Papers and Articles

Tonsillar biopsy and PrP^{Sc} detection in the preclinical diagnosis of scrapie

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Preliminary findings have indicated that in naturally infected sheep, fully susceptible to scrapie (VRQ-homozygous), PrP^{Sc} can be detected in the tonsils approximately one year before the expected onset of clinical disease, whereas no immunostaining can be detected in animals with a semi-resistant genotype. This paper describes the technique for taking tonsillar biopsies from sheep and gives the results of the completed experiment. In another experiment PrP^{Sc} was detected even earlier in comparable VRQ-homozygous sheep born and raised in different surroundings. At three-and-a-half months of age no PrP^{Sc} could be detected in three homozygous susceptible sheep (VRQ/VRQ), but PrP^{Sc} was detected at four months in one similar sheep. At eight months of age all seven sampled VRQ/VRQ sheep showed positive immunostaining in the biopsies, but none of the biopsies from three VRQ/ARQ heterozygotes showed any immunostaining; they were positive when sampled at 14 to 15 months of age. Biopsies from VRQ/ARR sheep were negative throughout this period. On the basis of the established or expected incubation period, PrP^{Sc} could thus be detected in the tonsils of live susceptible animals at between one-third and a half of the incubation period, more than one-and-a-half years before clinical signs normally appear in both these genotypes.

THE recent recognition that there is a link between bovine spongiform encephalopathy (BSE) in cattle and a new variant of Creutzfeldt-Jakob disease (v-CJD) in human beings (Will and others 1996, Bruce and others 1997, Hill and others 1997a) highlights the urgent need to find a method for the preclinical diagnosis of transmissible spongiform encephalopathies (TSEs), or prion diseases.

The preliminary results of an experiment which could lead to an early preclinical diagnostic method for scrapie and possibly for other TSEs, using tonsillar biopsies and scrapie in sheep as a model, have recently been reported by Schreuder and others (1996). This study observed that in sheep with a genetically determined high susceptibility and born in a flock where natural scrapie occurred, the disease-associated form of the prion protein, PrP^{Sc}, could be detected in the tonsils approximately one year before the expected onset of clinical disease. This paper describes the procedure for taking tonsillar biopsies from sheep and gives the results of the completed experiment.

The search for a practical preclinical diagnostic test for TSEs continues to be a main topic of research. Because TSE-infected animals and humans have neither a disease-specific immune response

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nor consistent biochemical, haematological, or gross pathological abnormalities associated with the disease, early diagnosis depends on the recognition of clinical signs, electroencephalography or magnetic resonance imaging techniques, or the more invasive method of taking brain biopsies.

Confirmatory diagnosis of TSEs still depends on the histological examination of the brain of suspected cases. The neuropathological lesions, mainly consisting of vacuolation (spongiform change) of the grey matter associated with gliosis and neuronal loss, are generally sufficiently characteristic. Further confirmation can be obtained by demonstrating the presence of scrapie associated fibrils, PrP^{Sc}, or infectivity. Infectivity is detected in a mouse bioassay, but this, in spite of being the most sensitive detection method available, is too cumbersome and time-consuming ever to become a practical diagnostic method. Numerous studies have confirmed PrP^{Sc} as a sensitive and specific marker for TSEs (Prusiner 1992). PrP^{Sc} is detected by immunochemical techniques such as Western blotting or immunohistochemistry. The latter technique has become an accepted diagnostic tool for diagnosing clinical CJD, scrapie and BSE, and is less cumbersome than Western blotting.

In attempts to develop an early detection method, similar techniques have been applied to blood, urine, tissue fibroblasts and, particularly in the animal field, to lymphoid tissue. Most of these attempts have given either negative or inconclusive results (Schreuder 1994). Reports have started to appear of the presence of disease-specific proteins in cerebrospinal fluid (CSF) (Hsich and others 1996). They were based on work done in the 1980s by Harrington and others (1986), in which brain proteins of the 14-3-3 group were related to the presence of CJD. The initial optimism about the use of CSF in clinical cases of BSE (Jones and others 1996, Lee and Harrington 1997) has declined and, because the markers are probably connected with pathological changes in the central nervous system itself, it is unlikely that this approach will be successful in preclinical stages, though it may have potential in the early clinical stages (Jones and others 1996).

Hadlow and colleagues (1980, 1982) used a mouse bioassay to show that infectivity was detectable in the lymphoid tissue of naturally infected scrapie sheep as early as 10 to 14 months of age, well before it occurred in the central nervous system (CNS). From this work, the present authors concluded that lymphoid tissues offered the best possibilities for the development of a preclinical test based on the detection of PrP^{Sc}. This was further corroborated by the immunohistochemical examination of a range of lymphoid tissues from a group of 55 naturally infected, clinically positive scrapie sheep (confirmed histologically at postmortem) in which PrP^{Sc} was detected in the spleen, retropharyngeal lymph nodes, mesenteric lymph nodes and palatine tonsils, in all but one of the animals (van Keulen and others 1996). The tonsils were found to have the highest 'infection rate' per follicle; in 93 per cent of the sheep with scrapie, more than 80 per cent of the follicles stained positive for PrP^{Sc}. For this reason, and because they are relatively easily accessible, the tonsils appeared to offer the best prospect

for a preclinical diagnostic test. Sequential biopsies were therefore taken from the tonsils of two groups of sheep of known genotypes, born and raised at two different locations but both in a scrapie-infected environment, and they were examined immunohistochemically.

Materials and methods

Animals

Experiment A. – Eleven purpose-bred lambs predominantly of the Swifter breed, a synthetic breed built up from crosses from Flemish and Texel breeds, were used. Seven lambs were homozygous for the PrP^{VRQ} allele (with the residue valine [V] at position 136 of the ovine PrP amino acid sequence, arginine [R] at position 154, and glutamine [Q] at position 171). The remaining four lambs were heterozygous, possessing one PrP^{VRQ} allele and one PrP^{ARR} allele (with alanine [A] at position 136 and arginine [R] at positions 154 and 171). The PrP^{VRQ} allele has been found to be significantly associated with an increased susceptibility to scrapie in several breeds of sheep including that used in this experiment, whereas the PrP^{ARR} allele is significantly associated with increased resistance to scrapie (Belt and others 1995). The 11 lambs were born and raised on a farm where scrapie had been occurring for several years. In this flock it was observed that sheep with the genotype PrP$^{VRQ/VRQ}$ died from scrapie when they were about 25 months old (range 23 to 29 months) (Bossers and others 1996), but that most of the sheep with the genotype PrP$^{VRQ/ARR}$ were still healthy at 70 months. Maintained in this environment, the PrP$^{VRQ/VRQ}$ sheep would be expected to develop signs of disease at about 25 months old, whereas the PrP$^{VRQ/ARR}$ sheep should remain healthy and serve as negative controls. These two groups of sheep were therefore regarded as a suitable model for studying changes at known stages of the incubation period.

The lambs were transferred to the ID-DLO at six months old. Tonsillar biopsies were taken sequentially from 10 of the 11 animals, starting at about nine months of age, with intervals of about three months between biopsies in the susceptible sheep. One animal served as an additional control to assess the incubation period; it was not biopsied and was allowed to incubate the disease naturally.

Experiment B. – The lambs used in experiment B were Swifter × Texel crossbreds and were, unlike those in experiment A, born and raised on ID-DLO premises. On these premises, scrapie suspected animals from all over the Netherlands have been gathered, suspected and positive ewes have lambed, and scrapie has been occurring for several years. Eleven purpose-bred lambs were selected, seven of them homozygous for the PrP^{VRQ} allele. The remaining four lambs were heterozygous; three of them were of the genotype PrP$^{VRQ/ARQ}$, and the other had one PrP^{VRQ} allele and one PrP^{ARH} allele (with histine [H] at position 171 instead of glutamine).

In the breeds investigated in the Netherlands, the PrP^{ARQ} allele is associated with an intermediate susceptibility to scrapie, whereas the PrP^{ARH} allele is not significantly associated with increased resistance or susceptibility (Belt and others 1995). Under the conditions of experiment A, animals with the VRQ/ARQ genotype were expected to become clinically positive at about three years of age (Bossers and others 1996).

Tonsillar biopsies were taken at irregular intervals, starting at an age of about four months in the homozygous animals and at eight months in the heterozygous animals.

Tonsillar biopsy

The sheep were anaesthetised by the intravenous administration of a combination of 4 to 8 mg/kg ketamine-HCl (Ketalar; Alfasan NL), 0·05 mg/kg xylazine (Rompun; Bayer) and 0·1 mg/kg atropine. Alternatively, medetomidine-HCl (Domitor; SmithKline Beecham) at a rate of 0·1 to 0·14 ml/10 kg was used, which allows the use of the antidote atipamezole (Antisedan; SmithKline Beecham) at 0·1 ml/10 kg. As experience in performing the biopsy increased, anaesthesia became unnecessary in the younger, more easily handled animals, reducing the time involved in the collection of a sample to less than five minutes. Using full anaesthesia took about 15 minutes and required the usual precautions during recovery. The biopsy was collected by using a mouth gag, a laryngoscope, and biopsy forceps. A headlamp helped to make it easier to see the tonsils. The tongue was depressed with the laryngoscope and a pinch biopsy was taken from the edge of the entrance to the palatine tonsil.

Rectal biopsy forceps, as used in human medicine, with a head approximately 4 mm in diameter were used. The biopsy material was fixed in 10 per cent neutral buffered formalin (4 per cent formaldehyde). The forceps were decontaminated between uses by exposure to 3 M sodium hydroxide for at least one hour, followed by two cycles of porous-load autoclaving for 60 minutes at 134°C. A more detailed account of the technique of taking a tonsillar biopsy has been given by Schreuder and others (1997).

In sheep, the tonsils are encrypted, surrounding a small cavity, the tonsillar sinus. A biopsy from the edge of the entrance (usually more than one entrance is visible) to the sinus usually yields sufficient lymphoid follicular material to be examined. With more than 80 per cent of the follicles staining positive in a large majority of the confirmed clinical scrapie cases, a minimum number of three follicles was estimated to be sufficient for a proper assessment. Samples immunostained for the presence of PrP^{Sc} could thus have three possible interpretations:

1) positive: immunostaining indicating the presence of PrP^{Sc}, in any number of follicles;
2) negative: no detectable immunostaining in at least three follicles;
3) inconclusive: less than three follicles in the sample with no detectable immunostaining.

PrP^{Sc} immunohistochemistry

Details of the immunoperoxidase staining method for PrP^{Sc}, using antibodies directed against selected, synthetic, ovine PrP-based peptides, have been described for staining brain sections (van Keulen and others 1995) and lymphoid tissue sections (van Keulen and others 1996). Slight changes have been made to the latter procedure. The tonsillar biopsy material was fixed in 10 per cent buffered formalin for four to six hours, 5 µm thick sections, mounted on silane-coated glass slides, were dried for at least 48 hours at 37°C and deparaffinised. After pretreatment with formic acid for 30 minutes and hydrated autoclaving, the sections were immunostained with the antipeptide antiserum R521, which is raised in rabbits and based on the ovine PrP sequence 94 to 105. As substrate for the peroxidase reaction, diaminobenzidine (Sigma) was used and 0·1 M imidazole was added to darken and increase the intensity of the staining. Sections were mounted in Eukitt (Kindler).

Results

Experiment A

Homozygous susceptible sheep. – As previously reported by Schreuder and others (1996), clear, extensive PrP^{Sc} immunostaining was found in tonsillar biopsies from all six sampled susceptible PrP$^{VRQ/VRQ}$ sheep, collected for the first time at approximately 10 months of age (range nine-and-a-half to 10 months), when none of the sheep showed clinical signs of scrapie (Fig 1a). Four of the six susceptible animals were removed from the group at 10, 13, 17 and 20 months of age for the bioassay of tonsils (results not yet available) but the remaining two were allowed to incubate the disease fully (numbers 1994 and 2005). They were both killed at 26 months of age, after having shown clear signs of scrapie for one and one-and-a-half months (Fig 2a). The one susceptible
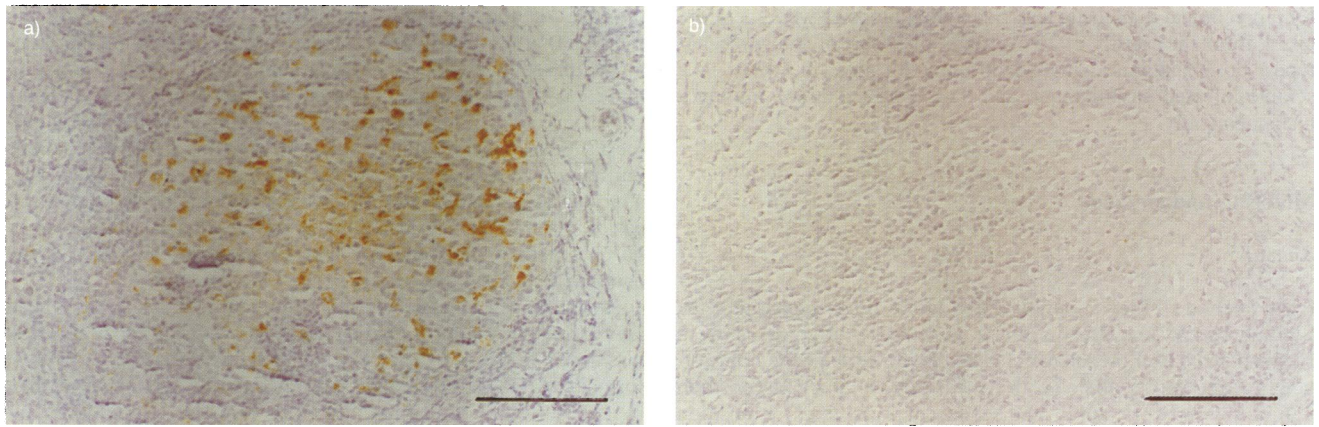
FIG 1: a) Positive PrP^{Sc} immunostaining in lymphoid follicles in a tonsillar biopsy from a scrapie-susceptible sheep (PrP$^{VRQ/VRQ}$) aged 10 months. Peroxidase-labelled streptavidin-biotin staining. b) Negatively staining tonsillar biopsy from a PrP$^{VRQ/ARR}$ sheep of the same age. Bar = 100 μm

PrP$^{VRQ/VRQ}$ sheep which was not biopsied (2008) was also killed at 26 months, after having shown signs of scrapie for about two months.

The clinical signs in the last three animals included progressive emaciation and pruritus, and in the final stages of the disease, fine tremors were noted. Scrapie was confirmed by standard histological procedures of brain sections and by immunohistochemistry.
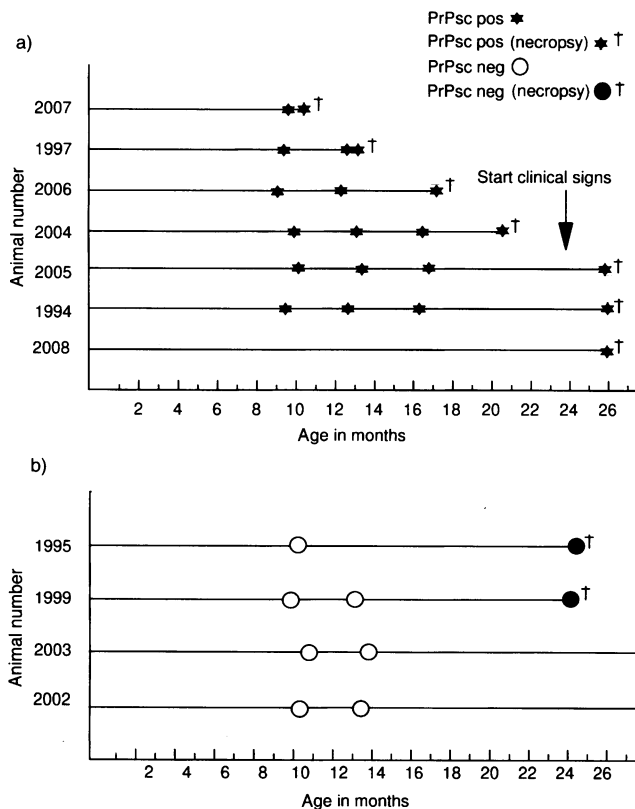
For all the susceptible animals, immunostaining in the tonsillar biopsies remained positive throughout their respective observation periods and PrP^{Sc} was detected in the tonsils in all cases (Fig 2a).

Heterozygous resistant sheep. – No PrP^{Sc} immunostaining was detected in biopsies from any of the four resistant PrP$^{VRQ/ARR}$ sheep (Fig 1b). Two of them were killed at 24 months of age and examined for the presence of PrP^{Sc} in a number of lymphoid tis-

sues, and no immunostaining was detected. The remaining two sheep have remained negative in the tonsils (Fig 2b) (presently 34 months).

Experiment B

Homozygous susceptible sheep. – Three of four samples collected at three-and-a-quarter to three-and-a-half months contained sufficient lymphoid follicles to allow interpretation (animals 2838, 2847 and 2848); no PrP^{Sc} immunostaining was detected in any of them. Two animals were sampled later in the first round, one at an age of exactly four months (animal 2863) and one at five months (animal 2839) (after two inconclusive samples): both showed positive PrP^{Sc} immunostaining. These two were removed from the group for confirmation of infectivity by bioassay. The remaining five susceptible PrP$^{VRQ/VRQ}$ lambs were successfully sampled in a second round at seven-and-a-half to eight months, all with positive results (Fig 3a).

FIG 2: Age of sampling and results of tonsillar biopsies in experiment A: a) in VRQ/VRQ sheep, b) in VRQ/ARR sheep
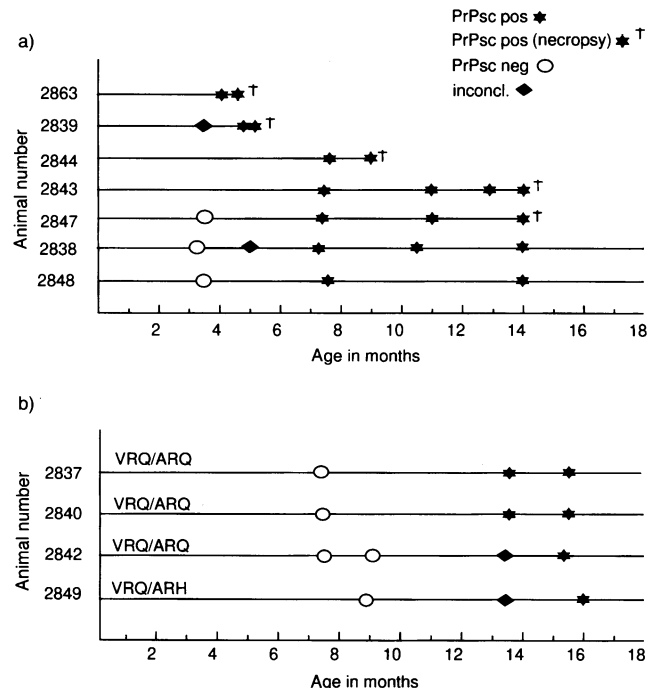
FIG 3: Age of sampling and results of tonsillar biopsies in experiment B: a) in VRQ/VRQ sheep, b) in VRQ heterozygous sheep

Tonsillar biopsies from these animals remained positive from then on, during their respective observation periods. In all cases PrP^{Sc} was detected in the tonsils postmortem. Clinical signs indicative of scrapie have so far not been observed in the two sheep allowed to incubate the disease fully (animals 2838 and 2848) after 18 months of observation.

Heterozygous susceptible sheep. – The four heterozygous lambs were sampled at longer intervals. When sampled for the first time at about eight months of age, none of the three PrP$^{VRQ/ARQ}$ lambs showed any PrP^{Sc} immunostaining in the tonsillar biopsies. However, two were positive at 14 months (animals 2837 and 2840), and the third was positive at 15 months and three weeks (animal 2842) (Fig 3b).

The lamb with genotype PrP$^{VRQ/ARH}$ (animal 2849) did not show any PrP^{Sc} immunostaining at nine months, but after two inconclusive samplings it was found positive at 16 months and one week old. So far no clinical signs of scrapie have been detected in this group after 18 months.

Discussion

The results of experiments A and B demonstrate that in this model a reliable diagnosis of scrapie could be made in live animals incubating the disease, at less than half the incubation period. The animals in the two experiments were born and raised at two different locations, at which scrapie had been present for several years. The expected incubation period for PrP$^{VRQ/VRQ}$ homozygous animals was confirmed under the conditions of experiment A. Two regularly sampled PrP$^{VRQ/VRQ}$ animals and one additional PrP$^{VRQ/VRQ}$ sheep that had not been biopsied in experiment A were allowed to incubate the disease fully and were killed at 26 months of age, after having shown clear signs of scrapie for one to two months. The procedure of collecting biopsies did not appear to interfere with the length of the incubation period. The survival time of these three animals was consistent with previous findings on the incubation period for this susceptible genotype in an infected environment (range 23 to 29 months) (Bossers and others 1996). Two PrP$^{VRQ/VRQ}$ animals from experiment B were also allowed to incubate the disease fully, they are 18 months old and still healthy.

In experiment A, the scrapie associated PrP^{Sc} was detected in the tonsils of sheep at about 10 months of age, which is less than half the expected and now confirmed incubation period of about two years, and more than one year before the onset of clinical disease. This PrP^{Sc} protein was not detected in sheep expected to develop scrapie at a much later stage or in animals expected to remain healthy throughout their lives. It should be stressed that a PrP^{Sc}-negative tonsillar biopsy does not imply that an animal is free of infection (see also below).

Because there was already extensive PrP^{Sc} staining in the susceptible PrP$^{VRQ/VRQ}$ animals in the first samples taken at about 10 months of age, accumulation of PrP^{Sc} had probably started earlier in this genotype. This was demonstrated in experiment B, in which the data obtained in experiment A were reproduced in sheep born and raised at a different location. In addition, it was shown that in the tonsils of fully susceptible animals there was no detectable accumulation of PrP^{Sc} for at least the first three months. All the tested PrP$^{VRQ/VRQ}$ animals accumulated PrP^{Sc} in their tonsils at seven to eight months of age, at about 30 per cent of the expected incubation period. The data from animals 2863 and 2839 suggest that this accumulation started even earlier, between four and five months, which is at about 20 per cent of the expected incubation period. Too few sheep were biopsied between four and seven months, however, to draw firm conclusions about when PrP^{Sc} begins to accumulate.

In the heterozygous susceptible PrP$^{VRQ/ARQ}$ animals, PrP^{Sc} was detected in the tonsils at 14 to 16 months of age whereas no PrP^{Sc} accumulation was detectable at eight months of age. These animals are expected to become clinically positive at about three years old, and the present results indicate their tonsils were already positive at less than 50 per cent of the expected incubation period. More intensive sampling between nine and 14 months might have yielded a more accurate 'conversion period' for this genotype.

There was a difference in the time of appearance of PrP^{Sc} in the biopsies from the VRQ-homozygous and VRQ-heterozygous animals; at eight months of age positive results were found in all the PrP$^{VRQ/VRQ}$ animals but in none of the PrP$^{VRQ/ARQ}$ or PrP$^{VRQ/ARH}$ animals, which had all been born and raised under the same conditions. From experimental work with scrapie strains injected intraperitoneally into different strains of mice, it was concluded that the incubation periods depend mainly on the dynamics of the replication of the agent within the nervous system and not the lymphoreticular system (Kimberlin and Walker 1988), because the nervous system had in all cases been invaded within a few days or weeks. Kimberlin (1990) suggested that the innate resistance of certain genotypes could also be due to a barrier preventing or delaying the invasion of the CNS. From the above difference in temporal development in the lymphoid tissue, it can be concluded that – under the circumstances of a natural infection – at least part of the relative resistance or susceptibility lies in events at the level of the lymphoreticular system or earlier in the pathogenesis. Such events may have a molecular basis in the gene-dose effect, that is the fact that VRQ-homozygous animals have double the expression level of the PrP^{VRQ} allele, compared with the heterozygous animals.

Application to scrapie control

Because immunohistochemistry is a standard procedure in most research and routine diagnostic laboratories, these results could have several applications in the field of diagnosing TSEs, particularly with respect to future control programmes for scrapie. Control programmes could consist of a combination of breeding programmes that make use of the linkage between PrP genotype and increased resistance for scrapie, and the above method that detects the pathognomonic presence of PrP^{Sc} in the tonsils of infected animals in the preclinical stage. Screening tonsillar tissues will yield far more positive cases than any technique using brain material or markers formed by changes in the CNS, because the changes start much earlier in the tonsil and, furthermore, remain positive throughout the remainder of the incubation period – even when the infectivity level may have dropped (Hadlow and others 1974).

Further research should define at what stage the tonsils become positive in the various other PrP genotypes and breeds, under different conditions of infective load and different strains of the agent. Once this information has been established, tonsillar biopsies combined with genotyping could be used to determine the infection status of a flock by sampling a representative number of selected sheep with susceptible genotypes. In the authors' opinion this would be a more accurate method for establishing a flock's freedom from infection than other methods, for example farmers' statements on the absence of clinical signs or monitoring the scrapie status by a histopathological examination of one brain per hundred animals per year.

A possible drawback was the earlier finding that the tonsils from one of 55 clinically positive animals did not stain positively for PrP^{Sc} (van Keulen and others 1996); the animal had a semi-resistant genotype (PrP$^{VRQ/ARR}$) and no positive immunostaining was found in the tonsils in spite of it having had clinical and histopathologically confirmed scrapie. A similar result was obtained by Western blotting techniques of spleen samples from various experimentally infected sheep (Somerville and others 1997); the same genotype escaped detection in the spleen when the animals had been inoculated by the intracerebral route, but not when they had been inoculated subcutaneously. In the same study, other genotypes inoculated with the notorious isolate CH1641 and animals challenged orally with BSE-infected material, proved mostly negative in the spleens. Conversely, in another study infectivity and PrP^{Sc} were detected in the spleens of sheep infected orally with BSE (Foster and others 1996, Somerville and others 1997), but unfortunately the tonsils were not available for immunohistochemistry.

Proper validation is therefore required with regard to these varying conditions, after which the test may be suitable for the routine screening of slaughter animals, although it would need to be converted into a rapid and sensitive antigen detection assay for use on homogenised tissue.

Application to other TSEs

BSE. – The BSE agent is not distributed as widely in tissues outside the CNS as is the scrapie agent in sheep; experimental transmission to mice succeeded only when brain and other CNS material was used (Fraser and others 1988, 1991) and mice inoculated with other materials, including lymphoid tissue, remained healthy (Fraser and Foster 1994). The distal ileum has been added to the list of BSE-positive materials, but this was after a massive oral challenge (Wells and others 1994). These largely negative results may have favourable implications for the assessment of the risks to consumers, but the prospects for a preclinical test for BSE using less invasive techniques than, for example brain biopsies, are less favourable. However, the bioassay of infectivity may also be less sensitive for BSE than for scrapie.

What has been said about infectivity also applies to the presence of PrP^{Sc}; in clinical cases of BSE in cattle, PrP^{Sc} has so far been detected only in brain material and other parts of the CNS, and not in the spleen, lymph nodes or white blood cells (Farquhar and others 1994). The present technique was therefore unlikely to work in the case of BSE. However, because the immunohistochemical technique had not been tried in depth in the preclinical stages of BSE (Mohri and others 1992), and because BSE might behave like experimental scrapie infection in goats, with the infectivity disappearing from the lymphatic tissues (Hadlow and others 1974), and because the mouse bioassay might not be sensitive enough for various non-neural tissues of BSE-infected cattle, it was considered worth investigating. The tonsillar tissues from the two clinically positive and histologically confirmed cases of BSE in the Netherlands were examined, but no PrP^{Sc} immunostaining was detected in the samples (L. J. M. van Keulen, unpublished observations).

Creutzfeldt-Jakob disease (CJD). – Schreuder and others (1996) indicated that their test was likely to be applicable to human TSEs such as CJD or v-CJD, because infectivity had been detected in various lymphoid tissues of patients with classical CJD (Brown and others 1994) and tonsillar biopsy is a relatively non-invasive, simple technique. Its applicability has been confirmed in a clinical case of v-CJD, albeit in material obtained postmortem (Hill and others 1997b).

A diagnosis made less than halfway through the incubation period undoubtedly has advantages, but the possibility of early detection also has adverse implications; if the tonsils of CJD patients should, as in the case of scrapie, prove to harbour the disease-specific form of PrP long before the clinical signs have developed, there would be a danger of iatrogenic transmission not only in neurosurgery, but also in 'simple' surgical procedures like tonsillectomies.

For the immediate future the major contribution of this test will undoubtedly be in the field of scrapie diagnosis in live susceptible sheep, for which it is – apart from the bioassay in mice – the only true preclinical test, with the disease being detected at least one-and-a-half years before clinical signs develop. Although these results indicate the presence of infectivity (or at least PrP^{Sc}) in the lymphoid tissues of scrapie-infected sheep less than 12 months old, the age that was stipulated for public health measures (Commission Decision 1997), it is not suggested that this age should be reduced. It should be taken into account that the study was intentionally carried out with the genotype that had the shortest incubation period, a genotype that is expected to be rare in field material.

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