

# Autoantibodies to Brain Components and Antibodies to *Acinetobacter calcoaceticus* Are Present in Bovine Spongiform Encephalopathy

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**Bovine spongiform encephalopathy (BSE) is a neurological disorder, predominantly of British cattle, which belongs to the group of transmissible spongiform encephalopathies together with Creutzfeldt-Jakob disease (CJD), kuru, and scrapie. Autoantibodies to brain neurofilaments have been previously described in patients with CJD and kuru and in sheep affected by scrapie. Spongiform-like changes have also been observed in chronic experimental allergic encephalomyelitis, at least in rabbits and guinea pigs, and in these conditions autoantibodies to myelin occur. We report here that animals with BSE have elevated levels of immunoglobulin A autoantibodies to brain components, i.e., neurofilaments ( $P < 0.001$ ) and myelin ( $P < 0.001$ ), as well as to *Acinetobacter calcoaceticus* ( $P < 0.001$ ), saprophytic microbes found in soil which have sequences cross-reacting with bovine neurofilaments and myelin, but there were no antibody elevations against *Agrobacterium tumefaciens* or *Escherichia coli*. The relevance of such mucosal autoantibodies or antibacterial antibodies to the pathology of BSE and its possible link to prions requires further evaluation.**

Bovine spongiform encephalopathy (BSE) is a recently discovered neurological disorder of cattle which was first reported in the United Kingdom after 1985, following a change in the preparation of "meat and bone meal" (MBM) feeds used especially during the winter months (1). The disorder has attracted public concern lest it be transmitted to humans following consumption of meat or other animal products (20). It has been suggested that BSE is caused by either abnormal prions (PrP<sup>sc</sup>) (11, 12) or exposure to organophosphates (13) and belongs to the group of transmissible spongiform encephalopathies (TSEs) together with kuru, Creutzfeldt-Jakob disease, and scrapie, conditions in which autoantibodies to brain neurofilaments have been described by Gajdusek's group (2, 15).

A characteristic histopathological feature of BSE is a spongiform appearance, which also occurs in chronic but not acute experimental allergic encephalomyelitis (10, 14), a condition in which autoantibodies to myelin occur (17), but its possible link to BSE has so far not been examined. A short sequence of bovine myelin (RFSWGAEGQK) resistant to denaturation by heating to 100°C for 1 h or by treatment with 8 M urea (a resistance which it shares with prion molecules) was reported over 25 years ago to produce ataxia, hind quarter paralysis, tremors, and eventually death following inoculation into guinea pigs (5). These features resemble, to some extent, those observed in cattle affected by BSE. This sequence was used as a computer probe to search protein databases for bacterial and viral proteins which may show molecular mimicry to bovine brain tissues. Analysis of proteins in databases (GenBank and SwissProt) revealed that three microbes showed molecular mimicry with brain tissues, the best one being found in 4-carboxy-muconolactone-decarboxylase of *Acinetobacter calcoace-*

*ticus* (4), a common saprophytic microbe found in soil and water supplies (19) which also possesses sequences resembling bovine neurofilaments (Table 1). Furthermore, another common environmental microbe, *Agrobacterium tumefaciens*, also showed some similarities to bovine myelin, although not to the same extent as *A. calcoaceticus*. Further probing with published prion sequences (7) revealed similarities with three molecules (recognition protein, colicin M, and maltodextrin-glucosidase), all of which are found in *Escherichia coli* (4).

BSE-affected cattle and healthy controls have been tested by enzyme-linked immunosorbent assay (ELISA) for the presence of autoantibodies to bovine neurofilaments and myelin and to these three common microorganisms (18). Since BSE was thought to be caused by consumption of MBM winter feeds, it was believed that the mucosal immunoglobulin A (IgA) isotype was more likely to show any possible differences in the titer of autoantibodies to brain components. Molecular modelling suggested three possible microbes which showed cross-reactivity, and these were tested by using a total Ig (IgG + IgA + IgM) assay in an endeavor to detect any immunological signal.

## MATERIALS AND METHODS

**Sera from animals with or without BSE.** Sera from 29 animals (mean age, 74.4 months; range, 44 to 122 months) which were found at postmortem to satisfy the criteria of having BSE and 18 animals which did not have the disorder were supplied by the Central Veterinary Laboratory (CVL) (New Haw, Addlestone, Surrey, England), 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 behavior involving ataxia and suggesting a neurological disease. Postmortem examinations were carried out to exclude BSE. The BSE and control sera (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.

**Sera from animals from an organic farm.** In addition, sera were obtained from an additional 58 healthy animals to act as extra controls: 30 serum samples from animals aged less than 30 months (8 Friesians and 21 Hereford-Friesian and 1

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TABLE 1. Comparison of similar sequences in bovine neurofilaments compared with *A. calcoaceticus* molecular sequences

Sequence <sup>a</sup>	Source <sup>b</sup>
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 .....	$\beta$ -Carboxy- <i>cis</i> -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)

<sup>a</sup> Sequences retrieved from the Protein Information Resource database release 44. Identical amino acids are shown in boldface.

<sup>b</sup> Amino acid positions in brackets.

Charolais-Friesian crossbreeds, the crossbreeds being raised for meat production) and 28 serum samples from animals aged more than 30 months, all of which were dairy Friesians. The animals were raised on a farm 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 annual herd testing for brucellosis.

**Bacterial cultures.** *A. calcoaceticus* (NCIMB 10694) and *A. tumefaciens* (NCIMB 9036) were obtained from National Collections of Industrial and Marine Bacteria, Ltd. (Aberdeen, Scotland), and *E. 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-liter flasks on an orbital shaker for 16 h at 37°C for *E. coli* and for 2 days at 30°C for *A. calcoaceticus* and *A. tumefaciens* in 200 ml of nutrient broth (Oxoid; 25 g/liter). 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 6,000 rpm for 20 min at 4°C (Beckman JA-20 rotor, six 250-ml cuvettes). The pellets of cells were then washed three times 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.** ELISAs were carried out as previously described (20). Briefly, ELISA plates were coated (5  $\mu$ g/well) with neurofilaments prepared from bovine spinal cord (Sigma), myelin basic protein obtained from bovine brain (Sigma), or bacterial suspension (200  $\mu$ l/well) overnight at 4°C, and the nonspecific sites were blocked with PBS containing 0.1% Tween and 0.2% ovalbumin (Grade III; Sigma), plates were washed, and a 1/200 dilution of test or control serum was added. The plates were incubated at 37°C for 2 h, were washed, and rabbit anti-bovine alpha-chain-specific horseradish peroxidase conjugate (1/3,000) (Bethyl Laboratories, Ltd.) or rabbit anticow Ig (IgG + IgA + IgM)-horseradish peroxidase conjugate (1/4,000) (Dako Ltd.) was added. The plates were reincubated for 2 h, were washed, and a substrate solution of 0.5 mg of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS; Sigma) per ml in citrate-phosphate buffer (pH 4.1) containing 0.98 mM H<sub>2</sub>O<sub>2</sub> (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 microtiter plate reader (Dynatech MR 600), and results were expressed as OD units  $\pm$  standard errors (SE). Each serum sample was tested in duplicate. All studies were 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 *A. calcoaceticus*.

**Absorption studies.** Serum samples from six animals with BSE and high antibody levels to *A. calcoaceticus*, bovine myelin, and neurofilaments were selected for absorption studies. A suspension of *A. calcoaceticus*, OD 1.60 at 540 nm, was sonicated using an MSE Soni prep 150 with a 1/2-in probe, amplitude 10 to 14, for five 1-min bursts. Serum samples (200  $\mu$ l) were absorbed with sonicated bacteria (25  $\mu$ l) in a plastic tube and were rotated gently overnight at 4°C. The absorption was repeated until the antibacterial antibody levels for each sample were below the mean value for healthy controls when measured by ELISA (mean OD  $\pm$  SE). Absorbed sera were then retested for reactivity against bovine myelin and neurofilaments, as previously described.

## RESULTS

**Measurement of autoantibodies to brain components.** Elevated levels of IgA autoantibodies to bovine neurofilaments (Fig. 1a) and bovine myelin (Fig. 1b) were found in the 29 animals with BSE (respective mean ODs  $\pm$  SEs,  $0.451 \pm 0.029$  and  $0.260 \pm 0.019$ ) when compared to 18 animals free of BSE ( $0.149 \pm 0.009$ ;  $P < 0.001$ ) ( $0.100 \pm 0.0012$ ;  $P < 0.001$ ), 30 organically raised cows less than 30 months of age ( $0.149 \pm 0.007$ ;  $P < 0.001$ ) ( $0.078 \pm 0.005$ ;  $P < 0.001$ ), and 28 organically raised cows greater than 30 months of age ( $0.157 \pm 0.006$ ;  $P < 0.001$ ) ( $0.078 \pm 0.005$ ;  $P < 0.001$ ).

Elevated levels of IgA antibodies to whole *A. calcoaceticus* bacteria (Fig. 1c) were found in the 29 BSE-affected cattle ( $0.737 \pm 0.022$ ) when compared to 18 animals free of BSE ( $0.416 \pm 0.024$ ;  $P < 0.001$ ), 30 organically raised cows less than 30 months of age ( $0.409 \pm 0.009$ ;  $P < 0.001$ ), and 28 organically raised cows greater than 30 months of age ( $0.432 \pm 0.029$ ;  $P < 0.001$ ). Absorption of BSE sera with sonicated *A. calcoaceticus* reduced autoantibodies to bovine myelin and neurofilaments almost to the levels found in control sera (Table 2), although some activity to neurofilaments remained.

**Measurement of antibacterial antibodies.** Antibodies to *A. calcoaceticus* of total Ig (IgG + IgA + IgM) were significantly elevated in the sera from animals with BSE ( $0.99 \pm 0.05$ ) (Fig. 2a) compared to CVL controls ( $0.65 \pm 0.06$ ;  $P < 0.001$ ) and organic farming controls, either in animals greater than 30 months of age ( $0.57 \pm 0.03$ ;  $P < 0.001$ ) or in animals less than 30 months of age ( $0.53 \pm 0.02$ ;  $P < 0.001$ ). There was no significant difference between the CVL controls and the organic farming controls aged more than 30 months, 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-*A. 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-*A. tumefaciens* (Fig. 2b) or anti-*E. coli* antibodies (Fig. 2c).

**Measurement of 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. 3a), bovine myelin (Fig. 3b), and *A. calcoaceticus* (Fig. 3c).

In each case, the high-titer serum reacted with a dilution of up to 1/6,400 of its respective antigen, whereas the medium- and low-titer sera gave lower readings.

## DISCUSSION

Elevated levels of autoantibodies to bovine neurofilaments and myelin, as well as elevated levels of specific antibodies to *A. calcoaceticus*, have been shown to be present in BSE-affected cattle when compared to three different groups of controls, whilst no such elevations have been seen against either *E.*

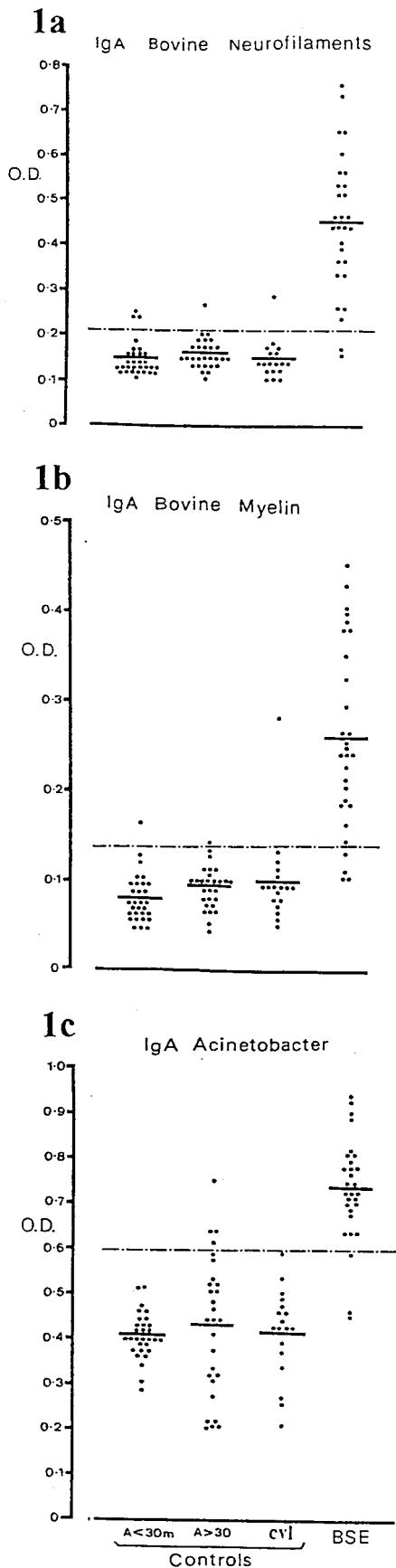


TABLE 2. Levels of IgA before and after ELISA absorption with bacteria (mean OD ± SE)

Source	IgA levels	
	Preabsorption	Postabsorption
<i>A. calcoaceticus</i>	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

*coli* or *A. tumefaciens*. This is clearly a specific observation, since the other two species of microorganisms tested did not show such elevations in their antibody levels. The agent responsible for the production of these specific autoantibodies is unclear, but it would seem that BSE cattle have been exposed to *A. calcoaceticus*. Whether this implies a link to 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 with the analysis carried out with different species of *Acinetobacter*. Furthermore, such sera should be tested against other bacteria commonly present in the bowel flora, as well as against peptides derived from the cross-reacting sequences resembling bovine neurofilaments, myelin, and other brain tissues.

*A. 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, and body secretions from both animals and humans. It is relevant to note that *A. 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 (4), and 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.

One clear result from these studies is that in at least one TSE disease, namely BSE, specific immune responses predominantly involving IgA, suggesting antigenic exposure across a mucosal surface such as the gut, can be demonstrated against a microbe that is found readily in the environment of cattle and which also happens to possess molecular sequences resembling bovine neurofilaments and myelin. Determinations of whether this microbe was introduced into the food chain of cattle following changes in the preparation of winter feeds or has any pathological significance in the development of BSE await further studies.

Autoantibodies to neuronal components have previously been reported in TSEs, especially in patients with kuru and Creutzfeldt-Jakob disease (15) and in animals with natural scrapie (2). 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 (*Streptococcus pyo-*

FIG. 1. IgA antibody titers (bar = mean) for 30 control serum samples from cows aged less than 30 months (A<30m), 28 control serum samples from cows aged more than 30 months (A>30m), and 18 control serum samples from cows not having BSE at postmortem compared to 29 serum samples from cows with BSE at postmortem when tested against bovine neurofilaments (a), bovine myelin (b), and *A. calcoaceticus* (c). Dashed line represents 95% confidence limits for mean of control as given by A<30M + A>30M - results of the one-tailed test.

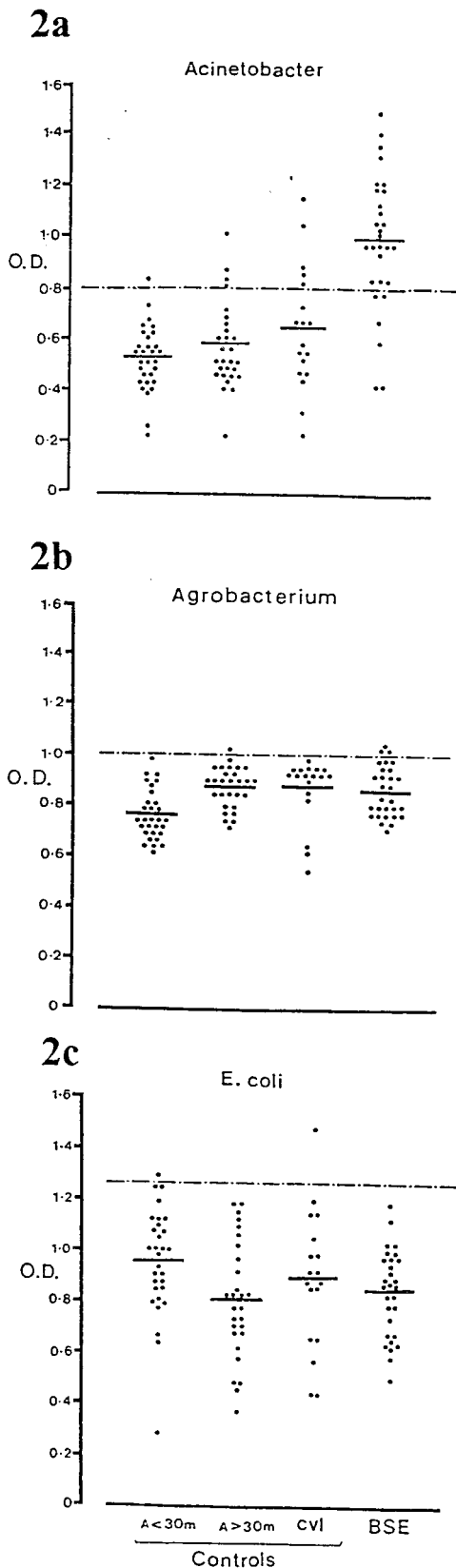


FIG. 2. Total antibody titers (bar = mean) for 30 control serum samples from cows aged less than 30 months (A<30m), 28 control serum samples from cows aged more than 30 months (A>30m), and 18 control serum samples from cows not having BSE at postmortem compared to 29 serum samples from cows with BSE at postmortem. Total antibody titers were measured against *A. calcoaceticus* (a), *A. tumefaciens* (b), and *E. coli* (c). Dashed line represents 95% confidence limits for mean of controls by the same formula as in the legend to Fig. 1.

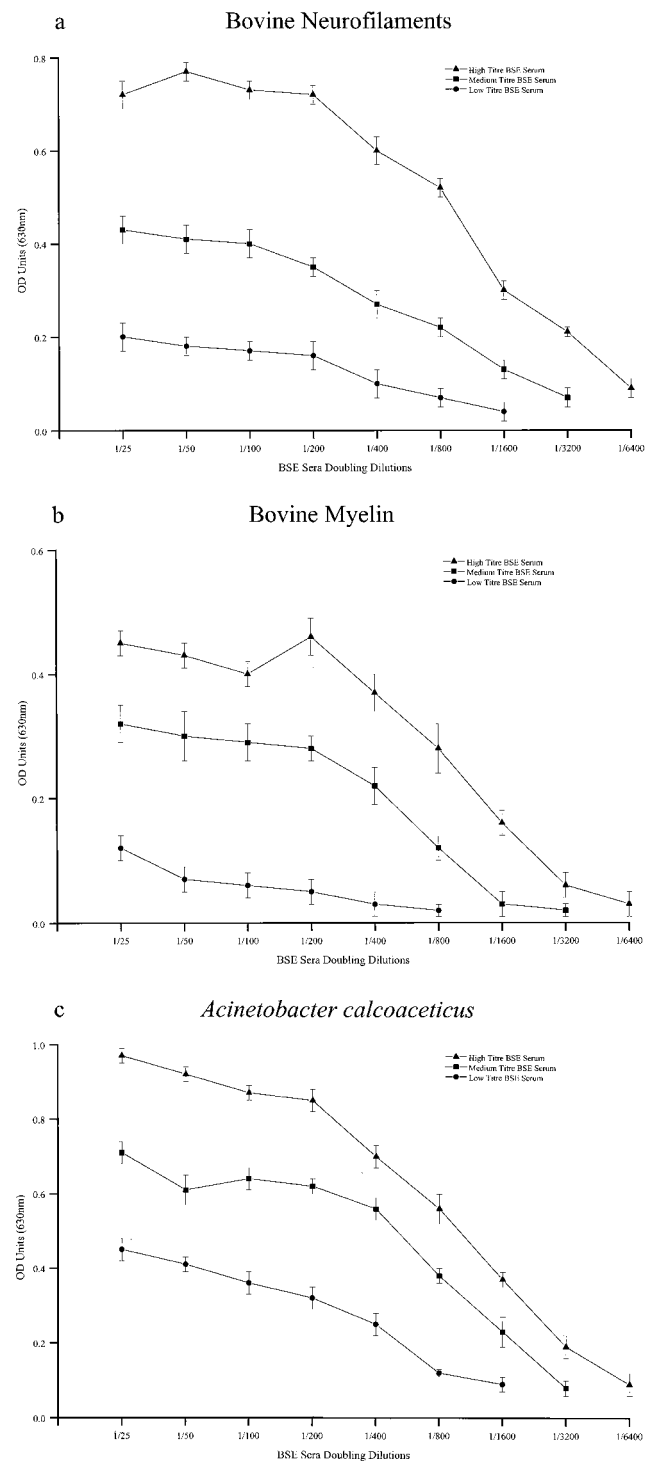


FIG. 3. Serial doubling dilutions (mean  $\pm$  SE) of high-, medium-, and low-reactivity BSE sera against bovine neurofilaments (a), bovine myelin (b), and *A. calcoaceticus* (c).

*genes*) (9), rheumatoid arthritis (*Proteus mirabilis*) (18, 21), and ankylosing spondylitis (*Klebsiella*) (3, 6).

Rheumatic fever is the classic model of an autoimmune disease caused by an infection. A bacterial infection of the tonsils by *S. pyogenes* evokes the formation of antibodies which bind to heart tissue, resulting in acute rheumatic fever, because there is molecular mimicry or similarity between cardiac tissues and streptococcal antigens. Furthermore, antistreptococcal an-



tibodies can also bind to the basal ganglia of the brain, thereby evoking abnormal gait movements, and this is known as rheumatic fever chorea or Sydenham's chorea (8). Injection of antistreptococcal antibodies into rabbits will produce abnormal neurological features of disordered gait and postmortem elution from the rabbit basal ganglia will lead to recovery of an antibody with specificity for streptococcal antigens.

A similar neurological disorder could occur in cattle with BSE following the production of anti-*A. calcoaceticus* antibodies, since this microbe possesses antigens resembling brain tissue. Another possibility is that these anti-*A. calcoaceticus* antibodies appeared following damage to brain tissues by prions, a situation that frequently occurs in patients with burns who develop antiskin antibodies or following a myocardial infarction, when anticardiac autoantibodies can be detected. A third possibility is that direct infection of brain tissues could occur, similar to the recent observation that *Chlamydia* microbes can be isolated from the cerebrospinal fluid of patients with multiple sclerosis (16). Further studies are required to determine whether anti-*A. calcoaceticus* antibodies exhibit cytotoxic responses against neurons, involving complement activation and NK cells, and to assess the possible relationships between normal (PrP<sup>c</sup>) and abnormal (PrP<sup>sc</sup>) prions, *A. calcoaceticus*, and brain autoantibodies in BSE. The mechanism responsible for these serological observations remains unclear, but at least these results confirm and extend the observations of Gajdusek's group that autoantibodies to brain components are present in TSEs.

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