Papers and Articles

Preliminary observations on the pathogenesis of experimental bovine spongiform encephalopathy (BSE): an update

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Further preliminary observations are reported of an experiment to examine the spread of infectivity and the occurrence of pathological changes in cattle exposed orally to infection with bovine spongiform encephalopathy. Calves were dosed at four months of age and clinically monitored groups were killed sequentially from two to 40 months after inoculation. Tissues were collected for bioassay, for histopathological examinations and for the detection of PrP. Previous reported observations have included the presence of infectivity in the distal ileum of cattle killed after six to 18 months, the earliest onset of clinical signs in an exposed animal after 35 months, and diagnostic histopathological changes in the brain, in association with clinical disease, after 36, 38 and 40 months. In spite of the relative inefficiency of the bioassay of scrapie-like agents across a species barrier the new observations confirm that the onset of clinical signs and pathological changes in the central nervous system (CNS) occur at approximately the same time. The earliest pathological change, the presence of abnor-

PrP 32 months after inoculation, coincided with the earli- detected infectivity in the CNS and occurred shortly before there was evidence of typical spongiform changes in the brain 36 months after inoculation. Infectivity has now been demonstrated in the peripheral nervous system, in the cervical and thoracic dorsal root ganglia 32 to 40 months after inoculation and in the trigeminal ganglion 36 and 38 months after inoculation. At the time of writing evidence of infectivity in other tissues is confined to the distal ileum, not only after six to 18 months but also after 38 and 40 months, but these findings may be supplemented by the results of further mouse assays. Nevertheless, they are in general agreement with current knowledge of the pathogenesis of scrapie.

PRELIMINARY findings from a long-term and unfinished experiment to determine the spatial and temporal development of infectivity and pathological changes in cattle exposed to a single large oral dose of bovine spongiform encephalopathy (BSE)-affected brain homogenate have been described previously (Wells and others 1994a, 1996). The first major finding was the presence of infectivity in the distal ileum (the inoculum comprising the wall of the intestine including Peyer's patches) of cattle killed six and 10 months after inoculation but not in those killed after two months (Wells and others 1994a). Subsequent bioassay results provided evidence of infectivity in the distal ileum of cattle killed 14 and 18 months after inoculation, and of a reduction in incubation period in the mice inoculated after each successive kill, at least up to 14 months after inoculation, suggesting that the agent replicated at this site (Wells and others 1996). At the time of reporting (January 1995), only the assays of tissues from cattle killed two and six months after inoculation were complete, and at two months there was no evidence of infectivity in any of the tissues, and at six months there was no evidence of infectivity in any tissue other than the distal ileum. All the other bioassays were incomplete.

Unequivocal clinical signs of BSE were first observed in one of the challenged cattle 35 months after inoculation, and by 37 months unequivocal, or early clinical signs were present in the remaining cattle (Wells and others 1996). Some results of the histopathological examination of the brains of the challenged cattle for BSE status have been given in the report of a study to examine correlates of protein markers in the cerebrospinal fluid of BSE-affected cattle (Jones and others 1996).

This paper provides further details of the occurrence of pathological changes in the cattle and reports additional preliminary results of the mouse bioassays on cattle tissues.

Materials and methods

The design of the study has been described by Wells and others (1996). Briefly, 40 Friesian/Holstein calves, born in 1991, were assembled from farms with no history of BSE. At four months of age, 30 were each dosed orally with 100 g of pooled brain stems from 75 cases of BSE. Ten calves received no treatment and served as controls.

The cattle were monitored clinically during daily husbandry routines, by weekly veterinary inspections, by the recording of responses during blood sampling and other procedures, by behavioural observations over 24 hour periods, by recording their performance in an improvised open field test (Austin and others 1997) and, finally, by detailed clinical neurological examinations made within seven days of their being killed. The onset of clinical disease was defined as the time when convincing signs consistent with BSE first became regularly observable. The variability of the initial clinical signs between individual cattle prevented any single clinical sign being used as the indicator of the onset of the disease.

Starting at six months of age, two months after inoculation, and then at four month intervals until 22 months after inoculation,

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three of the challenged calves and one control calf were killed. Thereafter challenged and control cattle were killed at discretionary intervals until 40 months after inoculation (Table 1).

Samples of brain and spinal cord (Table 2) were taken for examination for BSE fibrils, equivalent to scrapie-associated fibrils (SAF). All tissues taken for assays of BSE infectivity in mice were sampled aseptically. A wide range of tissues was collected for histopathological examinations, although only the results of the examinations of brain and spinal cord are presented here. Selected blocks (Table 2) were examined for immunohistochemical evidence of the presence of disease-specific PrP.

After completion of each sequential kill inocula were prepared from 44 tissues, representing principally the lymphoreticular system (LRS), the peripheral nervous system (PNS) and the central nervous system (CNS), the alimentary tract, striated muscles and major viscera (see Table 3.1, Wells and others 1996). They were prepared as 10 per cent suspensions in saline, with antibiotics being included for certain tissues. Single tissue inoculum pools were made from the groups of exposed cattle killed at each time point, and inocula were prepared similarly from single tissues of each control animal. Test and control inocula were injected intracerebrally (20 µl) and intraperitoneally (100 µl) into inbred mice for a standard qualitative assay of infectivity. The inocula prepared from cattle killed up to 18 months after inoculation were injected into RIII mice, but C57BI-J6 mice were used thereafter. The strain of mouse was changed because an intercurrent disease problem in the breeding colony reduced the longevity of the RIII mice to such an extent that many were unlikely to have lived to the endpoint of the bioassay, as determined from dose-response data for the BSE agent in these mice (Dr H. Fraser, personal communication). C57BI mice were chosen as the alternative strain because the titre of BSE infectivity in affected bovine brain was the same when measured in RIII and C57BI mice (Fraser and others 1992).

From each of the cattle killed brain and spinal cord were collected into 10 per cent formol saline for histopathological examination. After two weeks primary fixation the brains were cut into 12 coronal blocks, representing seven levels of the brain as in previous studies (McGill and Wells 1993, Simmons 1994), and fixed for a further week in fresh fixative. The blocks were then processed over a three day schedule, embedded in paraffin wax, and 5 µm sections were cut and stained with haematoxylin and eosin.

After three weeks in formol saline, the spinal cords were cut to provide both transverse and frontally oriented blocks at C6, T6 and L5-6. The blocks were then processed, and stained sections were prepared as for the brains.

The PrP immunohistochemistry (IHC) techniques used were modifications of those described by Haritani and others (1994) and Bell and others (1997). Formalin-fixed, paraffin-embedded, 4 µm sections of medulla at the obex, and C6, T6 and L5-6 spinal cord, were immunostained with rabbit polyclonal antiserum 971, and normal rabbit control serum. Serum 971 was prepared at the Central Veterinary Laboratory to a synthetic peptide equivalent to bovine PrP residues 230-244. The peptide was conjugated to two carriers: multiple antigen presenting and mycobacterial purified protein derivative. The whole serum and IgG fractions recognise PrP in Western blots of fibril preparations and in formalin-fixed, paraffin-embedded, immunostained sections prepared from CNS tissues of BSE-affected cattle (P. M. Edwards, Y. I. Spencer, unpublished observations).

Epitopes were demasked by immersing the sections in 96 per cent formic acid for 20 minutes, followed by a wash in tap water and hydrated autoclaving at 122°C for 30 minutes. Immunolocalisation was amplified by a peroxidase-anti-peroxidase technique, and was visualised with citrate-buffered diaminobenzidine. Sections were counterstained with Mayer's haematoxylin.

All the sections were examined by light microscopy. The presence of abnormal PrP was confirmed when the configurations and distribution of immunostaining were consistent with those previously described in BSE (Wells and others 1994b, Wells and Wilesmith 1995). Where there was insufficient immunostaining to indicate a defined morphological pattern, the result was declared inconclusive.

The biochemical extraction of BSE fibrils has been described by Scott and others (1992) and Stack and others (1993). Essentially, 1 g aliquots of the frontal cerebral cortex and spinal cord at cervical (C1-C2), thoracic (T9-T10) and lumbar (L2-L3) regions from each animal were homogenised in 10 per cent N-lauroylsarcosine detergent and, after differential centrifugation, digested with proteinase K enzyme at a concentration of 10 µg/ml. For electron microscopy, formvar and carbon-coated grids were subjected to a plasma glow discharge to impart a hydrophilic charge before they were used. The tissue extracts were dried on to these sample grids and negatively stained with 2 per cent phosphotungstate. The grids were then examined for the presence of BSE fibrils in a Philips CM10 electron microscope with an accelerating voltage of 80 kV and at magnifications greater than x 25,000. At least 20 grid squares were examined for a minimum of 20 minutes before a sample was considered negative for the presence of BSE fibrils (Scott and others 1990). The morphological criteria for the identification of BSE fibrils were those defined for SAF (Merz and others 1981).

Results

The schedule of sampling of the cattle in this study has been completed (Table 1), but some of the bioassays for tissue infectivity are incomplete. The clinical status and the CNS pathology and infectivity data of the cattle killed 32 to 40 months after inoculation are given in Table 2.

Profound changes in emotionality, constant sensory abnormalities and motor disorders (in the form of adventitious muscle activity or ataxia) were the main clinical signs observed, usually in combinations which varied between individual animals. Episodic, inconsistent sensory signs of low intensity were not regarded as indicating the unequivocal onset of clinical disease and neither were subtle changes in temperament, although all these changes were recorded as part of the insidious prodromal progression of the disease.

As reported by Wells and others (1996), no significant vacuolar changes were observed in the brain and spinal cord of cattle killed from two to 26 months after inoculation. However, vacuolar changes confined to the vestibular nuclear complex of the medulla oblongata were observed in one of the two cattle killed 32 months after inoculation, although neither showed clinical signs, and a mild spongiform encephalopathy, typical of BSE, was present in one of the three cattle killed 36 months after inoculation, and it showed equivocal early clinical signs (Table 2). All the confirmed histopathological diagnoses were based on a pattern of distribution of lesions typical of natural cases of BSE. Table 2 also shows the IHC and BSE fibril results from 32 months after inoculation, the time after which abnormal PrP was detected in the CNS. This change preceded the pathognomonic histopathological changes in the brain, and the onset of clinical signs, and was most consistently detected in the lumbar spinal cord. The yield of fibrils was low in most of the extracts, with the exception of those from the

TABLE 1: Numbers of challenged and control animals killed at intervals after oral inoculation with bovine spongiform encephalopathy

Time after inoculation (months)	Age (months)	Number of challenged animals killed	Number of control animals killed
2	6	3	1
6	10	3	1
10	14	3	1
14	18	3	1
14·5*	18·5	1	0
18	22	3	1
22	26	3	1
26	30	1	0
28	32	0	1
32	36	2	0
35	39	0	1
36	40	3	0
38	42	3	1
40	44	2	1

*Killed as a casualty and not included in tissue inoculum pools

TABLE 2: Summary of the clinical status and the pathological changes and presence of infectivity in CNS of cattle killed 32 to 40 months after inoculation with bovine spongiform encephalopathy

Animal	Months	Clinical status	Spongiform encephalopathy	Immunohistochemical PrP: Brain medulla	Spinal cord C6	Spinal cord T6	Spinal cord L5-6	Fibril detection: Brain cortex	Spinal cord C1-2	Spinal cord T9-10	Spinal cord L2-3	Infectivity*: Brain cortex	Brain medulla	Spinal cord C2-3	Spinal cord T10-11	Spinal cord L3-4
254	32	–	–	–	–	+	+	–	–	–	+	–	+	+	+	+
232		–	Vacuolar change (vestibular nuclei)	+	+	+	+	–	–	+	+					
299	36	+/–	+	+	+	+	+	+	+	+	+	–	+	+	+	+
194		+/–	–	+	+/–	–	+/–	+	–	–	–					
231		–	–	+	+	+	+	–	–	–	–					
261	38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
192		+/–	+	+	+	+	+	–	+	+	+					
300		+/–	–	–	–	–	–	–	–	–	–					
277	40	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+
296		+/–	–	–	–	–	–	–	–	–	–					

*Infectivity assays are incomplete, negative results are therefore only interim findings (see text and Table 2). Infectivity results are derived from a pool of tissue from the animals in each group

+ Confirmed finding, +/– Probable, but equivocal finding, – Negative finding, C Cervical, T Thoracic, L Lumbar

lumbar spinal cord. No PrP-immunostaining or fibrils were found in any of the control samples.

The bioassays in RIII mice of the tissues collected from cattle killed up to 18 months after inoculation are complete. As reported by Wells and others (1996), the only tissue in which infectivity has been detected is the distal ileum of cattle killed six, 10, 14 and 18 months after inoculation. The bioassays in C57Bl-J6 mice of tissues collected from cattle killed up to 22 months after inoculation are also complete (950 days after inoculation). No evidence of infectivity was found in any of the tissues, including the distal ileum. The bioassays in C57Bl-J6 mice of tissues from cattle killed more than 22 months after inoculation are incomplete. Interim data on the occurrence of infectivity in the CNS of cattle killed 32 to 40 months after inoculation are given in Table 2 for comparison with the pathological observations. The current status of the assay results for all the tissues found to contain infectivity from cattle killed 32 to 40 months after inoculation is given in Table 3.

Discussion

With experimental models of scrapie, in rodents inoculated by non-neural routes, including gavage and oral dosing, the infective agent replicates in the LRS before it can be demonstrated in the CNS (Kimberlin and Walker 1988, 1989, Scott 1993, Beekes and others 1996, Baldauf and others 1997). A very similar pattern, with the LRS becoming infected before the CNS, has been recorded in natural scrapie of sheep (Hadlow and others 1982), and in experimental studies the evidence indicates that the agent spreads to the CNS by neural pathways, involving the peripheral nerve innervation of the LRS (Kimberlin 1986, Scott 1993, Beekes and others 1996, Baldauf and others 1997).

The findings in the present study are broadly consistent with our understanding of the pathogenesis of scrapie from these earlier studies. Infectivity has been demonstrated in the CNS and the PNS (trigeminal ganglion and dorsal root ganglia) (Table 3) before the onset of clinical signs but, unlike the experimental models of scrapie, no infectivity has been demonstrated in lymphoreticular organs, apart from the presumed involvement of Peyer's patches which are concentrated in the distal ileum. One reason for this result may be limitations in sensitivity of the assay.

The calculated limit of detectability of infectivity in mice is approximately $10^{1.4}$ intracerebral LD50/g for 20 µl (intracerebral) and 100 µl (intraperitoneal) doses of a 10 per cent tissue homogenate (Kimberlin 1996). In practice, the sensitivity of detection of infectivity by bioassay is influenced by the extent of the species barrier, and further experiments are in progress to measure the effect of the cattle/RIII mouse species barrier in relation to BSE by a comparative titration of a BSE inoculum by the intracerebral route in mice and cattle. The titration in cattle is incomplete but the results suggest that the assay of BSE infectivity in cattle is at least 1000 times more sensitive than the assay in RIII mice (G. A. H. Wells, S. A. C. Hawkins, unpublished observations). Additional infectivity assays of selected tissues from the present study, by intracerebral inoculation into cattle, are thus in progress.

In the present study it is therefore not surprising the infectivity has not been detected in the CNS of cattle before the recognition of pathological changes (Table 2), but infectivity may yet be detected at an earlier stage. The detection of infectivity in the distal ileum of cattle during the clinical phase of the disease (38 and 40 months after inoculation) contrasts with the lack of detectable infectivity in this tissue in confirmed natural cases of BSE (Fraser and Foster 1994). However, Wells and others (1996) have suggested that the failure to detect infectivity in the distal ileum in natural cases of BSE could be due to the low doses of the agent which are considered to have sustained the major proportion of the BSE epidemic (Kimberlin and Wilesmith 1994) in comparison with the large dose administered to the calves in this study. The minimum incubation period of BSE observed in this study lies in the lower part of the distribution range of values of incubation periods estimated from age-specific incidence in the epidemic of

TABLE 3: Summary of bioassay results for confirmed infectivity in tissues collected from cattle slaughtered 32 to 40 months after inoculation with bovine spongiform encephalopathy

Tissue	Number of positive mice/number of mice surviving when the first mouse was confirmed positive: 32 months	36 months	38 months	40 months
Frontal cortex	–	–	1/18	–
Caudal medulla	7/16	8/19	11/19	7/20
Spinal cord C2-3	4/20	6/14	6/18	3/16
Spinal cord T10-11	5/19	11/18	1/13	7/19
Spinal cord L3-4	8/19	10/19	10/20	4/17
Dorsal root ganglia C3-6	1/10	3/16	–	–
Dorsal root ganglia T5-8	1/9	7/14	1/18	–
Tr-geminal ganglia	–	2/18	2/16	–
Distal ileum	–	–	1/12	2/19

– Infectivity not detected, assay incomplete, C Cervical, T Thoracic, L Lumbar

BSE (Wilesmith and Ryan 1992); over the course of the epidemic only 1·22 per cent of affected cattle have been less than 41 months old at the onset of the disease (J. W. Wilesmith, personal communication). Furthermore, samples of distal ileum from only three natural cases of BSE have been examined. The variations between cases and the relatively low titre of infectivity in this tissue at the stage of clinical disease may well therefore account for these previous results.

The failure to detect infectivity in the distal ileum of cattle killed 22 months after inoculation in C57BI-J6 mice conflicts with previous data from assays of this tissue from cattle killed earlier in the study, when there was a progressive reduction of the incubation period in RIII mice, at least in cattle killed up to 14 months after inoculation. The interpretation of this finding is at present confounded by the change in the strain of mouse from RIII to C57BI for all the assays of tissues from animals killed more than 18 months after inoculation. The C57BI-J6 mice appear, unexpectedly, to be less susceptible to BSE, despite the comparability of data from the titration of brain material of high titre in RIII and C57BI mice (Fraser and others 1992).

It also appears that in BSE overt clinical signs and diagnostically significant pathological changes in the brain may develop after a similar period (Table 2). Although PrP was demonstrable in the spinal cord of both the cattle killed 32 months after inoculation, which did not show clinical signs, it was demonstrated in the medulla oblongata of only one of them and no vacuolar changes were demonstrable in the sites in the medulla examined routinely in the statutory diagnosis of BSE (Bradley and Matthews 1992). By 36 months after inoculation, when early clinical signs were emerging in two of the three cattle killed, only one had spongiform encephalopathy with a typical distribution pattern in the medulla. However, given the small number of animals per group the result cannot be interpreted as reflecting the invariable sequence of changes in natural cases of BSE. It has been previously commented that this result is quite different from observations in rodent models of scrapie, in which the development of vacuolar changes relative to incubation period has been studied after infection with rodent-adapted agents and is usually evident substantially before the onset of clinical disease (Wells and others 1996).

Addendum

Since finalising the time point in the mouse bioassay results for inclusion in this paper, further results have become available indicating infectivity in bone marrow from cattle killed 38 months after inoculation. A short communication providing details of this additional finding is in preparation.

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Abstracts

Effects of otitis on the hearing of dogs

THE hearing of normal dogs and dogs with otitis was assessed by testing the responses of their brainstems to auditory stimuli. Data were obtained from 86 normal ears and 105 ears with otitis ranging in severity from mild to severe. Although the dogs with more severe otitis had more severe hearing loss, only two dogs were completely deaf in the affected ear, and cleaning the ear canal improved the hearing of several of them, indicating the serious effect that a physical obstruction can have. The process of cleaning and inspection did not impair the hearing of any of the dogs. It is concluded that most dogs with otitis are not totally deaf, and that the problem is largely restricted to the middle and external cavities of the ear.

EGER, C. E. & LINDSAY, P. (1997) *Journal of Small Animal Practice* 38, 380

Dermatosis associated with *Acarus farris* in gerbils

THREE gerbils developed alopecia, scaling and thickening of the skin of the tail. Hair samples contained large numbers of an immature, non-feeding stage (hypopi) of the mite *Acarus farris*. Treatment with ivermectin failed to prevent the condition from spreading and worsening, probably because the mites were not feeding, but it responded gradually to treatment with fipronil applied topically with a wet swab, combined with the replacement of the bedding, better ventilation, and the placement of all new bedding and food in a freezer for 24 hours to kill any mites present.

JACKSON, M. R. (1997) *Journal of Small Animal Practice* 38, 410