Neurobiology of Disease **11,** 386–393 (2002)
doi:10.1006/nbdi.2002.0558

Astrocytes Accumulate 4-Hydroxynonenal Adducts in Murine Scrapie and Human Creutzfeldt–Jakob Disease

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Received July 21, 2002; revised September 18, 2002; accepted for publication September 25, 2002

Scrapie-infected mice are considered a model for study in prion diseases, which are characterized by the progressive accumulation in the brain of an abnormal isoform (PrPsc) of the normal cellular prion protein (PrPc). Increasing data suggest that the neurodegenerative process in prion diseases may result, at least partially, from a defect in antioxidant function, but so far *in vivo* oxidative stress remains poorly documented. We report here that 4-hydroxynonenal, a lipid peroxidation by-product, forms protein adducts in brains of scrapie-infected mice and of Creutzfeldt–Jakob disease affected patients. In scrapie mice, studies on the progression of PrPsc accumulation, glial activation, ubiquitin deposition, and 4-HNE adduct formation allowed us to conclude the late occurrence of oxidative damage in the course of the disease. Massive 4-HNE accumulation was identified in astrocytes, but not in neurons or microglial cells. These findings suggest an important oxidative stress (and subsequent lipid peroxidation) in astrocytes, with possible consequences on their neuronal trophic function.

INTRODUCTION

Creutzfeldt–Jakob disease (CJD) in humans and experimental scrapie in rodents are neurodegenerative disorders known as prion diseases. According to the theory formulated by Prusiner, these diseases are caused by nonconventional infectious agents, corresponding to the pathological form (PrPsc) of the widely expressed normal host prion protein (PrPc) (Prusiner, 1982). The disease process is characterized by the accumulation of this altered form in the brain (Lantos *et al.*, 1992), associated with astrogliosis, spongiosis, and neuronal apoptosis (Hafiz and Brown, 2000).

Prion protein expression is a prerequisite for the development of the disease since PrPc knockout ($PRNP^{-/-}$) mice are resistant to scrapie infection (Bueler *et al.*, 1993). Although the precise physiological functions of PrPc remain unclear, accumulating data indicate that this protein may play a role in redox homeostasis (Hornshaw *et al.*, 1995; Brown *et al.*, 1997). *In vivo* studies indicate that $PRNP^{-/-}$ cerebellar cells exhibit a greater sensibility to oxidative stress when compared to wild-type cells (Wong *et al.*, 2001c). Moreover, PrPc antioxidant activity could be impaired when the isoform PrPsc accumulates in neurons (Milhavet *et al.*, 2000). These data suggest an important role for oxidative stress in prion disease pathogenesis, as reported in other neurodegenerative diseases (Mark *et al.*, 1997; Markesbery and Carney, 1999). Recently,

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0969-9961/03 $35.00

3-nitrotyrosine accumulation, a consequence of the irreversible nitration of tyrosine residues by peroxynitrite, in brain from scrapie-infected mice at the terminal stage of the disease was reported, thus confirming the occurrence of *in vivo* oxidative damage (Guentchev *et al.*, 2000). However, no information is currently available about the temporary occurrence of oxidative stress nor are damaged cellular subsets identified.

Oxidative stress in several neurodegenerative diseases results in lipid peroxidation products such as 4-hydroxynonenal (4-HNE), acrolein, or malondialdehyde (Esterbauer *et al.*, 1991). These compounds covalently react with nucleophilic side-chains of some amino acid residues (lysine, cysteine, and histidine) (Uchida and Stadtman, 1993) which alter cellular protein functions (Mark *et al.*, 1997). 4-HNE is one of the most bioactive aldehydic by-products and may potentiate neuronal oxidative stress and induce cell activation or death (depending on the cell type and 4-HNE concentration) (Esterbauer *et al.*, 1991).

In this study we investigated *in vivo* lipid peroxidation (4-HNE accumulation) in brains from scrapie-infected mice and human sporadic CJD cases. We (i) established a spatial and chronological scheme of PrPsc accumulation, glial cell activation, and 4-HNE accumulation, but also (ii) identified astrocytes as the main 4-HNE-accumulating cells. Consequences of astrocyte impairment and a potential role for 4-HNE in the pathogenesis of prion diseases are discussed.

MATERIALS AND METHODS

Mice and TSE Agent Strain

Eight- to 12-week-old C57/BL mice, maintained under pathogen-free conditions, were inoculated intracerebrally (ic) with 20 μl of a 1% (W/V) brain homogenate in 5% glucose solution from terminally scrapie-affected mice inoculated with the mouse-adapted C506-M3 scrapie strain. Negative control mice were inoculated under the same conditions with a normal brain suspension. Mice were sequentially sacrificed by carbon dioxide suffocation at different time points: 38, 54, 86, 112, 140, and 155 days postinoculation (dpi). In addition, a few mice were allowed to live until the terminal stage of the disease (160 dpi.). At each time point, brains were formalin fixed before being paraffin embedded ($n = 3$ infected + 3 controls) or snap-frozen in liquid nitrogen and stored at −80°C ($n = 4$ infected + 2 controls). Immunolabeling and observations were performed on coronal brain sections at the diencephalon level.

Sporadic Creutzfeldt–Jakob Disease Cases

Five brains from patients who died from sporadic CJD between 1997 and 2001 were collected, kept at −80°C, and used for 4-HNE immunostaining. Two brains from patients who died from nonneurodegenerative diseases were used as negative controls. Formalin-fixed, paraffin-embedded tissues from the same patients were examined for PrPsc accumulation and glial activation. Immunolabeling was performed on sections from the frontal cortex region.

Immunohistochemistry

Paraffin-embedded tissue sections were first incubated in 98% formic acid (Merck) for 30 min at room temperature and then autoclaved for 30 min at 121°C in 10 mM citrate buffer (pH 6.10). A mouse monoclonal antibody (mAb) 2G11 (IgG2a, 1:350 ascites dilution, kindly provided by J. Grosclaude, INRA-VIM, Jouy en Josas) was used for PrPsc labeling in mice while 12F10 mouse mAb (IgG2a, 1:5000 ascites dilution, kindly provided by J. Grassi, CEA Saclay) detected PrPsc accumulation within human tissues.

All other immunolabelings were performed using frozen sections. Endogenous peroxidase activity was eliminated by incubating sections with 0.3% H_2O_2 in PBS for 30 min, and nonspecific binding sites were blocked with 20% goat serum in PBS for 20 min. Neurons were identified with a mouse mAb raised against the DNA-binding, neuron-specific protein NeuN (Chemicon, MAB377, 1:8000 IgG1 purified dilution). Astrocyte proliferation and activation were evaluated using the mouse mAb anti-glial fibrillary acidic protein (GFAP) (Sigma, G3893, 1:8000 ascites dilution). Ubiquitin deposits were characterized with a pAb (Dako, Z 0458, 1:700 diluted). Microglial activation was detected using a rat mAb anti-F4/80 (Serotec, MCAP497, 1:50 IgG purified dilution). Finally, 4-HNE accumulation in mouse and human frozen brain tissues was investigated using 4-HNE rabbit pAb (Calbiochem, 393205, 1:250 serum dilution). Secondary antibody consisted of (i) peroxidase-conjugated EnVision+ reagent (Dako, goat anti-mouse, K4001, or goat anti-rabbit, K4003) when the primary antibody was produced in mouse or rabbit, respectively, or (ii), a biotinylated secondary antibody anti-rat immunoglobulin (IgG) (Dako, E 0468) was applied before incubation with a streptavidin–peroxidase complex (Dako, P 0397) when it was produced in the rat. Revelations were performed with 3,3′-diaminobenzidine (DAB) as chromogen (brown final product).

In mouse sections, labeling intensity was scored using a semi-quantitative scale (from 0 to 5) by two independent pathologists.

Double immunohistochemical stainings were performed using immunofluorescence techniques. Briefly, mouse frozen sections were first incubated with anti-4-HNE pAb and (i) anti-NeuN mAb, (ii) anti-GFAP mAb, or (iii) anti F4/80 mAb. Revelation was subsequently performed using a rhodamine-conjugated anti-rabbit IgG (Rockland, 611-100-122) and (i and ii) FITC-conjugated anti-mouse IgG (Rockland, 610-102-121) or (iii) a biotinylated anti-rat IgG followed by an incubation with fluorescein-conjugated streptavidin (Dako, F 0422, 1/100 diluted)

Each immunohistochemical run included a negative serum control in which the primary antibody was omitted or replaced by a normal rabbit or mouse serum to characterize nonspecific immunolabeling. For double-immunohistochemical stainings, cross-reactivity control was performed to check the absence of interspecies reactivity of secondary antibodies to primary antibodies. The absence of a possible affinity between secondary antibodies was also checked.

RESULTS

PrPsc Immunoreactivity and Glial Cell Activation in Mice

In infected mice, PrPsc was detected as early as 38 dpi at the inoculation point, in the right dorsal region of the thalamus (Fig. 1). PrPsc staining first spread asymmetrically to the hippocampus (54 dpi) and then to the piriform area and cortex at 86 dpi. At 112 dpi, an intense and widespread PrPsc distribution was observed, which still increased at 140 and 155 dpi (Figs. 1 and 2A). Microglial activation, as demonstrated by F4/80 labeling, was delayed in time compared to PrPsc deposition but showed a similar pattern. A significant increase in intensity of F4/80 immunostaining was mostly found at 140 and 155 dpi (Figs. 1 and 2C). GFAP staining was also linked and delayed compared with that of PrPsc and found to be intense in the late stage of the disease (Figs. 1 and 2D).

4-HNE Detection in Scrapie-Infected Mice

4-HNE labeling was detectable only in the late stages of the incubation period (after 112 dpi). The extent of 4-HNE immunostaining correlated with that of microglia and astrocyte activation regions (Fig. 1). At 140 dpi, the most intense spider-like staining, strongly resembling astrocytes, was found in the hippocampal formation (Figs. 1, 2E, and 2F). Nevertheless, a weaker but significant staining was also detectable within some cortical regions. At 155 dpi, 4-HNE immunoreactivity increased in intensity and distribution and was widespread through the whole cortex. Double labeling demonstrated that positive 4-HNE cells (Figs. 3B, 3E, and 3H) were exclusively astrocytes (Fig. 3F), and neither neurons (Fig. 3A) nor microglia (Fig. 3G).

4-HNE Detection in Human Sporadic CJD

In sporadic CJD brain sections, the pattern of PrPsc deposits in the frontal cortex (Fig. 4A) strongly resembled that of ubiquitin, astrocytosis, and microgliosis. 4-HNE-positive cells were detected in brains from sporadic CJD (Fig. 4B) but not in controls (Fig. 4C). The 4-HNE labeling was mainly located in neuron-surrounding cells, suggesting that these cells were subjected to oxidative damage.

Ubiquitin Deposits in Humans and Mice

Ubiquitin immunoreactivity in infected mice was detected long before 4-HNE deposition, in a chronological and spatial pattern similar to that of PrPsc (Fig. 1). The labeling appeared granular, mainly located in the neuropil, within spongiotic and PrPsc accumulating areas, in both mouse (Fig. 2B) and humans (data not shown). Ubiquitin immunoreactivity was observed within neuronal and glial cells. No obvious morphological changes were associated with these deposits.

DISCUSSION

The findings reported here demonstrate that a lipid peroxidation product, 4-HNE, is generated in the brains of scrapie-infected mice and of CJD patients, which provides further evidence for the occurrence of an *in vivo* oxidative stress in prion diseases. To our knowledge, there are very few studies reporting the accumulation of such bioactive and neurotoxic lipid peroxidation product in the course of prion disease (Wong *et al.*, 2001a,b).

Our results clearly show that PrPsc deposition and glial activation patterns are linked in a chronological and spatial manner, which is consistent with previous results indicating that PrPsc protein is localized in

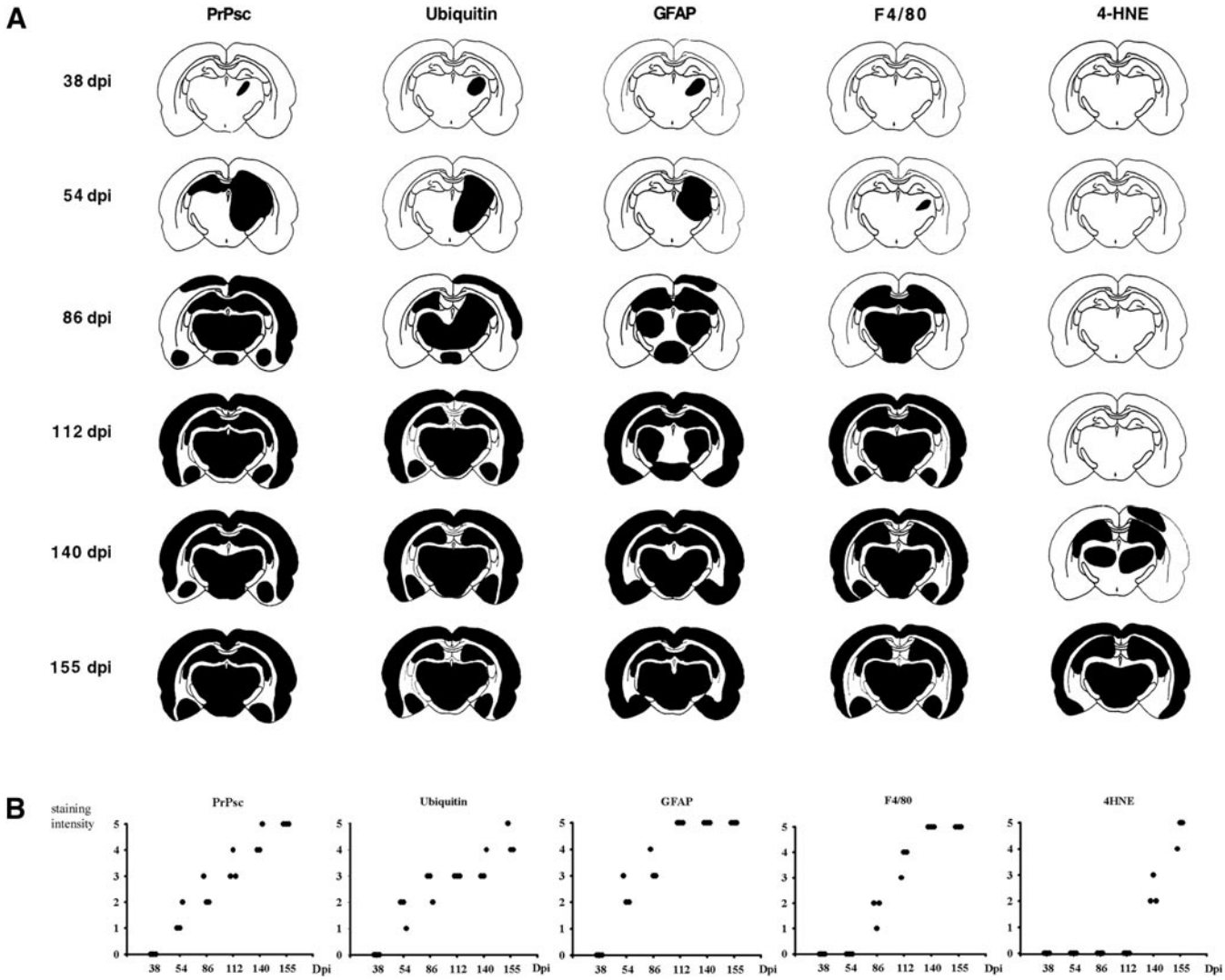

FIG. 1. C506-M3 intracerebrally inoculated mice succumb to disease 160 days postinoculation (dpi.). Each figure is representative of three infected mice, compared to three controls. (A) shows chronological distribution of abnormal prion protein (PrPsc), ubiquitin, astrocyte activation (GFAP), microglial activation (F4/80), and 4-HNE accumulation in the brain at the thalamus level. (B) shows scoring (semi-quantitative scale from 0 to 5) of immunolabeling intensity in the right hippocampus. Progressive PrPsc accumulation is accompanied by ubiquitin deposition and precedes astrocyte and microglial activation whereas 4-HNE accumulation is detected only in the final stages of the pathological process.

areas where there is neurodegeneration and astrocytosis (Giese *et al.*, 1998). Furthermore, it is generally thought that PrPsc is toxic for neurons and trophic for astrocytes (Giese *et al.*, 1998; Ye *et al.*, 1998). The microglial activation was slightly delayed by comparison to PrPsc deposition, but appeared long before neurodegeneration which occurred in the late stages of the disease. As documented by Hafiz and Brown (2000), astrogliosis is a hallmark of prion diseases, associated with (and potentially involved in) neurodegeneration. Such a relationship was established by *in vitro* studies indicating that neuronal degeneration is mediated by activated microglia in the presence of the HuPrP106-126 peptide, the biological active fragment of the human PrP protein (Brown *et al.*, 1996). This microglial activation is supposed to be directly due to contact with the human peptide fragment PrP106-126 and results in reactive oxygen species and cytokine release (Forloni *et al.*, 1994; Brown *et al.*, 1996; Peyrin *et al.*, 1999). Therefore, 4-HNE adduct accumulation in as-

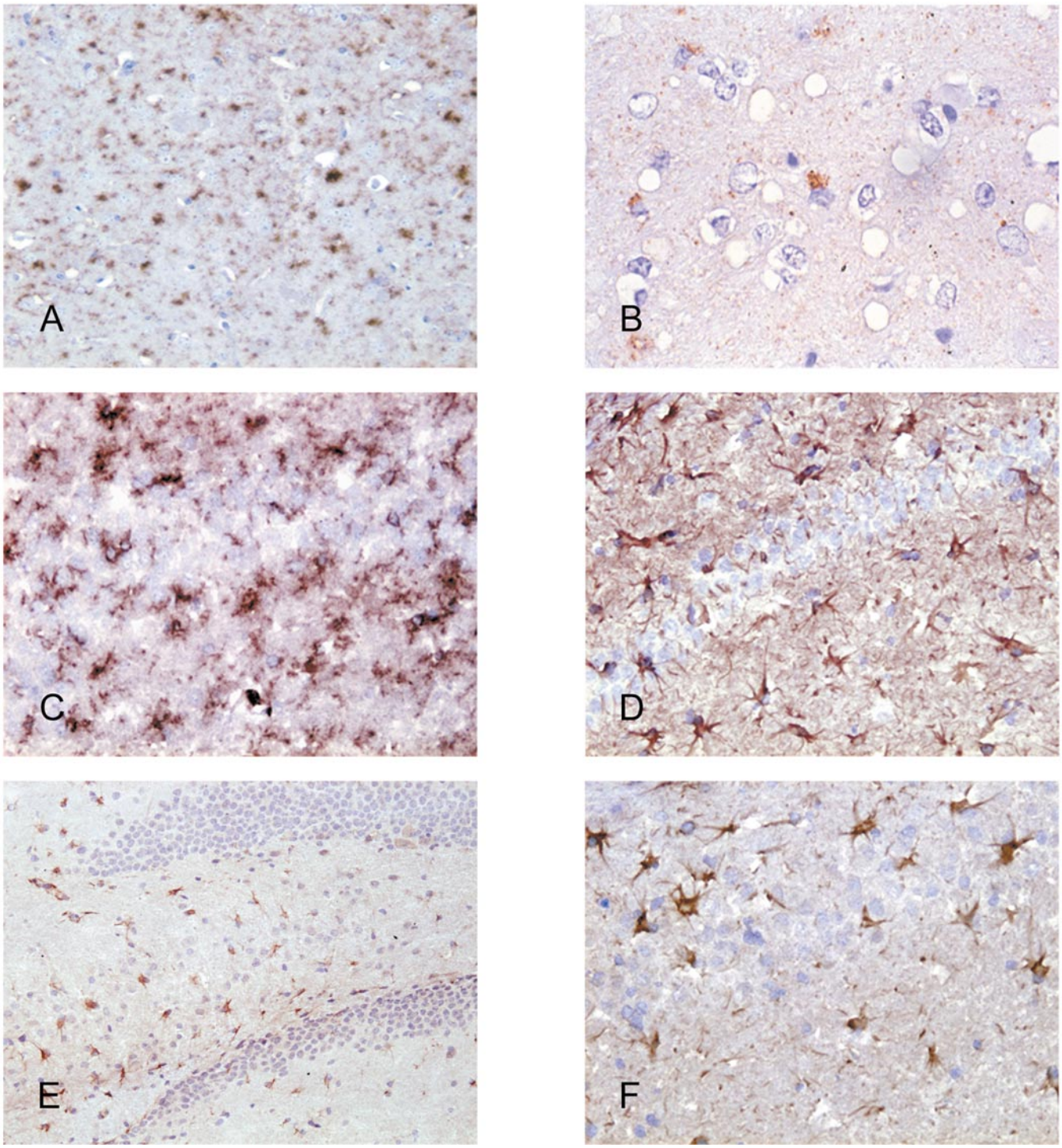

FIG. 2. PrPsc and ubiquitin deposits (A, ×200 and B, ×400, respectively) are detected in infected mice only. No prion protein staining was detected in control mice (not shown), indicating that no PrPc is stained after proteinase K pretreatment. Microglial activation (C, ×200), astrocyte activation (D, ×200), and 4-HNE accumulation (F, ×200), are strongly evidenced in the hippocampus (pyramidal layer) of infected mouse brain at 140 dpi. Note the high similarity between astrocyte and spider-like 4-HNE stainings (D and F). A low-power image of 4-HNE staining in the whole hippocampus region (E, ×100) shows the severity and extent of oxidative damage in brain of scrapie mice.

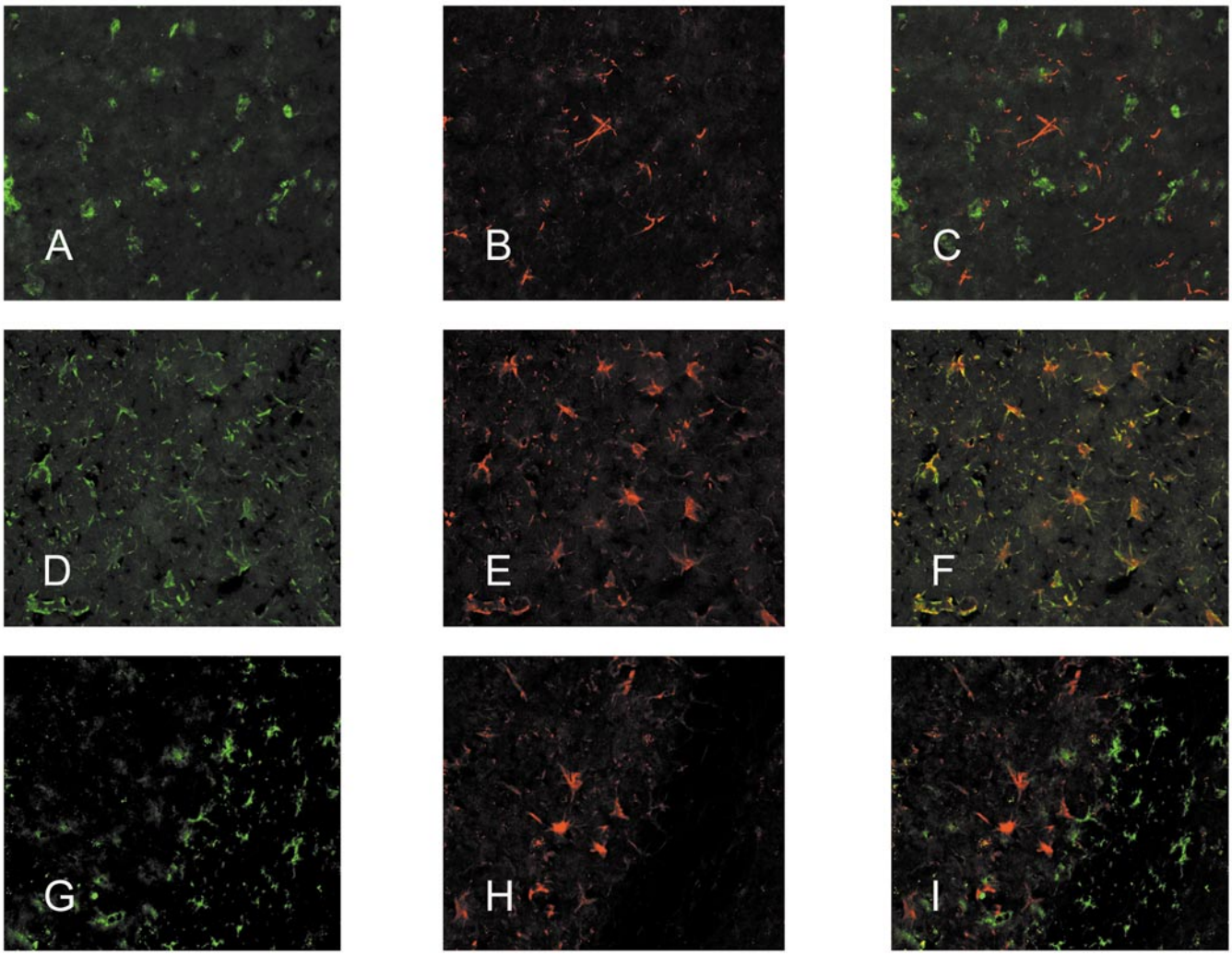

FIG. 3. Double labeling of neurons (A), astrocytes (D), or microglia (G), with 4-HNE (B, E, H), in mice. While neuron or microglial staining (green color) is clearly disctinct from that of 4-HNE (red color), the yellow staining in F indicates the colocalization of astrocytes with 4-HNE. Almost all astrocytes were 4-HNE positive, indicative of a major oxidative stress in these cells.

trocytes (as assessed by double labeling) could participate in astrocyte proliferation, since it is able to activate mitogenic signaling cascades including ERK, JNK, and p38 MAP kinases, and smooth muscle cell proliferation (Kakishita and Hattori, 2001).

Ubiquitin deposition was correlated to that of PrPsc in both scrapie-infected mice and CJD human cases and was detected long before 4-HNE adduct patterns. Ubiquitination is a prerequisite for the degradation of proteins through the ubiquitin / proteasome pathway, which plays a major role in the proteolytic degradation of abnormal proteins resulting from oxidative stress, neurotoxicity, and mutations (Grune *et al.*, 1997). The accumulation of ubiquitinated proteins associated with a failure in their degradation is a phenomenon shared by most neurodegenerative diseases including prion diseases (Lowe *et al.*, 1990; Ironside *et al.*, 1993) and may result from dysfunction of the ubiquitin / proteasome pathway (Okada *et al.*, 1999) or from structural changes in the protein substrates (Alves-Rodrigues *et al.*, 1998). The results reported here show that accumulation of ubiquitinated proteins occurs earlier than that of 4-HNE adducts, thus suggesting that 4-HNE-induced inhibition of proteasomes (Okada *et al.*, 1999) is not the primary cause of proteasome impairment.

In the brain, astrocytes play an important physiological role. These cells are involved in CNS homeosta-

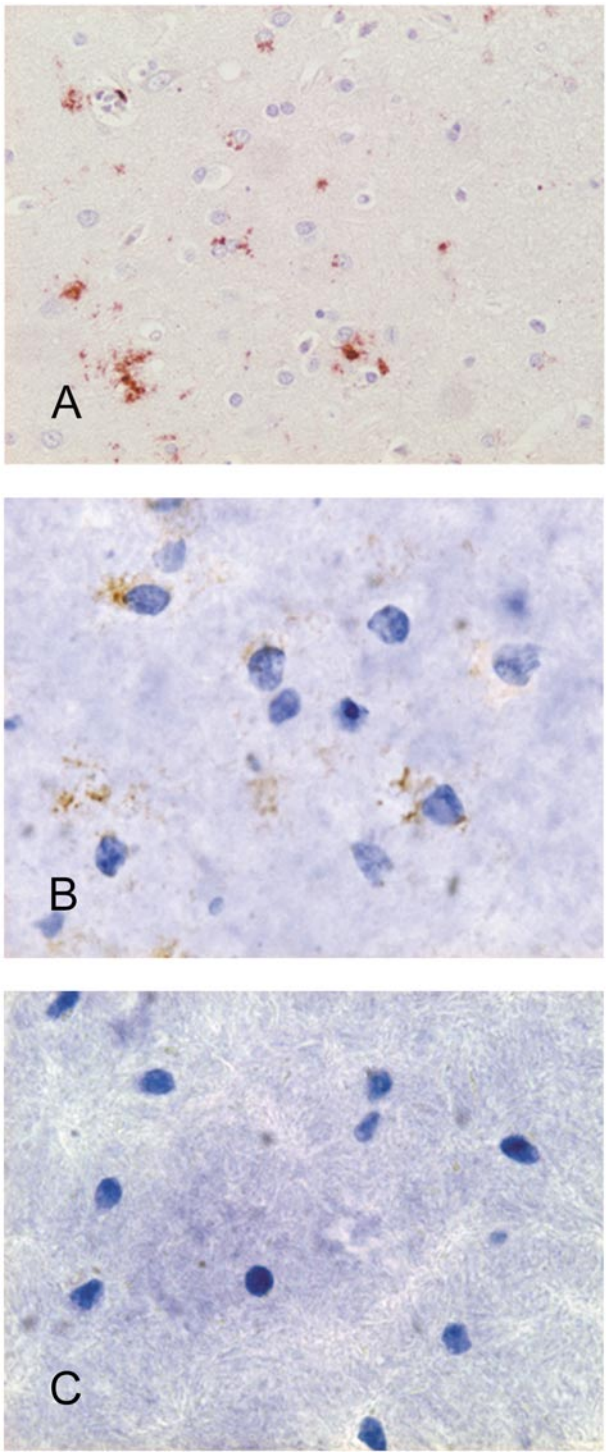

FIG. 4. PrPsc (A, ×200) and 4-HNE (B, ×400) accumulation is detected in human CJD brains. No 4-HNE staining was detected in brains of human control (C, ×400).

sis, controlling neuronal functions by the release of neurotrophic factors, and contribute to neurotransmitter metabolism. They also give physical support to neighboring neurons and provide nutrients to them (Tsacopoulos and Magistretti, 1996). As immunocompetent cells of the CNS, reactive astrocytes are thought to promote mainly Th2 responses, via PGE_2 or TGF-β production, that may downregulate microglial activation (Merrill and Benveniste, 1996).

In vitro studies using PrP106-126 suggest that astrocytes could become more susceptible to oxidative stress (Brown *et al.*, 1998) and suffer from cellular function alterations in prion diseases (Brown and Mohn, 1999; Hafiz *et al.*, 2000). The observed massive 4-HNE accumulation is consistent with an impairment of astrocyte cellular functions *in vivo*. Such an alteration is likely to participate in neurodegeneration.

Moreover, an increasing body of evidence has pointed out the role of 4-HNE in neuronal cell death, which involves different signaling mechanisms such as glutathione depletion and antioxidant enzyme inactivation (Tamagno *et al.*, 2000), activation of the caspase cascade (Zhang *et al.*, 2001), impairment of calcium homeostasis, and mitochondrial alterations (Carini *et al.*, 1996; Kruman and Mattson, 1999).

Interestingly, we found that 4-HNE-adduct labeling in the brain was detected only in the terminal stages of the disease incubation period. This result is consistent with recent studies, where 4-HNE was biochemically detected in brains from sporadic CJD patients (Wong *et al.*, 2001b) and scrapie mice (Wong *et al.*, 2001a). All together, these results point out a significantly elevated oxidative stress in prion diseases and argue for a saturation of antioxidant mechanisms.

Finally, these data highlight the occurrence of a severe *in vivo* oxidative stress associated with lipid peroxidation and 4-HNE adduct formation in astrocytes during the final steps of prion disease, which suggests a role for toxic lipoperoxidation products in neurodegeneration and provides new insights on potential therapeutical approaches.

ACKNOWLEDGMENTS

This work was supported by the French Interministerial Committee GIS “Prion Diseases”(Grant 2002) and the Midi-Pyrenees Region (Grant 2001). The authors thank Yvette Gras (UMR959 INRA-ENVT) for skillful assistance in preparation of the figures and P. L. Toutain (INRA UMR960) for critical reading of the manuscript. This work was presented at the Vth European Meeting on Glial Cell Function in Health and Disease in Rome, May 2002. E. Levavasseur is supported by a grant from the Region Centre and was a recipient of a fellowship from the International Society for Neurochemistry during the meeting.

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