

Limited detection of sternal bone marrow infectivity in the clinical phase of experimental bovine spongiform encephalopathy (BSE)

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A RECENTLY published report (Wells and others 1998) updated interim findings in a sequential time point study which is examining the spread of infectivity and development of pathological changes in cattle exposed orally to infection with the agent of bovine spongiform encephalopathy (BSE) (Wells and others 1994, 1996).

These previous results described the schedule of examination of cattle, killed from two to 40 months after oral exposure, and the development of clinical signs in cattle 35 to 37 months after the exposure. They also demonstrated infectivity by mouse bioassay in: distal ileum (sampled from cattle six to 18 months, 38 months and 40 months after exposure); central nervous system – brain and spinal cord (sampled from cattle 32 to 40 months after exposure); and sensory ganglia – dorsal root ganglia (sampled from cattle 32 to 40 months after exposure) and trigeminal ganglion (sampled from cattle 36 months and 38 months after exposure). No infectivity had been detected in any of the 35 remaining tissues for which assays were complete at June 1997 (that is, those sampled from cattle two to 22 months after exposure).

Mouse bioassays of a large range of tissues from all sequential kill time points of the study have now been completed (at December 1998) and will be reported in full elsewhere. This short communication reports additional data on the bioassay in C57B1-J6 mice of bone marrow, completing results for this tissue from all cattle in the study (Wells and others 1998).

Details of the experimental design of the study have been described previously (Wells and others 1996, 1998). Bone marrow from the sternum (cancellous bone from the centre of the third or fourth sternebra) was sampled, as for each of the tissues taken, using new sterile disposable instruments or instruments sterilised by porous load autoclaving at a holding temperature of 136°C for 18 minutes at 30 lb/in². The sternum was sawn sagittally and then transversely to isolate a portion of sternebra. With a new disposable scalpel and forceps a portion of cancellous bone containing marrow was removed from the sternebra. A weighed amount of the tissue was ground in a Griffiths tube and diluted with sterile saline to give a 10 per cent suspension. The suspension was filtered through sterile gauze before final aliquoting and freezing at -70°C. An aliquot was thawed just before inoculation. The samples were taken from BSE-exposed and control cattle at each sequential time point of the study. Pooled bone marrow from the BSE-exposed cattle was assayed in parallel with bone marrow from a clinically normal animal from undosed control groups. Mice were inoculated by intracerebral and intraperitoneal routes. Bioassays of tissues from cattle killed earlier than 22 months after exposure were conducted in RIII mice, whereas assays from 22 months and later times after exposure have been con-

ducted in C57B1-J6 mice. Surviving mice were killed at an experimental end point of 650 days (RIII) or 950 days (C57B1-J6) after inoculation (Wells and others 1996). Postmortem confirmation of disease in mice is routinely carried out by histopathological examination of the brain for morphological changes of spongiform encephalopathy.

Following the histopathological assessment of mice inoculated with bone marrow from cattle killed 38 months after inoculation, immunohistochemical (IHC) examination for the presence of disease-specific PrP was made of the brains of all mice in each of the groups inoculated with bone marrow from cattle killed 32 to 40 months after exposure. This was introduced to the standard protocol for assessment of bioassay results as it has the potential to provide improved specificity and sensitivity of detection of disease in experimental transmissions of scrapie and related agents. The principal justification for this in the present context was to confirm observations and resolve equivocal findings in the histopathological assessment. It was applied further to groups of mice inoculated with bone marrow from cattle killed at sequential time points throughout the immediate preclinical and clinical disease periods in an attempt to clarify the significance of the findings. The brains of mice inoculated with bone marrow of clinically normal cattle from appropriate control groups were examined in parallel. The IHC method used was essentially that applied previously to cattle central nervous system tissues (Wells and others 1998), with the following modification. In addition to serum 971, a rabbit polyclonal antiserum to mouse ME7 scrapie-associated fibrils, IA8 (courtesy of the Institute of Animal Health, Neuropathogenesis Unit, Edinburgh), previously characterised by immunohistochemistry (Bruce and others 1989) was used. Serum 971 was applied at dilutions of 1/8000 and 1/15,000 in an avidin-biotin-peroxidase complex (ABC) technique. Serum IA8 was applied at dilutions of 1/500 and 1/1000 in a peroxidase-anti-peroxidase technique. Normal rabbit serum was used as a control in both methods.

Evidence of infectivity in bone marrow by morphological and IHC criteria was confined to cattle killed 38 months after exposure (Table 1). The two mice assessed as clinically affected had incubation periods of 695 days and 842 days and were confirmed positive for spongiform encephalopathy by histopathological examination. In addition to these two mice, four additional mice in the group with survival times of 653, 792, 799 and 923 days had evidence of PrP immunostaining in the brain. In all five mice considered positive by IHC, detection of PrP was obtained with both antisera. Mice examined from the bioassays of the bone marrow completed from cattle killed 32, 36 and 40 months after exposure and from the clinically normal control cattle were negative clinically, histopathologically and immunohistochemically.

There have been few studies in which assays of infectivity in bone marrow of animals with natural infection with the agents of the transmissible spongiform encephalopathies (TSE) have been attempted. Hadlow and others (1982) detected a very low concentration of infectivity by mouse bioassay in bone marrow of one of nine Suffolk sheep clinically affected with natural scrapie from a flock assembled of sheep from infected farm flocks. In all nine sheep infectivity was widespread and in moderate to high titre in lymphatic and nervous system tissues. Bone marrow was not among the tissues assayed (W. J. Hadlow, personal communication) from any of the 34 lambs and sheep studied for evidence of preclinical infection in this flock.

Mouse assays of infectivity in bone marrow from three adult goats with natural scrapie failed to detect the agent (Hadlow and others 1980).

Examinations of bone marrow infectivity in experimentally induced TSE have been equally infrequent. In a study in mice (Eklund and others 1967) the inoculum was comprised

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TABLE 1: Summary of bioassay results* confirming infectivity in bone marrow of cattle 38 months after exposure

Clinical status (incubation period: days)	Histopathological examination (survival period: days)	PrP immunohistochemistry (survival period: days)
2/16 (695, 842)	2/16 (695, 842)	6/16 (653†, 695, 792, 799, 842, 923)

* Number of mice positive/number of mice surviving when the first mouse was confirmed positive by histopathology or PrP immunohistochemical examinations

† One mouse was considered inconclusive (on histopathological examination) had mild or equivocal spongiform change confined to medulla, hypothalamus and lateral thalamus

of whole femur derived from random-bred Swiss mice infected by subcutaneous injection with the Chandler strain of mouse-adapted scrapie agent. Infectivity was detected at a low concentration from early in the clinical disease and throughout it, with increasing but variable concentrations comparable with those found in non-neural lymphoreticular tissues.

In their study of the distribution of scrapie agent in goats experimentally infected with the Chandler strain, Hadlow and others (1974) did not detect infectivity in bone marrow at any time through the incubation or clinical course of the disease.

In all of these assays, the calculated limit of detectability of infectivity by intracerebral inoculation of mice is approximately $10^2 \log_{10}$ mouse intracerebral LD_{50}/g of tissue (Kimberlin 1994). In the single sheep in which infectivity was detected in bone marrow (Hadlow and others 1982) the titre of agent was close to this limit of detectability.

Similarly, mouse assays of infectivity of bone marrow from two naturally affected cattle with BSE have given negative results, as indeed have all assays of non-neural tissues from natural cases of the disease (Fraser and Foster 1994; H. Fraser, personal communication). These assays used a combination of intracerebral and intraperitoneal injections with a calculated limit of detectability of $10^{1.4} LD_{50}/g$ (Kimberlin 1996).

In studies to examine the tissue distribution of infectivity in human spongiform encephalopathies by inoculation of primates (mostly squirrel monkeys) with 1 to 20 per cent tissue suspensions, no infectivity was found in the bone marrow of the only two patients (disease unspecified) from which the tissue was examined (Brown and others 1994).

The involvement of bone marrow in the course of scrapie would seem to be a phenomenon occurring rarely and then only at the end of the incubation period. In the present study also, only one of the three exposed cattle need have contributed bone marrow with detectable infectivity to the tissue pool assayed. Although this possible dilution effect would be inconsequential, the results do not allow an estimation of the concentration of infectivity in bone marrow since the C57Bl/16 mice used throughout this series of bioassays have given a wide incubation period range for any given inoculum (Wells and others 1998 and unpublished data) and a dose response curve for BSE infectivity in bone marrow has not been obtained.

The authors have previously compared results of studies of BSE and transmissible mink encephalopathy (TME) (Wells and others 1996) with respect to possible similarities in pathogenesis which contrast with that of scrapie. In TME, involvement of extraneural tissues is confined to low concentrations of agent in the lymphoreticular tissues before detection of infectivity in central nervous system (CNS) and in some other extraneural tissues only after replication in the CNS can be detected. The detection of infectivity in bone marrow in experimental BSE in cattle is broadly consistent with this pattern since infectivity was initially detected in the CNS of cattle killed 32 months after exposure (Wells and others 1998).

It is difficult to draw conclusions on the origin of the infectivity in bone marrow in experimental BSE, but three possi-

bilities must be considered. The first is that it is the result of the spread of infectivity from the CNS to the peripheral nervous system (PNS) in the clinical phase of disease. Studies of experimental scrapie in mice have demonstrated the spread of the infection from CNS to peripheral nerves (Kimberlin and others 1983). Since bone marrow does have an autonomic innervation, largely supplying smooth muscle of blood vessels (Wickramasinghe 1992), BSE infection could theoretically spread directly from the CNS via the PNS. While infectivity in this study has been demonstrated in dorsal root ganglia, concurrent with infectivity in the CNS, the present failure to detect evidence of infection in those autonomic ganglia or peripheral nerve trunks assayed (Wells and others 1996, 1998) may be a consequence of the concentration being below the limit of detectability. Also, for this hypothesis to be tenable, the tissue innervated must contain cells which support the replication of the agent. While, as discussed later, this might be feasible for bone marrow, it would seem that replication within blood vessels per se might be expected to lead to a more widespread distribution of infectivity in the clinical phase. Failure to detect infectivity in bone marrow from cattle killed at 40 months after exposure might also argue against the phenomenon being related to a progression of tissue involvement by spread along peripheral nerves in the clinical phase of disease.

The second possibility is that infection reaches the bone marrow (albeit undetected) via the circulation. Haematogenous dissemination of infectivity to lymphoreticular sites is an accepted feature of pathogenesis early in the incubation period of experimental rodent scrapie, when infection has been by non-neural routes (for reviews see Kimberlin and Walker 1988, Scott 1993). BSE infectivity may be transported to bone marrow, via blood, early in the incubation period or only at the clinical stage of disease. However, in both situations, the evidence from studies of scrapie and TME predicts that if BSE infectivity can be detected in bone marrow, it should also be detected in spleen and lymph nodes in equal, if not greater, concentrations. This is supported not only by data on infectivity at these latter sites in previously cited studies which have included assays of bone marrow (Eklund and others 1967, Hadlow and others 1974, 1980, 1982) but by the fact that bone marrow is, in common with spleen and lymph nodes, a location of the long-lived cells considered to be crucial to replication of scrapie agents (Fraser and others 1986). However, in the present study of experimental BSE in cattle (Wells and others 1998) and in studies of confirmed field cases of BSE (Fraser and Foster 1994; H. Fraser, personal communication) infectivity has not been detected in spleen or lymph nodes. It is also interesting that in a further study, still in progress, cattle inoculated intracerebrally as calves with pools of spleen or multiple regional lymph nodes from cattle terminally infected with BSE are all surviving (as of December 1998) at 72 months after inoculation (G. A. H. Wells, S. A. C. Hawkins, unpublished observations). This period is approximately 40 months greater than the apparent end point of an as yet incomplete titration of BSE infected brain stem by intracerebral inoculation in cattle (G. A. H. Wells, S. A. C. Hawkins, unpublished observations). This suggests that any infectivity in these lymphoreticular tissues, at least at the terminal stage of disease in natural BSE is below detectability even by within species assay.

This inconsistency raises a third possibility, namely that the detection of BSE infectivity in bone marrow taken 38 months after infection was the result of contamination of one (possibly more) of the samples used to make the pooled inoculum. Given the large number of tissues collected from each animal and the fact that sternal bone marrow was the last tissue to be taken in the sequence of harvesting tissues at necropsy, it is difficult to exclude all possibilities of contamination, even with the extreme care exercised in the present

studies. The risk of BSE contamination from a small amount of, for example, CNS is greatest when animals are clinically affected. That this result is related to an episode of contamination is consistent with the absence of detectable infectivity in bone marrow samples from clinically affected cattle killed at 40 months after inoculation. Nevertheless, the result is also consistent with the limited evidence of previous studies that the occurrence of infection in bone marrow in these diseases is a rare event and probably not part of the general pathogenetic pattern.

The application of PrP detection to morphological assessment of brains of mice in qualitative mouse bioassays for TSE infectivity has considerable implication for this and future similar studies. Clear distinctions must be drawn between its application for the most sensitive available means of detection of disease in recipient mice used in a qualitative assay as here, and application to a quantitative bioassay for detection of the concentration of infectivity in a tissue. The latter changes the mathematical basis of the assay. Limited experience to date of the former application using PrP IHC indicates that it is a useful adjunct in the assay, especially to resolve results equivocal on the basis of morphological observations alone. The retrospective inclusion of this method for bioassays in this study is being considered.

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Rupture of the gracilis muscle in Iberian pigs at slaughter

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MYOPATHIES in pigs, as a primary affection of muscle fibre, can be classified according to their aetiology as nutritional, endocrinological, toxic, infectious, or myositic and traumatic (Hulland 1993, Banker and Engel 1994, McGavin 1995). Traumatic injury may cause a total rupture of fibres or only a partial muscle fibre tear. In domestic animals, traumatic myopathies of different origins affecting different muscles have been described (Hulland 1993, McGavin 1995). These may occur spontaneously in competition animals during strenuous exercise and, in livestock, adductor tears have been reported when animals have slipped on the ground during activities such as being loaded for transportation (Hulland 1993, Banker and Engel 1994, McGavin 1995); this is of particular importance in the case of the Iberian pig in view of the financial losses involved (Rouco Yañez and Rodríguez Mora 1995).

The Iberian pig is an autochthonous breed reared in a free-roaming system in grasslands with distinct characteristics: a sparse grass covering, which grows according to the meteorological season; and arboreal flora, mainly acorn-bearing and cork oaks, to which the possibility of cereal crops can be added. This ecosystem has such a close soil-plant-animal relationship that any factor acting upon any of these can cause an alteration in the system as a whole.

This short communication describes, for the first time, a nutritional myopathy of the gracilis muscle in Iberian pigs

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