THE WELFARE PROBLEMS ASSOCIATED WITH USING TRANSGENIC MICE TO BIOASSAY FOR BOVINE SPONGIFORM ENCEPHALOPATHY

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Abstract *Animal Welfare 1999, 8:* 421-431

Prion diseases are fatal neurodegenerative disorders, epitomized by the recent bovine spongiform encephalopathy (BSE) epidemic in cattle and the emergence of a novel variant of Creutzfeldt-Jacob disease (vCJD) in humans. In prion disease, the agent of infection is believed to be composed of proteinaceous particles, termed prions, which are converted from a normal isoform into a pathogenic isoform during pathogenesis. A bioassay to detect pathogenic prions of BSE in bovine products consumed by humans was unattainable until the development of transgenic mice, due to the significantly lower susceptibility of wild-type mice to BSE. Transgenic mice have now been generated which express the bovine prion protein and are susceptible to BSE. Following an intracerebral injection with brain homogenate of BSE-infected cattle, transgenic mice develop numerous clinical signs of prion disease, including truncal ataxia (inability to coordinate the torso's muscular activity), increased tone of the tail, generalized tremor, and lack of a forelimb extensor response.

In this study, the ethical score system devised by Porter (1992) was applied to the BSE bioassay as a tool for identifying welfare issues affecting animals used in the bioassay. We acknowledge that there are limitations to the use of the information arising from the application of the Porter scoring scheme for assessing the justification to proceed with any animal experiment; notwithstanding these problems, however, our application of the Porter model to the BSE bioassay enabled us to identify potential targets for refinement: pain involved, duration of distress and the duration of the experiment. This was despite lenient scoring for the duration of distress and pain experienced by the mice, and optimal scoring for the quality of animal care. The targets identified for refinement are discussed in relation to the method of inoculation, the duration of the bioassay, and the duration of the clinical phase, with the objective of exploring ways of reducing the severity of the bioassay.

Keywords: *animal welfare, BSE bioassay, prion disease, transgenic mice*

Introduction

Prion diseases are fatal neurodegenerative disorders affecting both humans and animals (Moore & Melton 1997). They are epitomized by the recent bovine spongiform

encephalopathy (BSE) epidemic in cattle (Wells *et al* 1987), and by the emergence of a novel variant of Creutzfeldt-Jacob disease (vCJD) in humans (Collinge *et al* 1996). Both conditions are believed to have arisen through the consumption of infected meat products: BSE through the consumption of scrapie-infected ruminant feed by cattle (Wells *et al* 1987); and vCJD through the consumption of infected beef products (Williams 1997). Recent experiments have shown that the strain of infectious agent in vCJD is indistinguishable from that in BSE (Collinge *et al* 1996; Bruce *et al* 1997; Hill *et al* 1997). New variant CJD, unlike classical CJD, can affect teenagers and young adults. Clinical symptoms of vCJD include behavioural changes, ataxia (inability to coordinate muscular activity), progressive dementia and myoclonus (shock-like muscular contractions), and last for 13 months on average, prior to death. At present, the UK Creuzfeldt-Jakob Disease Surveillance Unit has confirmed 40 cases of vCJD in the UK (Anonymous, undated). In light of the high probability that these cases are the result of exposure to BSE prions, there is concern over the number of people who have been exposed to BSE, and the possibility of a future epidemic of vCJD.

The BSE epidemic and its apparent transmission to humans has intensified research on prion diseases. It has been proposed that the agent of infection is composed of proteinaceous particles, termed prions, which exist in two isoforms (Prusiner 1982). The normal isoform of the protein, termed PrP^{C}, is present in healthy brain tissue. During pathogenesis, PrP^{C} is converted into an infectious form of the prion protein, termed PrP^{Sc} (Prusiner *et al* 1981; 1984) by a conformational change in its secondary structure (Pan *et al* 1993). Differences in the amino acid sequences of infecting PrP^{Sc} and endogenous PrP^{C} have been associated with an initial barrier to infection, known as the species barrier (Scott *et al* 1989). This species barrier is demonstrated by the significantly lower susceptibility of wild-type mice to BSE (Fraser 1992).

The first transgenic mice generated to investigate the species barrier expressed hamster prion protein – PrP (Scott *et al* 1989). Studies with these mice showed that efficient infection with prions depended on the expression of PrP^{C} from the same species as the source of infecting prion. This knowledge has been applied to produce transgenic mice which express bovine PrP and are more susceptible to bovine prions than wild-type mice.

Transgenic mice providing a sensitive bioassay for BSE have the potential to be used extensively. Availability of this bioassay will allow epidemiological studies to be conducted on the frequency of BSE in cattle. The transgenic mice could be utilized to monitor prion contamination in an extensive range of products consumed by humans. These products include meat and offal, and also medicinal products derived from cattle such as collagen, used widely in plastic and reconstructive surgery, and gelatine used in the production of drug capsules. The bioassay could be crucial in the detection of subclinical cases of BSE due to the 5-year incubation period of BSE in cattle (MacKenzie 1998a). As reported recently (MacKenzie 1998b), the European Commission (EC) wants European Union countries to test cattle for BSE in abattoirs. As this paper goes to press (September 1999), four rapid tests for the diagnosis of BSE in bovines have been evaluated by the EC and a decision will be made shortly regarding the test that will be used.

Ethical scoring systems have been developed to evaluate the acceptability of proposed animal experiments with respect to the potential benefits of the research and likely costs to animals. A number of these models have now been developed, for example the Institute of Medical Ethics (IME) model (Smith & Boyd 1991), the Dutch model (Theune & de Cock Buning 1991; 1993) and the Porter model (Porter 1992). These systems have been developed for the ethical evaluation of animal experiments at different levels. The European Centre for

the Validation of Alternative Methods (ECVAM) 1997 workshop focusing on the use of transgenic animals in the European Union recommended that the use of ethical scoring systems be applied to transgenic research (Mepham *et al* 1998).

The IME and Dutch models were designed for the evaluation of projects at the level of local ethics committees and institutionally based committees. Therefore, these systems examine an extensive range of issues including the quality of the facilities, animal technicians, housing and husbandry conditions, and the credentials of the research group. Retrospective analysis of projects using these models is not possible, since their application requires information which cannot be subsequently derived from published literature. An ethical evaluation of the BSE bioassay with such schemes would require a substantial number of assumptions, and would, therefore, make the assessment invalid.

The Porter model was originally intended as an ethical scoring system for use by individual scientists, as a tool for minimizing animal suffering. This scoring system is based on the premise that every experiment on a sentient animal represents a departure from the Schweitzerian ideal that one should avoid harming animals whenever possible. Albert Schweitzer states in his *Ethic of Reverence for Life* (Schweitzer 1989) that scientists who experiment upon animals in order to help mankind, have a duty to ponder in every separate case whether it is really and truly necessary to sacrifice an animal for humanity. Schweitzer (1989) adds that scientists ought to be filled with anxious care to alleviate, as far as possible, the pain that they cause. Therefore, it follows that the Porter model is based on a utilitarian ethical standpoint. Utilitarianism in its original and simplest form maintains that actions are right or wrong in proportion to the total amount of pleasure or pain that they produce. This requires that the predicted costs and benefits of a study must be weighed against each other. The Porter model is restricted to eight questions, two of which are related to the potential benefits of the study, and six to animal welfare issues (Table 1). The information that is required to apply this system is available in the published literature, with the exception of the quality of animal care. Therefore, an ethical evaluation of the BSE bioassay with this system requires only one assumption, rather than the many assumptions required for the application of the other ethical evaluation schemes.

Comparison of the categories relating to animal welfare between the Porter, IME and Dutch models reveals a greater subdivision of the adverse effects caused by scientific procedures in the Porter model. The pain induced during an experiment, duration of distress and duration of the experiment are distinguished in the Porter model. In the Dutch model, the extent and duration of discomfort are used to assess the cost to animal welfare. In the IME model, a score is assigned to the likely severity of the procedures involved, and this score must consider all aspects of the adverse effects. Therefore, the Porter model is the only one that directly identifies several individual issues influencing animal welfare.

The Porter model has received a large degree of criticism compared with other ethical scoring systems. It categorizes the aims of animal experiments, placing greater importance on the alleviation of substantial pain. However, there are many elements, including socio-economic factors, which need to be considered. In the case of vCJD, this disease is not considered to be painful, however, it does incite intense fear in patients, and is severely distressing to family members. The category evaluating the duration of the experiment with respect to the lifespan of the animal has also raised concern (De Cock Buning & Theune 1994). When taken to the extreme, this approach may stimulate researchers to use animals with longer lifespans, such as primates instead of mice, in order to decrease their overall

Table 1 **Representation of the ethical scoring system proposed by D G Porter to minimize suffering in animal experiments (Porter 1992). The scores we assigned to the transgenic mice used to bioassay BSE are indicated by emboldened text. A score of 1 represents the minimum unavoidable score. A score of 5 represents a major departure from the ideal that one should not harm sentient animals. In the animal welfare categories C-H, note the following: in category C, 'sentient, conscious' species (score 4) includes all mammals (except primates, carnivores and cetaceans) and birds. Category D includes the use of analgesics, post-operative pain, and the skill of the experimenter in the proposed procedures. Category E considers all aspects of the procedure. Category F considers estimated normal lifespan (LS)[1]. Category G considers the numbers of animals involved[2]. Category H includes all aspects of the animals' environment and quality of care.**

Category	Score				
	1	2	3	4	5
A – Aim	Alleviate substantial pain	Alleviate moderate pain	**Clear benefit to human health**	Some benefit to human health	Fundamental research
B – Potential to achieve objective	**Excellent**	Very good	Average	Limited	Very limited
C – Species	Low sensibility	Some sensibility	Sentient, limited consciousness	**Sentient, conscious**	Sentient, intelligent, precognitive
D – Pain	None	**Minimal**	Moderate	Considerable	Severe
E – Duration of distress	None	Short	**Moderate**	Long	Very long
F – Duration of experiment[1]	Very short 10^{-7} LS	Short $2x10^{-4}$ LS	Moderate $2x10^{-2}$ LS	Long $2x10^{-2}$ LS	**Very long > $2x10^{-1}$ LS**
G – Number of animals[2]	1–5	**5–10**	10–20	20–100	> 100
H – Quality of animal care	**Excellent**	Very good	Average	Good	Poor

[1] Although this Table reproduces the error published by Porter (1992), in that both moderate and long lifespan durations are allocated identical figures, this had no influence on the score derived for our ethical evaluation of the BSE bioassay.

[2] Table 1 reproduces the original Porter (1992) score categories, which overlap (see footnote, p 426). In our assessment, these categories were effectively 1–5, >5–10, >10–20, >20–100 and >100.

score under this scheme. Clearly, this is not in concordance with the principle of refinement (Russell & Burch 1959). Assigning a numerical score with this model has also been criticized, since a project may still be approved when the score does not exceed the maximum permissible score, even though the rationale is inadequate. In addition, it has been

argued that the validity of calculating a final score is questionable as the categories in the Porter model do not have common units. Furthermore, the compact nature of this model has raised concerns that many factors are missing, such as a justification for why in vitro methods are not possible. It is also recognized that the Porter model, as with all ethical scoring systems, does not provide consideration for issues specific to transgenic animals, such as the possible, random side-effects of the transgene (Mepham *et al* 1998).

However, if taken into consideration and addressed when using the model, all these criticisms (which are specific to the Porter model) do not impinge on its use to identify welfare issues. Therefore, we chose the Porter model as the most applicable model to provide a rapid, ethical evaluation of the BSE bioassay derived from the published literature. Welfare problems associated with prion infectivity bioassays and potential targets for refinement, identified with the Porter system, will be discussed with regard to the necessity and scientific justification for a BSE bioassay.

Transgenic models expressing bovine PrP

Previous studies revealed that efficient transmission of prions was dependent on the expression of PrP transgenes from the same species as the source of the prion agent. Therefore, transgenic mice expressing bovine PrP transgenes were generated to develop a sensitive bioassay for BSE prions (Scott *et al* 1997). Two different lines of transgenic mice were generated, producing either high or low levels of bovine PrP. The levels of bovine PrP per gram of protein in the brains of these transgenic mice were, respectively, greater than 8 times, or greater than 4 times, more than levels found in control bovine brains.

The mice were inoculated intracerebrally with a 10 per cent brain homogenate derived from cattle infected with BSE (n = 10). Clinical signs of prion disease developed in all inoculated mice, notably truncal ataxia, increased tone of the tail, generalized tremor and a lack of the forelimb extensor response when lifted by the tail. Additional clinical signs associated with prion disease included aggression, hunching, head bobbing, difficulty in righting, disorientation, chronic convulsions, slow movements, kyphosis (deformity of the spine), deep loss of pain sensation, circling and partial or complete paralysis (G Telling personal communication 1998). The onset of symptoms occurred within 250 days of inoculation in mice expressing high levels of bovine PrP, or approximately 320 days in mice expressing intermediate levels of bovine PrP (Scott *et al* 1997). It is likely that the mice experienced a clinical phase of less than 2 weeks, although this information is not provided by Scott *et al* (1997). In comparison, BSE transmission to wild-type mice was variable, with incubation periods typically exceeding 1 year (Lasmézas *et al* 1997).

The transgenic mice exhibited many pathological characteristics of BSE disease in cattle, including a similar distribution of spongiform degeneration and fragments of PrP^{Sc}, which were indistinguishable from those found in infected cattle (Scott *et al* 1997). However, Scott *et al's* transgenic model failed to reproduce all aspects of the neuropathological profile of BSE in cattle.

Welfare implications associated with a bioassay for BSE

We applied the ethical scoring system devised by Porter (1992) to the transgenic murine model used to bioassay BSE. The scheme (reproduced in Table 1), is based on a system that involves assigning scores from 1 to 5 in each of eight categories (A–H). When the entire

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Porter system is applied to a study, a score of between 8 and 40 is obtained. The maximum score for the six categories relating to animal welfare (C–H) is 30 points (Table 1).

Our assessment of the BSE bioassay with the Porter model is based on information derived from Scott *et al* (1997). The scores we assigned are indicated by the emboldened categories in Table 1. The transgenic mice used to bioassay for BSE have a clear benefit to human health, as they will enable prion contamination to be monitored in an extensive range of products consumed by humans. The potential to achieve this objective was deemed to be excellent, based on the successes of previous similar studies. Thus, categories A and B together generated a score of 4 – well within what Porter (1992) considered the acceptable score of 7 for these categories – indicating that the rationale was acceptable for this experiment.

The animal welfare categories, C–H were assessed as follows. Mice were considered to be sentient and conscious animals (score 4). Pain induced during the study was considered to be minimal (score 2), with reference to intracerebral inoculation (discussed further in the following section of this paper). The duration of distress was ranked as moderate (score 3). This could be considered an underestimation of the duration of the distress experienced by these mice, considering the symptoms that they develop – and is discussed further (see, *Duration of distress*). The duration of the experiment, estimated to range from 264–334 days, exceeded 20 per cent (2×10^{-1} LS) of the normal lifespan of a mouse (score 5). This calculation was based on the normal lifespan of a laboratory mouse being 2 years. Ten animals were used in the study (see note 2, Table 1), generating a score of 2[1]. In order to complete the evaluation of the BSE bioassay, an assumption about the quality of animal care was required. The quality of animal care was assumed to be excellent, (score 1), and therefore represented the best-case scenario for this study. As Table 1 indicates, a total score of 17 was derived for the categories relating to animal welfare (C-H) This exceeded the maximum score of 15 which Porter (1992) considered acceptable for these categories. A score of this level should evoke concern for the welfare of the animals in the study.

Assessment of the BSE bioassay with the Porter ethical scoring system identified potential targets for refinement: pain induced during the experiment (D), the duration of distress (E) and the duration of the experiment (F). Each of these areas is discussed below, including suggestions for their refinement, with a view to reducing the severity of the bioassay.

Pain induced during the bioassay

During a BSE bioassay, it is not known whether the intracerebral injection causes pain or distress for the animal. Intracerebral inoculation is the preferred method for efficient transmission of prion disease. The procedure requires administration of a general anaesthetic and involves puncturing the skull bone over the right parietal lobe with a 27-gauge needle to inject infected brain homogenate into the cerebellum. The requirement for a general anaesthetic is an additional source of stress for the animal, and could be avoided by intraperitoneal (Kimberlin & Walker 1986; Baldauf *et al* 1997), or intravenous (Kimberlin & Walker 1979) injections. This observation is confirmed by the LASA Working Party report on severity assessment for the administration of substances (Wallace *et al* 1990). Prion inoculation at peripheral sites would reduce the severity of the procedure, although these

[1] In Porter (1992), the score categories 1 (1–5 animals), 2 (5–10), 3 (10–20) and 4 (20–100) overlap, so the BSE assay could have been assigned a score of either 2 or 3. In Scott *et al* (1997) one data set was obtained from 8 mice and one from 10, so a score of 2 was chosen.

methods are less efficient at transmitting prion disease compared with intracerebral inoculation (Farquhar *et al* 1996). However, we recognize that a less efficient procedure for transmitting prion disease would increase the number of animals required in a study, and hence produce a less favourable score in category G.

After recovery from the anaesthetic, pain or discomfort could be caused by an augmentation of pressure on the brain due to the volume of brain homogenate injected. Typically, 30µl of brain homogenate is injected into the brain, which equates to a minimal increase in pressure on the brain of an adult mouse. However, the pressure increase would be proportionally greater in the brain of a neonatal mouse. At this stage of development the skull is still soft, which simplifies penetration of the skull. The age at inoculation depends on the requirement for genetic screening of the transgenic offspring. No reference is given to the age at which the mice were inoculated in the paper by Scott *et al* (1997), and this is typical of publications in this field of research. The average age of inoculation for mice in other (unspecified) studies has been reported as 10 weeks (G Telling personal communication 1998).

Duration of distress

Modelling prion disease in animals is distressing for the animals. Ataxia, tremor, convulsions, kyphosis and paralysis are just some of the symptoms experienced by the mice, together with substantial weight loss of up to 25 per cent of their liveweight from time of onset of the neurological symptoms. All these symptoms can only be regarded as causing substantial distress to the animal (Baumans *et al* 1994). Some of the symptoms experienced by the mice infected with prion disease are listed in the OECD guidelines on humane end points in experimental animals (OECD 1998) and are indicative of a moribund condition.

The duration of the distress experienced by the mice in the BSE bioassay was probably less than 2 weeks, based on the length of the incubation period, although Scott *et al* (1997) did not provide this information. When modelling prion disease in mice, the progression of the disease needs to be monitored as the clinical signs of prion disease, particularly in aged mice, can be confused with other neurological diseases. The mice are killed according to *Schedule 1* of the UK *Animals (Scientific Procedures) Act 1986* (Home Office 1986) after diagnosis of prion disease has been verified by the presence of at least two signs of neurological dysfunction, and the progression of these signs observed for up to a maximum period of 3 weeks (G Telling personal communication 1998). The importance of observing the animals regularly and recording the onset of clinical signs and progression of disease is highlighted in the OECD guidance document on humane end points (OECD 1998).

Very few studies have been conducted to investigate the verification of prion disease earlier during the incubation period. This situation reflects a lack of research as a dominant factor impeding the advancement of refinement in general (Smaje *et al* 1998). In mice intracerebrally inoculated with BSE, PrP^{Sc} accumulation and histological changes were first detected approximately at 75 days and 140 days post-inoculation, respectively; and death occurred at 180 days (Lasmézas *et al* 1996). Thus, markers of prion disease were detected approximately 105 days (PrP^{Sc}) and 40 days (histological changes) prior to death of the animal. Lasmézas *et al* have, therefore, shown that prion disease can be verified in mice at an earlier stage of infection than is currently practised. The ability to use an earlier end point for prion infectivity studies would reduce the duration of distress, and should provide a more humane end point to the study.

Duration of the experiment

The duration of the BSE bioassay from inoculation until the diagnosis of prion disease was considered to constitute a significant proportion of the normal lifespan of a mouse, and this is typical of studies modelling prion disease in mice. Potential strategies have been identified to generate transgenic mice with short incubation periods and clinical phases, including: high levels of expression of the PrP transgene (Prusiner *et al* 1990), and the use of chimeric PrP constructs (Telling *et al* 1994), ie mice expressing segments of PrP from several different species. The incubation period for prion disease, and the duration of the clinical phase, have been shown to be inversely proportional to the level of expression of the PrP transgene (Prusiner *et al* 1990). Therefore, transgenic mice expressing high levels of PrP will develop prion disease faster, and will have a shorter clinical phase, compared with transgenic mice expressing low levels of the PrP transgene. This inverse relationship is demonstrated in the paper by Scott *et al* (1997) in which mice expressing high levels of bovine PrP developed symptoms 70 days earlier than mice which expressed only moderate levels of transgene.

An additional approach to reducing the incubation period and generating an earlier and shorter clinical phase is to generate transgenic mice expressing chimeric PrP constructs. Telling *et al* (1994) produced transgenic mice expressing a chimera of human and mouse PrP proteins, which resulted in an incubation period of 230 days, and a shorter clinical phase of less than 48h, following inoculation with human prions. In the chimeric transgene, nine amino acids in the central region of murine PrP were substituted for homologous human amino acids, and the resulting transgenic mice expressed the transgene at an equivalent level to human PrP expression in the human brain (Telling *et al* 1994). Initial attempts to replicate this strategy with transgenic mice expressing high levels of chimeric transgenes, in which eight amino acids of murine PrP were substituted for bovine amino acids, failed to generate mice susceptible to BSE (Scott *et al* 1997). Nevertheless, the utilization of chimeric PrP constructs, and the production of lines with high expression, are both potential strategies for the generation of transgenic mice with short incubation periods and clinical phases, thereby reducing the severity of prion bioassays.

The necessity for a BSE bioassay

The use of transgenic mice to provide a sensitive bioassay for BSE will facilitate monitoring levels of BSE contamination in bovine products consumed by humans. This bioassay is needed principally to detect contamination derived from subclinical cases of BSE. The bioassay will not yield results fast enough to directly exclude BSE-infected carcases from entering the food chain, although, indirectly, it could be used to reduce future exposure of humans to BSE.

Immunologically based assays for the pathogenic prion protein have recently been developed by the biomedical company Prionics Inc (Zurich) (MacKenzie 1998a) and by Safar *et al* (1998). These assays are based on differing principles: the Prionics assay is based on Western blot analysis of the prion protein following proteinase degradation, and the Safar assay on time-resolved fluorescent ELISA. These assays, although not as sensitive as the bioassay, would yield results fast enough to prevent BSE-infected carcases entering the food chain. Preliminary surveys are currently underway to ascertain the reliability of the Prionics assay compared with the bioassay for BSE (Mackenzie 1998a).

Conclusions and animal welfare implications

Bioassays for BSE using transgenic mice expressing bovine PrP, have an important role in providing information for reducing the future exposure of humans to BSE. In this paper, the Porter ethical scoring system (Porter 1992) was used as a tool to identify the welfare problems associated with one particular (Scott *et al* 1997) BSE bioassay. We acknowledge that there are limitations to the use of the information arising from the application of the Porter scoring scheme for assessing the justification to proceed with any animal experiment. Notwithstanding these limitations, our application of the Porter model to the BSE bioassay has highlighted several areas of concern regarding the welfare of the mice involved in the bioassay.

Intracerebral inoculation, duration of the incubation period, and especially the duration of the clinical phase of the disease were identified as areas for concern. Furthermore, this paper identifies areas where further research is needed: on the effect(s) of intracerebral inoculation and defining earlier humane end points. No studies have been conducted to establish the effect(s) of intracerebral inoculations on animal welfare. Earlier end points to prion infectivity bioassays need to be defined to reduce distress during the clinical phase. There also needs to be increased emphasis placed on the production of transgenic mice with short incubation periods and clinical phases. All the factors described above, as well as the potential role of immunologically based assays as alternatives to the bioassay, should be considered prior to the extensive use of transgenic mice in bioassays for BSE.

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