Chapter 7
Autoantibodies to Brain Components and Antibodies to *Acinetobacter* Are Present in Bovine Spongiform Encephalopathy

7.1 Introduction: Bovine Spongiform Encephalopathy or "Mad Cow Disease" Could Be Due to Environmental Factors

Bovine spongiform encephalopathy (BSE) or "mad cow disease" is a recently discovered neurological disorder of cattle which was first reported in the United Kingdom in 1985, after changes were introduced in the preparation of "meat and bone meal" (MBM) feeds. These supplementary feeds were used especially in the winter months.

It had been suggested that the bovine disease could be transmitted to humans (Will et al. 1996). This suggestion evoked widespread public concern because of its implications in possibly causing a similar disease in humans. There were several explanations proposed for the origin of this disease. One suggestion was that it was caused by abnormal prions (PrP^{SC}) and another one was that it was caused by exposure to organophosphates (Purdey 1994).

The disease belongs to a group of conditions labelled as transmissible spongiform encephalopathies which include kuru, Creutzfeldt-Jakob disease and scrapie. In these diseases both in affected animals and humans, autoantibodies to brain neurofilaments have been described by Gajdusek's group (Aoki et al. 1982).

A characteristic feature of BSE is a vacuolar appearance labelled as "spongiform changes" but a similar coalescence of vacuoles occurs in chronic experimental allergic encephalomyelitis (Raine et al. 1974). In experimental allergic encephalomyelitis autoantibodies to brain neurofilaments and myelin also occur.

Another possibility to explain the disease is that environmental bacteria may contain sequences which cross-react with brain tissues and following infection antibodies will be produced which will attack brain tissues.

A. Ebringer, *Multiple Sclerosis, Mad Cow Disease and Acinetobacter*,
DOI 10.1007/978-3-319-02735-7_7

7.2 Computer Analysis of a Short Sequence of Bovine Myelin Suggests "Molecular Mimicry" with 3 Common Bacteria: *Acinetobacter, Agrobacterium* and *Escherichia*

Workers from San Diego have identified a short sequence of bovine myelin (RFSWGAEGQK) which is resistant to denaturation by heating to 100 °C or by treatment with 8 M urea, These are properties which have also been described for prions.

It was reported over 40 years ago, that injection of this short peptide into experimental animals, such as guinea pigs will lead to ataxia, hind quarters paralysis, tremors and eventually death (Eylar et al. 1970).

These features resemble to some extent, those observed in cattle affected by BSE.

An analysis of proteins found in the GenBank and SwissProt databases was carried out and it showed that there were sequences in three microbes which exhibited molecular mimicry with some brain tissues.

The best sequence was found in 4-carboxy-muconolactone decarboxylase of *Acinetobacter calcoaceticus*, a common saprophytic microbe found in soil and water supplies and which also possesses sequences resembling bovine neurofilaments (Table 7.1).

Another common environmental microbe *Agrobacterium tumefaciens* also showed some similarities to bovine myelin although not to the same extent as *Acinetobacter calcoaceticus.* Further molecular analysis revealed similarities with

Table 7.1 Comparison of similar sequences in bovine neurofilaments compared with *Acinetobacter calcoaceticus* molecular sequences

Sequence	Source (amino acid positions in brackets)
NEALEK	Neurofilament (326–331)
KEALEK	Mercuric reductase (24–29)
LKKVHEE	Neurofilament (222–228)
IEKVEEE	RNA polymerase sigma-S4 factor (54–60)
EALEKQL	Neurofilament (327–333)
EALEYGL	Lysyl tRNA synthetase (471–477)
ELEDKQN	Neurofilament (335–341)
ALEDKSN	Protocatechuate 3,4-dioxygenase (212–218)
EALEKQL	Neurofilament (327–333)
EAYAKQL	β-carboxy-*cis*-muconate cyclomerase (218–224)
KKVHEE	Neurofilament (223–228)
KKVKEE	Regulatory protein (13–18)
EIRDLR	Neurofilament (141–146)
EIRDLE	Secretion protein (279–284)
EQEIRDLR	Neurofilament (139–146)
EQIVRDAR	Acyl coenzyme A dehydrogenase (174–181)

Sequences were retrieved from the Protein Information Resource Database release 44. Identical amino acids are shown in boldface

prion proteins found in three molecules of *Escherichia coli*: the three molecules were: recognition protein, colicin M and maltodextrose-glucosidase (Ebringer et al. 1997).

7.3 Sera from Animals with and Without BSE and from Healthy Animals from an Organic Farm

Sera from BSE affected cattle and healthy controls have been tested by enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to the three common microorganisms as well as autoantibodies to bovine neurofilaments and myelin.

Since BSE was thought to be caused by consumption of MBM winter feeds, it was considered that the "mucosal associated lymphoid system" present in the mucosal tissues of the respiratory and gastrointestinal tract would encounter such dietary antigens. Therefore mucosal immunoglobulin A (IgA) was more likely to show any possible differences in the titre of autoantibodies to brain components.

Molecular modelling suggested three possible microbes which showed cross-reactivity to brain tissues. Therefore a total Ig (IgG + IgA + IgM) assay was developed in an attempt to detect any immunological signal. The investigation was authorised and sponsored by the government, especially by the Ministry of Agriculture, Fisheries and Food (MAFF) of the U.K.

Sera from Animals with and Without BSE Supplied by the Central Veterinary Laboratory of the Ministry of Agriculture, Fisheries and Food (MAFF)

Animal sera were obtained from 29 animals (mean age 74.4 months, range 44–122 months) which were found at post-mortem to satisfy the criteria of having BSE and 18 animals which did have the disorder. These sera were supplied by the Central Veterinary Laboratory (CVL) (New Haw, Addlestone, Surrey, England), which is an executive agency of the Ministry of Agriculture, Fisheries and Food.

The 18 animals which did not have BSE had been referred to CVL because of abnormal behaviour involving ataxia and suggesting a neurological disease. The attending veterinarian decided that the limping and ataxic animal should be referred to government agencies and a post-mortem examination was carried out to exclude BSE.

The BSE and control sera provided by the CVL were obtained from animals raised on farms in different parts of England, each having its own water supply and belonging to separate herds. The majority of the BSE positive animals came from dairy Friesian herds.

Specifically there was no genetic or breeder link between the various animals that had developed BSE or the controls.

Control Sera from an Organic Farm Not Associated with MAFF

Another set of control sera were also investigated which came from animals unconnected with government departments.

These consisted of an additional 58 healthy animals to act as extra controls. This group consisted of 30 serum samples from animals aged less than 30 months (8 Friesians and 21 Hereford-Friesian and 1 Charolais-Friesian crossbreed, the crossbreed being raised for meat production).

There were also 28 serum samples from animals aged more than 30 months, all of which were dairy Friesians.

The animals were raised on a farm in Hampshire where no case of BSE had been reported and were kept under organic farming conditions, with winter feeds consisting of hay and grains but no MBM supplements.

Serum samples were obtained during yearly annual herd testing for brucellosis and were provided by the attending veterinarian.

7.4 Bacterial Cultures, ELISA and Absorption Studies

Bacterial Cultures

The microbes *Acinetobacter calcoaceticus* (NCIMB 10694) and *Agrobacterium tumefaciens* (NCIMB 9036) were obtained from the National Collections of Industrial and Murine Bacteria Ltd. (Aberdeen, Scotland) and *Escherichia coli* (NCTC 9002) was provided by the Department of Microbiology at King's College.

IgA and total Ig (IgG + IgA + IgM) antibodies were measured by ELISA.

Cultures were grown in 2 l flasks on an orbital shaker for 16 h at 37 °C for *Escherichia coli* and for 2 days at 30 °C for *Acinetobacter calcoaceticus* and *Agrobacterium tumefaciens* in 200 ml of nutrient broth (Oxoid 25 g/l).

Flasks were inoculated with 10 ml of the corresponding starter culture and were left shaking at 37 °C for 6 h. Batch culture cells were harvested by centrifugation at 6,000 rpm for 20 min at 4 °C.

The pellets of cells were then washed with 0.15 M phosphate-buffered saline (PBS; pH 7.4) before being finally resuspended in 20 ml of PBS.

A stock solution of the suspension was prepared by diluting in 0.05 M carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 (10^6 bacterial cells/ml) on the spectrophotometer (Corning Model 258).

ELISA

The ELISA method was carried out as follows: ELISA plates were coated (5 µg/well) with neurofilaments prepared from bovine spinal cord (Sigma), myelin basic protein obtained from bovine brain (Sigma) or bacterial suspension (200 µl/well) overnight at 4 °C.

Non-specific sites were blocked with PBS containing 0.1 % Tween and 0.2 % ovalbumin, plates were washed and 1/200 dilution of test or control serum was added.

The plates were incubated at 37 °C for 2 h, washed and rabbit antibovine alpha-chain-specific horseradish peroxidase conjugate (1/3,000) (Bethyl Laboratories Ltd.) or rabbit anti-cow Ig (IgG + IgA + IgM) horseradish peroxidase (1,4000) (Dako Ltd.) was added.

The plates were re-incubated for 2 h, washed and a substrate solution of 0.5 mg of 2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; Sigma) per ml in citrate-phosphate buffer (pH 4.1) containing 0.98 mM H_2O_2 (Sigma) was added to each well.

The reaction was stopped with a 2 mg/ml solution of sodium fluoride (Sigma), the plates were read at 630 nm on a microtitre plate reader (Dynatech MR 600). Results were expressed as OD units ± standard errors (SE). Each serum sample was tested in duplicate All studies were done blind in that the tester did not know which were test or control sera.

The mean OD units of IgA or total Ig antibodies in serum samples from BSE positive animals resulting from tests against the two autoantigens and three different microorganisms were compared to the corresponding control groups by using Student's *t*-test.

Furthermore, triplicate ELISA studies were carried out in serial doubling dilutions of three selected BSE serum samples which had high, medium and low reactivities to the respective antigens bovine neurofilaments, bovine myelin and *Acinetobacter calcoaceticus.*

Absorption Studies

Serum samples from six animals with BSE and high antibody levels to *Acinetobacter calcoaceticus,* bovine myelin and neurofilaments were selected for absorption studies.

A suspension of *Acinetobacter calcoaceticus,* OD 1.60 at 540 nm, was sonicated using an MSE Soni prep 150 with ½-in probe, amplitude 10–14, for five 1-min bursts. Serum samples (200 µl) were absorbed with sonicated bacteria (25 µl) in a plastic tube and rotated gently overnight at 4 °C.

The absorption was repeated until the antibacterial antibodies levels for each sample were below the mean value for healthy controls when measured by ELISA (mean OD ± SE). Absorbed sera were then retested for reactivity against bovine myelin and neurofilaments, as previously described.

7.5 Measurement of Autoantibodies to Brain Components

Elevated levels of IgA autoantibodies to bovine neurofilaments (Fig. 7.1a) and bovine myelin (Fig. 7.1b) were found in the 29 animals with BSE (respective mean ODs ± SEs, 0.451 ± 0.029 and 0.260 ± 0.019) when compared to 18 animals free of BSE (0.149 ± 0.009; $p < 0.001$) (0.100 ± 0.0012; $p < 0.001$), 30 organically raised

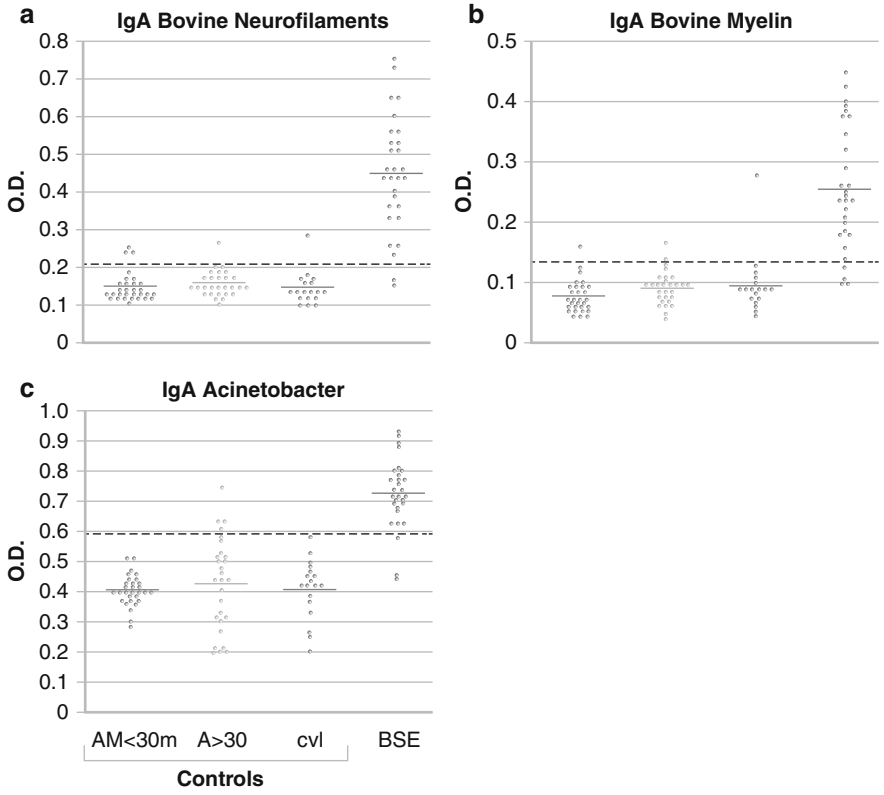

Fig. 7.1 IgA antibody titres (bar = mean) for 30 control serum samples from cows aged less than 30 months (A <30 m), 28 control serum samples from cows aged more than 30 months (A >30 m) and 18 control serum samples from cows not having BSE at post-mortem examination compared to 29 serum samples from cows with BSE at post-mortem examination against bovine neurofilaments (**a**), bovine myelin (**b**) and *Acinetobacter calcoaceticus* bacteria (**c**). *Dashed line* represents 95 % confidence limits for mean of control as given by A<30 M + A>30 M –results of the one tailed test (Copyright © American Society for Microbiology; Tiwana et al. (1999))

cows less than 30 months of age (0.149 ± 0.007; $p < 0.001$) (0.078 ± 0.005; $p < 0.001$) and 28 organically raised cows greater than 30 months of age (0.157 ± 0.006; $p < 0.001$) (0.078 ± 0.005, $p < 0.001$).

Elevated levels of IgA antibodies to whole *Acinetobacter calcoaceticus* bacteria (Fig. 7.1c) were found in the 29 BSE affected cattle (0.737 ± 0.022) when compared to 18 animals free of BSE (0.416 ± 0.024; $p < 0.001$), 30 organically raised cows less than 30 months of age (0.409 ± 0.0.009; $p < 0.001$) and 28 organically raised animals greater than 30 months of age (0.432 ± 0.029; $p < 0.001$).

Absorption of BSE sera with sonicated *Acinetobacter calcoaceticus* reduced autoantibodies to bovine myelin and neurofilaments almost to the levels found in control sera (Table 7.2), although some activity to neurofilaments remained.

Table 7.2 Levels of IgA before and after ELISA absorption with bacteria (mean OD ± SE)

Source	IgA levels	
	Pre-absorption	Post-absorption
Acinetobacter calcoaceticus	0.71 ± 0.02	0.13 ± 0.01
Bovine myelin	0.41 ± 0.01	0.22 ± 0.02
Bovine neurofilament	0.54 ± 0.06	0.28 ± 0.03

7.6 Measurement of Anti-Bacterial Antibodies

Antibodies to *Acinetobacter calcoaceticus* of total Ig (IgG + IgA + IgM) were significantly elevated in the sera from animals with BSE (0.99 ± 0.05) (Fig. 7.2a) compared to CVL controls (0.65 ± 0.06; $p < 0.001$) and organic farming controls, either in animals greater than 30 months of age (0.57 ± 0.03; $p < 0.001$) or in animals less than 30 months of age (0.53 ± 0.02; $p < 0.001$).

There was no significant difference between the CVL controls and the organic farming controls, but there was a small, statistically significant difference when compared with the sera from animals aged less than 30 months ($p < 0.05$).

However there was no significant difference in the level of anti-*Acinetobacter calcoaceticus* antibodies between organic farming animals aged more than 30 months when these animals were compared to those aged less than 30 months.

There was no significant difference between the BSE sera and the three control groups in the levels of either anti-*Agrobacterium tumefaciens* (Fig. 7.2b) and anti-Escherichia *coli* antibodies (Fig. 7.2c).

7.7 Measurement of Antibodies by Serial Dilutions

ELISA estimations of three BSE serum samples which had high, medium and low respective reactivities to the following antigens are shown: bovine neurofilaments (Fig. 7.3a), bovine myelin (Fig.7.3b) and *Acinetobacter calcoaceticus* (Fig. 7.3c).

In each case, the high titre serum reacted with a dilution of up to 1/6,400 of its respective antigen, whereas the medium and low titre sera gave lower readings (Tiwana et al. 1999).

7.8 Discussion and Conclusions

In these studies significantly elevated levels of autoantibodies to bovine neurofilaments and myelin were detected.

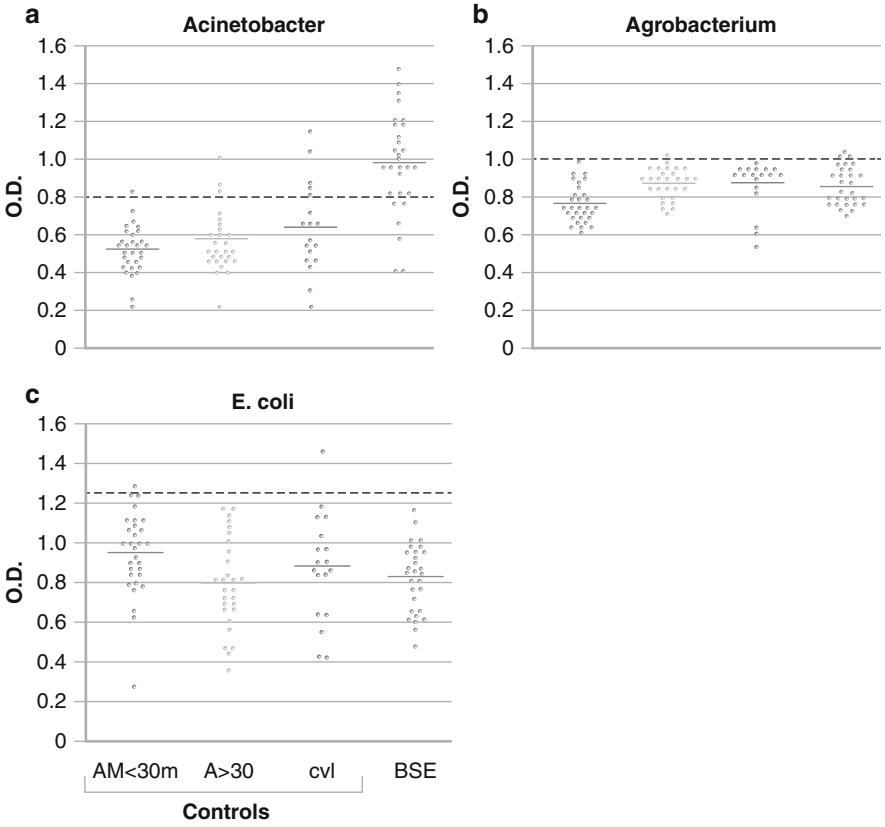

Fig. 7.2 Total antibody titres (bar = mean) for 30 control samples from cows aged less than 30 months (A < 30 m), 28 control samples from cows aged more than 30 months (A > 30 m) and 18 control samples from cows not having BSE at post-mortem examination compared to 29 serum samples from cows with BSE at post-mortem. Total antibody titres were measured against *Acinetobacter calcoaceticus* bacteria (**a**), *Agrobacterium tumefaciens* bacteria (**b**) and *Escherichia coli* bacteria (**c**). *Dashed line* represents 95 % confidence limits for mean of controls by the same formula as in the legend of Fig. 7.1 (Copyright © American Society for Microbiology; Tiwana et al. (1999))

Furthermore elevated levels of specific antibodies to *Acinetobacter calcoaceticus* have been shown to be present in BSE affected cattle when compared to the three different groups of controls. These elevations were statistically highly significant.

By contrast, no such antibody elevations were observed against either *Escherichia coli* or *Agrobacterium tumefaciens.* This is clearly a specific observation in that only one species of bacteria namely *Acinetobacter* would appear to have antibodies in BSE affected animals.

Clearly, the other two microbes, despite having cross-reacting sequences, did not evoke such specific elevations in their antibody levels and therefore were not involved in the causation of this disease.

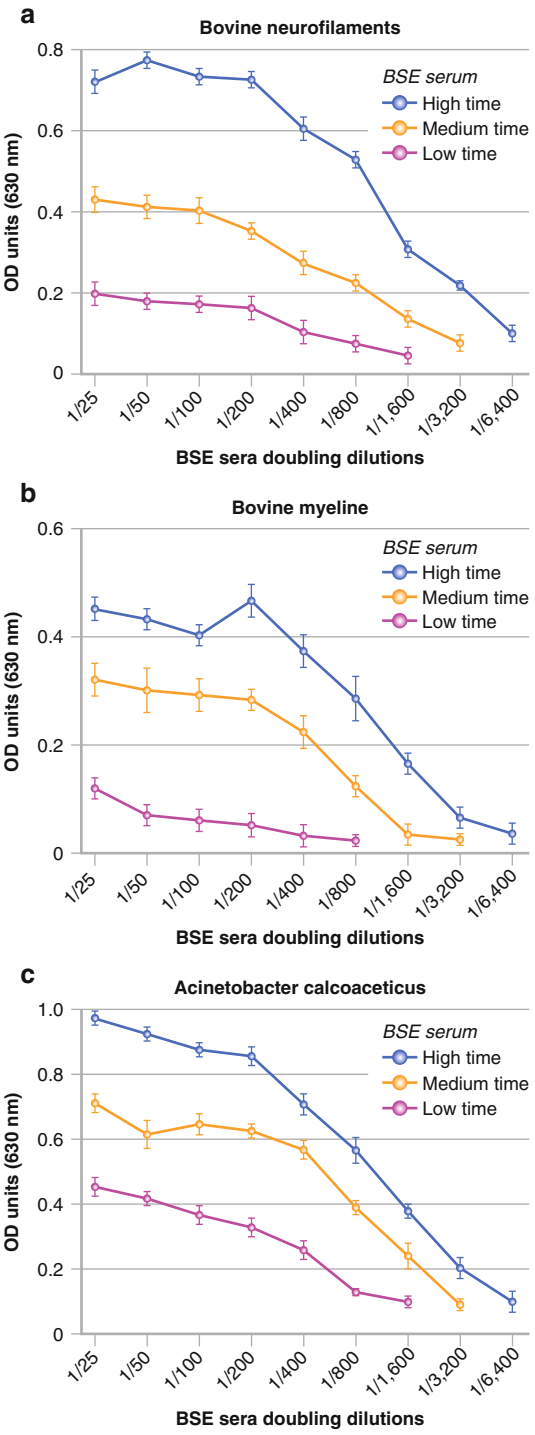

Fig. 7.3 Serial doubling dilutions (mean ± SE) of high, medium and low reactivity BSE sera against bovine neurofilaments (**a**), bovine myelin (**b**) and *Acinetobacter calcoaceticus* bacteria (**c**) (Copyright © American Society for Microbiology; Tiwana et al. (1999))

The agent responsible for the production of these specific autoantibodies is unclear but it would seem that BSE cattle have been exposed to *Acinetobacter calcoaceticus* bacteria, microbes which possess or carry antigens cross-reacting with brain tissues. Whether these bacteria are responsible for the neurological features of the disease remains to be determined.

This interesting observation requires confirmation with a larger sample of sera from animals with BSE selected from different parts of the United Kingdom and the analysis carried out with different species of *Acinetobacter.*

Furthermore, such sera should be tested against other bacteria commonly present in the bowel flora of ruminants, as well as against peptides derived from the cross-reacting sequences resembling bovine neurofilaments, myelin and other brain tissues.

Acinetobacter calcoaceticus is a species of saprophytic and aerobic Gram-negative bacteria that is widely distributed in soil and water supplies but can also be cultured from skin, mucous membranes, nasal cavities and body secretions from both animals and humans.

It is relevant to note that *Agrobacterium tumefaciens* antibodies are not elevated in animals with BSE. This microbe does not have glutamic acid in the cross-reacting epitope when compared to either *Acinetobacter* or bovine myelin (Ebringer et al. 1997). Furthermore, it is a plant pathogen of small trees and shrubs, which makes it unlikely that grass eating animals like cows would have been exposed to it.

It can be concluded from these investigations that in at least one "transmissible spongiform encephalopathy" disease, namely BSE, specific immune responses, involving predominantly the IgA system, would suggest that bacteria and bacterial antigens are acting across a mucosal surface of the gut. The microbe appears to be *Acinetobacter calcoaceticus,* one that is readily found in the environment of cattle. This microbe also happens to possess molecular sequences resembling bovine neurofilaments and bovine myelin. It is unclear why BSE animals have antibodies to this microbe but the possibility arises that it could have been introduced into the food chain of cattle, especially after changes were made in the preparation of winter feeds. Whether this has any pathological significance in the development of BSE awaits further studies.

Autoantibodies to neuronal components have previously been reported in patients with kuru and Creutzfeldt-Jakob disease (Sotello et al. 1980) and in animals with natural scrapie (Aoki et al. 1982). The pathological significance of these autoantibodies remains unclear but there are three human autoimmune diseases in which molecular mimicry occurs between bacterial antigens and self tissues: rheumatic fever, ankylosing spondylitis and rheumatoid arthritis. In rheumatic fever anti-streptococcal antibodies bind to the basal ganglia of the brain, thereby producing abnormal gait movements and the syndrome is described as Sydenham's chorea (Husby et al. 1976).

A similar neurological disorder could occur in cattle with BSE following production of anti-*Acinetobacter calcoaceticus* antibodies, since this microbe possesses antigens resembling brain tissues. The mechanism responsible for the neurological lesions remains unclear but at least these results confirm and extend the observations of Gajdusek's group that autoantibodies to neurofilaments and other brain components are present in BSE, a disease that belongs to the group of "transmissible spongiform encephalopathies".

References

Aoki T, Gibbs CJ, Sotello J, Gajdusek DC. Heterogenic autoantibody against neurofilament protein in sera of animals with experimental kuru, Creutzfeldt-Jakob disease and natural scrapie infection. Infect Immun. 1982;38:316–24.

Ebringer A, Pirt J, Wilson C, Cunnigham P, Thorpe C, Ettelaie C. Bovine spongiform encephalopathy: is it an autoimmune disease due to bacteria showing molecular mimicry with brain antigens? Environ Health Perspect. 1997;105:1172–4.

Eylar EH, Caccam J, Jackson JJ, Westfall FC, Robinson AB. Experimental allergic encephalomyelitis: synthesis of disease producing site of the basic protein. Science. 1970;168:1220–3.

Husby G, Van de Rijn I, Zabriskie JB, Abdin ZH, Williams RC. Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. J Exp Med. 1976;144:1094–110.

Purdey M. Are organophosphate pesticides involved in the causation of bovine spongiform encephalopathy (BSE)? Hypothesis based on literature review and limited trials on BSE cattle. J Nutr Med. 1994;4:43–82.

Raine CS, Snyder DH, Valsamis P, Stone SH. Chronic experimental allergic encephalomyelitis in inbred guinea pigs. An ultrastructural study. Lab Invest. 1974;31:369–80.

Sotello J, Gibbs CJ, Gajdusek DC. Autoantibodies against axonal neurofilaments in patients with kuru and Creutzfeldt-Jakob disease. Science. 1980;210:190–3.

Tiwana H, Wilson C, Pirt J, Cartmell W, Ebringer A. Autoantibodies to brain components and antibodies to *Acinetobacter calcoaceticus* are present in bovine spongiform encephalopathy. Infect Immun. 1999;67:6591–5, 0019–9567.

Will RG, Ironside JW, Zeidler M, Cousens SN, Estibeiro K, Alperovitch S, Poser M, Pocchiari M, Hoffman A, Smith PG. A new variant of Creutzfeldt-Jakob disease in the U. K. Lancet. 1996;347:921–5.