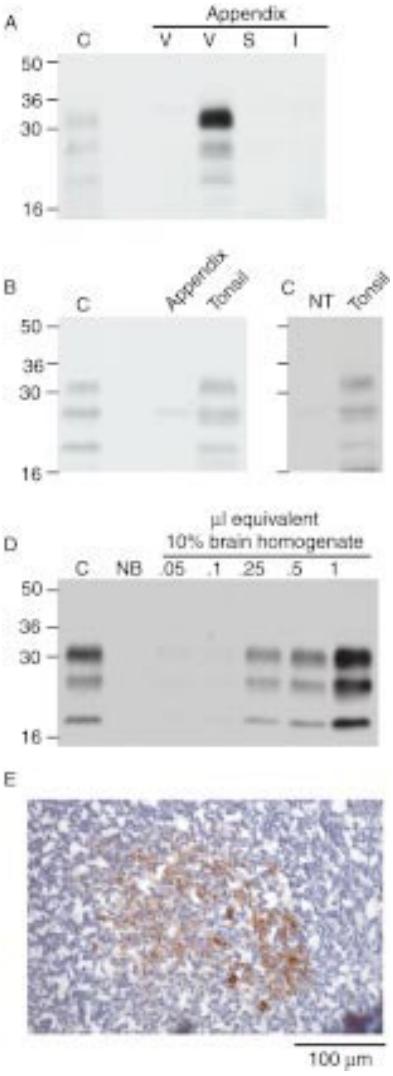

Figure 1 (A–D) Western blots of tissue homogenates with anti-PrP monoclonal antibody 3F4. Western blots were analysed by high sensitivity ECL.[3] The positions of molecular mass markers are indicated in kilodaltons (kDa). (A) Proteinase K (PK) digestion products from a sodium phosphotungstic acid pellet from 0.5 ml 10% normal human tonsil homogenate spiked with a control level of 10% vCJD brain homogenate (C) is compared with PK digestion products from sodium phosphotungstic acid pellets from 0.5 ml 10% appendix homogenates from vCJD (V), sporadic CJD (S) or inherited prion disease (I) cases. (B) PK digestion products from 20 µl 10% normal human tonsil spiked with a control level of 10% vCJD brain homogenate (C) is compared with PK digestion products from 20 µl of 10% appendix or 10% biopsy tonsil homogenate from the vCJD case with PrP^{Sc} positive appendix. (C) Proteinase K digestion products from 20 µl 10% normal human tonsil (NT) is compared with PK digestion products from 20 µl 10% necropsy tonsil homogenate from the vCJD case with PrP^{Sc} negative appendix. (D) The western blot shows PK digestion products from 10 µl 10% normal human brain homogenate obtained in the absence of spike (NB) or after spiking with either a control level of 10% vCJD brain homogenate (C) or 0.05, 0.1, 0.25, 0.5, or 1 µl of 10% brain homogenate from the vCJD case with PrP^{Sc} positive appendix. The control level of spike of 10% vCJD brain homogenate (C) shown in panels A, B, D is equivalent to 50 nl 10% brain homogenate from a vCJD case previously reported to have a maximal level of PrP^{Sc} in brain[3]; we estimate that an equivalent level of PrP^{Sc} is present in about 0.75 µl 10 % brain homogenate from the vCJD case with PrP^{Sc} positive appendix. (E) Photomicrograph of PrP^{Sc} positive appendix. Immunoreactivity for PrP^{Sc} in a lymphatic follicle of the appendix (anti-PrP monoclonal antibody 12F10). The immunostaining pattern is similar to that reported in tonsils in vCJD patients[2] and suggests deposition mainly in dendritic cells.

seen in normal tonsil and is attributable to weak cross reactivity of the secondary antibody with an immunoglobulin fragment. While this band is consistently observed after high sensitivity enhanced chemiluminescence of total lymphoreticular homogenate, it is not recovered after sodium phosphotungstic acid precipitation. We determined that the level of PrP^{Sc} present in the brain of the vCJD patient with PrP^{Sc} positive appendix patient is approximately 15-fold lower than the maximum level we have observed in vCJD brain[3] (fig 1D). Based upon these findings we estimate that biopsy tonsil and appendix, contain levels of PrP^{Sc} of about 4% and about 0.5%, respectively, of that found in the brain of the same vCJD patient (see legend to fig 1).

Importantly, we were able to correlate the detection of PrP^{Sc} by western blotting in vCJD appendix with the detection of abnormal prion protein staining by immunohistochemistry. Abnormal prion protein deposits were clearly observed on sections from the PrP^{Sc} positive vCJD appendix (fig 1E), while prion protein immunoreactivity was unremarkable on sections from the PrP^{Sc} negative vCJD appendix or on sections of appendix from the sporadic CJD or inherited prion disease cases (data not shown).

Discussion

Our findings, together with our previously reported inability to detect PrP^{Sc} in two other vCJD appendixes,[3] indicate that appendix does not reliably report vCJD infection even at the end stage of the disease. This observation must be considered when estimating the possible prevalence of vCJD based upon the analysis of archival appendicectomy tissues.[5] Although only a minority of appendixes in vCJD may contain detectable levels of PrP^{Sc}, surgical instruments used for appendicectomy should remain a cause of concern for potential iatrogenic transmission of vCJD prions.

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Cytokine profiles in HIV seropositive patients with tuberculous meningitis

The immunological response in pulmonary and pleural tuberculosis has been extensively studied. However, the response in tuberculous meningitis has not been well documented.[1] In pulmonary disease, exposure to tuberculous antigens results in a T cell and natural killer cellular response, elaborating various cytokines, mainly of T helper type 1 (Th1) origin. Stimulated macrophages elaborate tumour necrosis factor (TNF) α, interleukin (IL) 12, and IL 1, promoting further recruitment and activation of macrophages and lymphocytes.

TNF α correlates with disease severity and may contribute to tissue necrosis; however, TNFα has also contributed to survival in mouse studies.[2] Transforming growth factor β (Th3 cytokine) suppresses macrophage activation. IL 2 may be beneficial in promoting an immune response in HIV seropositive patients. Th1 and Th2 cytokine responses have been observed in cerebrospinal fluid (CSF) of HIV seronegative patients with tuberculous meningitis.[3 4]. Whether the response is similar in HIV seropositive patients with tuberculous meningitis is unknown.

We studied the cytokine response and its correlation with disease severity in HIV seropositive and HIV seronegative patients with tuberculous meningitis.

Tuberculous meningitis was diagnosed on clinical and CSF examination after exclusion of viral, acute bacterial, and other causes of aseptic meningitis. Disease severity was assessed according to the Medical Research Council stages 1 to 3. HIV ELISA was done on all patients. CSF samples were subjected to microscopy, culture, protein and glucose analysis, Venereal Disease Research Laboratory test, fluorescent treponemal antibody analysis, cryptococcal antigen analysis, viral studies, cysticercus ELISA, CD4 counts, and determination of concentrations of adenosine deaminase (ADA), CSF IgG, and albumin.

For cytokine assays, CSF was centrifuged at 3000 *g*, and supernatant was aliquoted and stored at –70°C. TNF α, interferon (IFN) γ, and IL 10 concentrations were measured by ELISA kits (Genzyme Diagnostics, Cambridge, Massachusetts, USA) with detection limits of 3 pg/ml, 3 pg/ml, and 5 pg/ml, respectively.

Data were summarised as medians and ranges. Non-parametric Wilcoxon rank sum tests were used to compare HIV seropositive groups with HIV seronegative groups, tuberculous meningitis severity groups, and groups derived according to the blood brain barrier index for cytokine concentrations. Spearman's rank correlation was used to derive correlations of cytokine concentration, ADA concentrations, and CD4 counts in CSF.

There were 27 patients: 18 (67%) women and 9 (33%) men. Seventeen were HIV seropositive and 10 HIV seronegative. The average interval between onset of symptoms and the first clinical assessment was 17 days (range 5–90 days) in 18 patients where this was recorded. The mean (SD) age was 26.8 (11.6) years. There was one patient aged 10 and one aged 60, and the rest were between 25 and 40. The cytokine concentrations were not analysed according to age, as this would make the categories too small and of little value. The IgG index was calculated for 23 patients. There was no significant difference between the HIV seropositive and HIV seronegative groups for ADA ($p = 0.4$) and CD4 counts ($p = 0.19$) in CSF and cytokine concentrations (table 1).

Ten patients (37%) were classified as having grade 1 tuberculous meningitis. Sixteen (59%) had grade 2 and one (4%) grade 3, which for analysis was considered to be grade 2. Table 1 summarises the cytokine concentrations for patients in stages 1 and 2.

Patients with stage 2 disease had significantly stronger Th1 responses. There was no difference in the IL 10 concentrations. The two patients with stage 2 disease who died had very high IFN γ concentrations, both greater than 2048 pg/ml.

IL 10 concentrations were moderately positively correlated with IFN γ concentrations ($r = 0.53$). The correlation coefficients were –0.18 for IFN γ, –0.33 for TNF α, and –0.34 for IL 10. Correlation coefficients between ADA and cytokine concentrations were 0.34 for IFN γ, 0.47 for TNF α, and 0.22 for IL 10. Cytokine concentrations correlated poorly with CD4 counts in CSF.

It is postulated that in HIV infection a predominant Th2 response accounts for extrapulmonary disease.[5] This study does not favour a predominance of either Th1 or Th2 in the CSF. It is possible that a Th0 response, which is a non-differentiated response seen early on in immune activation, was seen in our patients, as they were examined untreated and relatively early in the disease. Other investigators have also documented this phenomenon.[3] The positive correlation between IFN γ and IL 10 suggests that these were produced concurrently. This may reflect a control mechanism regulating Th1 and Th2 responses.

There was no difference in cytokine and ADA concentrations and CD4 counts between HIV seropositive and HIV seronegative patients. It is known that the clinical response to antituberculous treatment in both groups is similar.[5] Perhaps this similarity correlates with similar immune responses in both groups. The size of each group is small and a type 1 statistical error has to be considered.

Table 1 Differences between HIV seropositive and HIV seronegative groups and tuberculous meningitis severity

	HIV positive		HIV negative			Stage 1		Stage 2		
Cytokine	Median	Range	Median	Range	p Value	Median	Range	Median	Range	p Value
IFN γ (pg/ml)	569.9	16.0–2048	890.6	0–2048	0.9	184.5	0–1771.0	1000.0	16.0–2048	0.03
TNF α (pg/ml)	1.6	0–67.5	9.8	0–309.3	0.11	0.65	0–19.2	9.8	0–309.3	0.008
IL 10 (pg/ml)	24.6	0–127.9	17.3	0–296.3	0.9	3.68	0–53.0	27.4	0–296.4	0.97

IFN, interferon; Il, interleukin; TNF, tumour necrosis factor.