Detection of Bovine Spongiform Encephalopathy-Specific PrPSc by Treatment with Heat and Guanidine Thiocyanate

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The conversion of a ubiquitous cellular protein (PrPC), an isoform of the prion protein (PrP), to the pathologically associated isoform PrPSc is one of the hallmarks of transmissible spongiform encephalopathies such as bovine spongiform encephalopathy (BSE). Accumulation of PrPSc has been used to diagnose BSE. Here we describe a quantitative enzyme-linked immunosorbent assay (ELISA) that involves antibodies against epitopes within the protease-resistant core of the PrP molecule to measure the amount of PrP in brain tissues from animals with BSE and normal controls. In native tissue preparations, little difference was found between the two groups. However, following treatment of the tissue with heat and guanidine thiocyanate (Gh treatment), the ELISA discriminated BSE-specific PrPSc from PrPC in bovine brain homogenates. PrPSc was identified by Western blot, centrifugation, and protease digestion experiments. It was thought that folding or complexing of PrPSc is most probably reversed by the Gh treatment, making hidden antigenic sites accessible. The digestion experiments also showed that protease-resistant PrP in BSE is more difficult to detect than that in hamster scrapie. While the concentration of PrPC in cattle is similar to that in hamsters, PrPSc sparse in comparison.

The detection of PrPSc by a simple physicochemical treatment without the need for protease digestion, as described in this study, could be applied to develop a diagnostic assay to screen large numbers of samples.

Materials and Methods

Animals and brain material. Brain material was derived from 15 BSE-affected Swiss cattle. The diagnosis was based on the finding of spongiform change and neuronal vacuolation in susceptible areas and on immunocytochemical demonstration of PrPSc accumulation in the brain stem. Control samples included samples derived from 20 age-matched normal cattle from a BSE-free country. Brain tissue from the fish Salmo trutta was used in some experiments as a negative control and for dilutions.

Preparation of brain homogenates. Fragments of brain tissue (≥0.5 g) were homogenized in 320 mM sucrose solution (10 mlg [wet weight of brain tissue]) with an Ultra-Turrax T25 apparatus (Janke and Kungel, Staufen, Germany). The homogenate was cleared by a short (5-min) centrifugation at 7,000 × g. For some experiments, the pellets were resuspended and homogenized again in the same sucrose solution at a volume corresponding to that of the supernatant. The total amount of protein in most samples was measured by the bicinchoninic acid reaction (Pierce, Rockford, Ill.).
Anti-PrP antibodies. For detection of PrP in Western blots and for ELISA, three different anti-PrP antibodies were used, one monoclonal antibody and two rabbit antisera. Monoclonal antibody 6H4 was derived from PrP-null mice immunized with recombinant PrP of the bovine sequence (Prionics Ltd., Zürich, Switzerland). It binds to an epitope in the center of the protease-resistant core (7). A rabbit antisera was raised against full-length recombinant PrP (R#26; supplied by Prionics Ltd.). A second rabbit antisera, C15S, was raised against a peptide of the bovine PrP sequence (2), GQGGTHGQWNKPS, located near the N terminus of the putative protease-resistant core of the bovine PrP molecule. All antibodies used in this study could detect PrPSc in immunocytochemistry (data not shown) and Western blot analysis (Fig. 1).

Preparation and testing of the peptide antibody. The peptide was coupled to ovalbumin or keyhole limpet hemocyanin by using the Imject activated immunoconjugation kit (Pierce) and purified by gel filtration as specified by the supplier. Several rabbits were immunized by subcutaneous injection of coupled keyhole limpet hemocyanin. For the first immunization, the protein concentration was adjusted to 1 mg/ml and the protein was emulsified in a 1:2 ratio with complete Freund adjuvant. For the following four boosters, the protein was mixed 1:2 with incomplete Freund adjuvant.

The antisera were assessed by testing various dilutions in ELISA plates. The plates (Nunc-Immunoplate MaxiSorp; Nunc, Roskilde, Denmark) were coated by overnight incubation (4°C) with 200 ng of peptide-coupled ovalbumin per well in carbonate buffer (15 mM Na2CO3, 35 mM NaHCO3, 0.02% NaN3 [pH 9.6]). The plates were then blocked with 0.2 ml of 10% dry milk in RPB buffer (13.7 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 8.1 mM NaHPO4 [pH 7.3]) plus 0.01% Tween 20 (RBP-Tween) per well for 1 h at 37°C. The antisera were serially diluted in RPB-Tween containing 3.3% dry milk and added to the plates (100 μl/well). After incubation for 1 h at 37°C, the plates were washed with RPB-Tween and the antibodies bound were detected by incubation with a horse-radish peroxidase (HRP)-conjugated swine anti-rabbit antibody (Dako, Glostrup, Denmark) diluted 1:300 in phosphate-buffered saline–Tween (137 mM NaCl, 2.7 mM KCl, 1.4 mM KH2PO4, 8.1 mM NaHPO4 [pH 7.3]) containing 0.01% Tween 20 and 3.3% dry milk. After incubation for 1 h at 37°C, the plates were washed again with phosphate-buffered saline–Tween and filled with 0.2 ml of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) solution (Boehringer, Mannheim, Germany) per well for reaction with HRP. Oxidation of the ABTS substrate resulted in a change in the optical density, which was measured with an ELISA plate reader at 405 nm. Only antisera which had a half-maximal binding at dilutions of at least 1:16,000 were used.

Western blotting. Western blotting was carried out as specified for the Prionics BSE/scrapie test kit. Briefly, samples were first separated on sodium dodecyl sulfate–12.5% polyacrylamide gels and then blotted on polyvinylidene difluoride membranes (Millipore). The membranes were then blocked for 1 h in blocking buffer (Tropix, Bedford, Mass.). The first and second antibodies were diluted 1:1,000 to 1:5,000 in blocking buffer and successively incubated with the membranes after thorough washing with TBS-Tween (25 mM Tris base, 137 mM NaCl, and 0.1% Tween 20) containing 0.05% Tween 20. Second antibodies were labeled with either HRP or alkaline phosphatase. Detection was carried out with a chemiluminescent substrate, either ECL (Amersham) or CDP-Star (Tropix), according to the provider.

ELISA for PrP. ELISA plates were coated by overnight incubation with 0.1 ml of monoclonal antibody 6H4 diluted 1:100 in carbonate buffer at 4°C per well. The plates were blocked with 0.2 ml of RPB-Tween containing 10% dry milk per well for 1 h at 37°C and then incubated with 0.1 μl of bovine brain homogenate diluted 1:2 in RPB buffer per well in triplicate for 1 h at 37°C. After being washed with RPB-Tween, the bound PrP was quantified by successive incubation with two other antibodies, a rabbit PrP antibody (C15S or R#26) and an HRP-conjugated swine anti-rabbit antibody (each for 1 h at 37°C). The rabbit PrP antisera was diluted 1:500 in RPB-Tween containing 3% dry milk, the swine anti-rabbit antibody was diluted 1:300 in TBS-Tween containing 3% dry milk, and 0.1 ml/well was applied. After incubation, the plates were washed with PBS-Tween and filled with 0.2 ml of ABTS solution per well for reaction with HRP.

Quantification of the ELISA results. To test linearity, samples of bovine brain homogenates from healthy control animals were diluted with brain homogenates from the fish Salmo trutta, which has no proteins cross-reacting with the antibodies mentioned above. To calibrate the assay, different concentrations of recombinant PrP (Prionics) were mixed with fish brain homogenate and measured again in the ELISA with R#26 as a second antibody. The concentration of purified recombinant PrP was 3.07 ± 0.16 ng/ml (n = 3) as measured by the BCA protein assay (Pierce). It was diluted to 172 ng/ml. Appropriate volumes were further diluted with fish brain homogenate and pipetted into the wells of a coated immunoplate to an end concentration of 0.5 to 4 ng of recombinant PrP in 100 μl. A standard of bovine brain was equally diluted, and samples of 100 μl each containing 5 to 50 μl of the homogenate were distributed to other wells of the same plate (see Table 1). The PrP content of the bovine brain standard was calculated in terms of recombinant PrP from the slope of the two linear curves obtained from the optical density (OD)-concentration relationship. The values obtained for the concentration of PrP in this specific brain homogenate were 79 ng/ml in a first experiment (slope for recombinant PrP, 0.6686 OD unit/ng, and slope for the brain homogenate, 0.05267 OD unit/μl) and 78 ng/ml in a second (0.7484 and 0.0585 OD unit/μl). The concentrations of all other brain homogenates were obtained by calibration with this standard.

To create a standard, the bovine brain homogenate was diluted with fish brain homogenate to a concentration corresponding to 75 ng/ml. A 100-μl/well concentration of a 1:2 dilution of this standard in RPB buffer was included in each plate to determine the length of the HRP reaction and to allow quantification of unknown samples. The OD of the plates was read when the standard reached an OD between 0.900 and 1.100. To allow comparison of the results of different plates, zero values were subtracted first and then all results were divided by the value of the standard. Its OD (75 ng of PrP/ml) became 1.000 at this step.

A quadratic equation was fitted to the nonlinear dose-response curve of the peptide antibody C15S. The formula finally used for calculation in Microsoft Excel was OD = 1.887 x POWER(SORT(0.006 x + 1)−1/2.042,2), where x is the concentration of PrP in nanograms per milliliter.

Validation of the ELISA. To calculate the detection limit, the average standard deviation of the blank (0.003 ± 0.001, n = 24) was used. Accordingly, an OD of 0.006 ± 0.003 (average of the three wells of a plate) was found to be significantly different from zero, which corresponded to a detection limit of 4 ng/ml. The upper limit was given by the capacity of PrP binding of 6H4 bound to the plates (a 1:100 dilution) and was found to be about 6 ng/well, corresponding to 120 ng/ml.

The interassay variation coefficient (reproducibility, 100 ± 0.001, n = 3) as measured by the BCA protein assay (Pierce) was 1.9 ± 0.001. The upper limit was given by the capacity of PrP binding of 6H4 bound to the plates (a 1:100 dilution) and was found to be about 6 ng/well, corresponding to 120 ng/ml. The interassay variation coefficient (reproducibility, 100 ± 0.001, n = 3) as measured by the BCA protein assay (Pierce) was 1.9 ± 0.001. The upper limit was given by the capacity of PrP binding of 6H4 bound to the plates (a 1:100 dilution) and was found to be about 6 ng/well, corresponding to 120 ng/ml.

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RESULTS

Development of a PrP-specific ELISA. A quantitative PrP-specific immunocapturing ELISA was developed. The plates were coated with a PrP-specific monoclonal antibody (6H4) and incubated with cleared bovine brain homogenate. In a first step, the proteins bound to the plates were removed for analysis. PrP could be identified in Western blots.

For detection of PrP bound to the plate, one of two PrP-specific rabbit antisera was used as a second antibody. One (R#26) was raised against recombinant PrP, and the other (C15S) was raised against a synthetic peptide of the bovine sequence (see Materials and Methods). A PrP fraction could be detected in samples from both BSE-affected animals and in controls, independent of the second antibody used. The PrP fraction was identified as PrP<sup>C</sup> by protease digestion and ultracentrifugation (see below). Animals with BSE could not be distinguished from controls by ELISA of untreated preparations.

Heat treatment in the presence of GdnSCN exposed epitopes of BSE-specific PrP. The effect of GdnSCN on PrP detection was explored by adding GdnSCN to a standard of bovine brain homogenate. When C15S was used as the second antibody, little change in OD was observed for up to 0.1 M GdnSCN in comparison to a control. However, at 0.25 M GdnSCN, the OD was reduced by about 50%, and it was further decreased with increasing GdnSCN concentrations. No detection of a BSE-specific PrP fraction was achieved by adding GdnSCN alone (without subsequent dilution) (Fig. 2A).

To explore the effect of heat, brain homogenates were incubated for various times at 150°C (temperatures below 100°C had no effect) and the resulting OD was measured in the ELISA. The result was compared to the optical density of the corresponding unheated sample. Only reduction but no increase in the optical density could be observed by heat treatment alone (Fig. 2B).

The heat experiments were repeated in the presence of 0.1 M GdnSCN (Gh treatment). In heat-exposed samples from BSE-affected animals, a large increase in OD was detected, which reached a maximum at an incubation time of about 10 min (Fig. 2C). In some experiments, the signal was up to three times stronger than the OD of the unheated control.

The BSE-specific OD signal (i.e., PrP<sup>Sc</sup>; see below) could be detected only by using the peptide antiserum C15S as second antibody. R#26, raised against the recombinant protein, caused no difference in OD. In addition, no equivalent increase was obtained when other denaturing agents were present in addition to GdnSCN, e.g., Pefabloc or 0.05% sarcosine, as used for scrapie-associated filament (SAF) preparations.

Quantification of PrP<sup>Sc</sup>. When serial dilutions were measured, R#26 showed a linear dose-response curve (Table 1) and the peptide antiserum showed a nonlinear one. The nonlinear dose-response curve was obtained both with and without heat treatment (Fig. 3). It was not influenced by varying the antisem concentration. The cause of this nonlinearity is under investigation.

The assay was calibrated by comparing the ODs of different dilutions of a standard brain homogenate with that of recombinant PrP of known concentration (Table 1). The standard was included in all plates and used for calculation of the concentration of unknown samples. Compared to recombinant PrP, PrP<sup>Sc</sup> was less susceptible to spontaneous degradation and therefore was preferred as a standard. A quadratic equation was fitted to the nonlinear dose-response curve observed with C15S (see Materials and Methods). Thus, calculation of the concentration of both PrP<sup>P</sup> and PrP<sup>Sc</sup> was based on recombinant PrP.

Characterization of PrP<sup>P</sup> and PrP<sup>Sc</sup>. PrP<sup>Sc</sup> became obvious by a dramatic increase in OD following Gh treatment. For characterization of this PrP, samples of brain stem homogenates from BSE-affected animals were ultracentrifuged to sediment protein aggregates and cellular organelles. Samples of pellet and supernatant were Gh treated and analyzed by ELISA. All of PrP<sup>P</sup>, together with about half of the total PrP<sup>C</sup>, was found in the pellet fraction; i.e., PrP<sup>Sc</sup> either had been aggregated or was associated with cellular organelles before treatment (Table 2). In some experiments, the homogenates were digested with proteinase K before being subjected to Gh treatment. In samples from control animals, all of the protease-sensitive PrP measured by the ELISA disappeared with 20 μg of proteinase K per ml. In samples from BSE-affected animals, less than half of the initial PrP was digested when using 20 μg of proteinase K per ml, but the PrP disappeared proportionally when more proteinase K was added (Fig. 4). The samples were also analyzed by Western blotting. Protease-resistant PrP could be observed in proteinase K-digested fractions of all samples from BSE-affected animals (Fig. 1, lanes f to h) but not in control samples (lanes k to m).

PrP<sup>P</sup> and PrP<sup>Sc</sup> in different brain areas of BSE-affected and control animals. The PrP content of the medulla oblongata, thalamus, and cerebral cortex of several BSE-affected animals and controls was measured (Table 3). High animal-to-animal variation in both PrP<sup>P</sup> and PrP<sup>Sc</sup> was found. These variations were scarcely reduced when the PrP content was based on total protein, as measured by the BCA reaction.

A significant amount of PrP<sup>Sc</sup> was detected in the medulla oblongata of all BSE-affected animals used in this study. Its use as a BSE assay was investigated. For each animal, the OD difference between treated and untreated samples was calculated and results from 15 BSE-affected animals and the 20 negative controls obtained from a BSE-free country were compared. The range was 0.102 to 0.668 for BSE and ~0.214 to 0.077 for controls. We found that 83% of the control animals had either a negative difference or a value not significantly different from zero. Four control animals (17%) had values.
between 0.051 and 0.077. All of the BSE-affected animals had differences larger than 0.1, with no values overlapping (Fig. 5). Accordingly, all BSE-affected animals used in this study could be identified by the OD difference. However, the sample size was small and samples were not blinded.

A different result was obtained for the thalamus and cortex (Table 3). PrP<sup>Sc</sup> could be detected in only 20% of the samples obtained from the thalamus of BSE-affected animals. All other samples could not be distinguished from controls (Table 3). No PrP<sup>Sc</sup> was found in the cortex.

To improve sample handling and to reduce standard deviations, samples had been cleared by a short centrifugation step prior to the ELISA; i.e., some of the PrP was lost by this procedure. To estimate the extent of this fraction, pellets were resuspended and analyzed for PrP. On average, 49% ± 10% (n = 55) of total PrP could be recovered by this procedure. When corrected for this loss, the amount of PrP<sup>Sc</sup> detected in bovine brain was about the same as reported for hamster brain (1 to 5 μg/ml) (22) but the amount of PrP<sup>Sc</sup> was much smaller (0.5 to 1 μg/ml compared to 10 to 40 μg/ml in hamster brain).

FIG. 2. Effect of GdnSCN and heat on PrP<sup>Sc</sup> detection. (A) Increasing amounts of GdnSCN were added to samples of brain homogenate derived from either BSE-affected animals or unaffected controls. All samples were measured in the ELISA, and the resulting OD, given as a percentage of the untreated control, is plotted against the GdnSCN concentration. (B) Samples of brain homogenate from BSE-affected animals or controls were incubated for the indicated time (in minutes) at 150°C. (C) The experiment as described in panel B was repeated with samples containing 0.1 M GdnSCN. The data with standard error bars represent the mean of two independent experiments.
DISCUSSION

For the development of an ELISA to detect BSE, we used three different antibodies against bovine PrP in a variety of binding studies on normal and BSE-affected bovine brain tissues. All three antibodies were found to bind to PrP and its protease-resistant isoform PrP\text{Sc} following denaturing procedures as required for Western blotting (Fig. 1). They also bound to native PrP in fresh brain tissue, but we did not detect a significant quantitative difference between normal controls and BSE-affected animals in cleared untreated tissue homogenates. Very limited binding of PrP\text{Sc} to the first or second antibody used in the ELISA could explain this lack of difference between normal and BSE-affected tissue in native untreated preparations. Similar observations have been described before for other PrP antibodies (22, 26).

Following heat and GdnSCN treatment of the fresh tissue homogenate, a much stronger ELISA signal was obtained in the animals with BSE than in the controls. Thus, the treatment revealed a PrP fraction specific for BSE. To investigate whether this PrP fraction was related or identical to PrP\text{Sc}, ultracentrifugation and protease digestion studies with BSE-affected samples were performed. The PrP fraction detected by our ELISA did sediment by ultracentrifugation. This indicated that PrP was present either as large aggregates or complexed to cellular organelles. Both possibilities are characteristic of PrP\text{Sc}, which is either aggregated to SAFs or associated with the microsomal membranes (11). SAF formation is most effective in the presence of detergent (10), which in our ELISA interfered with PrP detection. In this respect, our brain homogenates were different from the detergent-treated brain fractions used for SAF isolation and were likely to contain little SAF. Digestion with 20 \(\mu\)g of proteinase K per ml destroyed much of the PrP\text{C} but not the PrP revealed by Gh treatment (Fig. 4). Protease-resistant PrP\text{Sc} could be demonstrated by Western blots in this fraction (Fig. 1, lane h). Both protease resistance and the relative amount of PrP\text{Sc} in BSE are limited compared to scrapie in hamsters (22). In BSE-affected tissue, 23\% \(\pm\) 13\% \((n = 4)\) of PrP\text{Sc} disappeared when the proteinase K concentration was increased from 20 to 100 \(\mu\)g/ml (Fig. 4).

Immunoprecipitation studies with a PrP\text{Sc}-specific antibody revealing multiple forms of disease-specific PrP with different sensitivities to proteinase K had been described (7). The ratio of protease-sensitive to protease-resistant pathological PrP has been used recently for characterization of a particular TSE strain (22). Our results suggest that the epitopes of PrP\text{Sc}, which are recognized by one or several of the antibodies used in our assay, become accessible only following Gh treatment. This could perhaps be related to complexing of PrP\text{Sc} with other fractions (14) or to a different conformation from PrP\text{C} (13). Significant conformational transitions and unfolding of PrP\text{Sc}...
by concentrated GdnHCl or GdnSCN with simultaneous loss of infectivity are well documented (19, 20). Hidden epitopes of PrP<sub>Sc</sub> could be exposed by treatment with GdnSCN (16). Treatment with GdnSCN had the additional advantage of PrP<sub>Sc</sub> solubilization (21). We compensated for the much lower concentration of GdnSCN (0.1 versus 4 M) used in our experiments by heating. Thus, the treatment increased the solubility of PrP<sub>Sc</sub> and reversed, at least partially, its folding and complexing, making hidden antigenic sites accessible (7).

The antibody R#26, which was raised against full-length PrP, did not reveal an increased signal following treatment. This indicates either that the chosen treatment was not sufficient to fully expose epitopes recognized by R#26, implying that Gh-treated PrP<sub>Sc</sub> is not completely reversed to PrP<sub>C</sub>, or that R#26 could immunodetect most if not all of the PrP present in the untreated BSE brain homogenates and further denaturation exposed no additional epitope(s).

The detection of protease-resistant bovine PrP<sub>Sc</sub> is difficult without extensive extraction and centrifugation (4). For diagnostic purposes, optimal incubation conditions have to be elaborated to minimize unintentional digestion of PrP<sub>Sc</sub> while eliminating PrP<sub>C</sub>. In contrast to Western blotting, in which both isoforms can be distinguished by size, any remaining PrP<sub>C</sub> could interfere with the diagnosis by ELISA. The Gh treatment approach had the advantage of not depending on protease digestion for discrimination of PrP<sub>Sc</sub> from PrP<sub>C</sub>. All BSE-affected animals used in this study could be distinguished from controls solely by the amount of their PrP<sub>Sc</sub>. The amounts of both PrP<sub>Sc</sub> and PrP<sub>C</sub> vary depending on the anatomic location of the sample, as described for experimentally infected Syrian golden hamsters (24). The former is restricted to well-confined neuroanatomic areas; the latter depends on the amount of gray matter in a sample, since PrP is expressed mostly in neurons. Our experiments show little effect of Gh treatment on samples of cortex or thalamic nuclei. Thus, the sample size and sample location must be optimized to reduce individual variations and to increase sensitivity.

We believe that the detection of PrP<sub>Sc</sub> by a simple physicochemical treatment, as used in this study, could be applied to develop a diagnostic assay for TSE. Trials to test large numbers of samples to validate the diagnostic usefulness of the technique are in progress. The detection of BSE and perhaps other TSE syndromes by ELISA may simplify mass screening by allowing automation.

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TABLE 3. Average concentration of PrP protein in cleared bovine brain homogenates as measured by ELISA using C15S as the second antibody

<table>
<thead>
<tr>
<th>Brain sample</th>
<th>PrP concn (ng/ml)&lt;sup&gt;a&lt;/sup&gt; for:</th>
<th>Differences (PrP&lt;sub&gt;Sc&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td>Gh treated (PrP&lt;sub&gt;Sc&lt;/sub&gt; and PrP&lt;sub&gt;C&lt;/sub&gt;)</td>
<td>Untreated (PrP&lt;sub&gt;C&lt;/sub&gt;)</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>51.7 ± 18.1 29.7 ± 11.7 21.9 ± 12.3</td>
<td>24.8 ± 15.4 24.1 ± 16.4 0.6 ± 3.5</td>
</tr>
<tr>
<td>BSE (n = 15)</td>
<td>Controls (n = 25)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.8 ± 12.7 69.9 ± 11.7 &lt;0</td>
</tr>
<tr>
<td>Thalamus</td>
<td>70.7 ± 12.6 72.8 ± 13.8 &lt;0</td>
<td></td>
</tr>
<tr>
<td>BSE (n = 15)</td>
<td>Controls (n = 20)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>71.2 ± 11.5 79.0 ± 7.2 &lt;0</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>68.5 ± 7.0 73.3 ± 6.0 &lt;0</td>
<td></td>
</tr>
<tr>
<td>BSE (n = 5)</td>
<td>Controls (n = 5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.5 ± 7.0 73.3 ± 6.0 &lt;0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± standard deviation.

<sup>b</sup> Healthy controls from a BSE-free country.

REFERENCES


