Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 375–379, January 1991
Neurobiology

Scrapie-associated prion protein accumulates in astrocytes during scrapie infection

(unconventional disease agents/amyloid)

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Communicated by Richard L. Sidman, October 24, 1990 (received for review September 17, 1990)

ABSTRACT **In the course of scrapie, a transmissible spongiform encephalopathy caused by an unconventional agent, a normal cellular protein is converted to an abnormal form that copurifies with infectivity and aggregates to form deposits of amyloid. We have used immunocytochemistry and methods that enhance detection of amyloidogenic proteins to investigate the types of cells in the central nervous system which are involved in the formation of the abnormal scrapie-associated protein. We show that this protein accumulates in astrocytes prior to the cardinal neuropathological changes in scrapie—astrogliosis, vacuolation, neuron loss, and amyloid deposition. These findings implicate the astrocyte in the formation of the scrapie isoform of the prion protein and amyloid in scrapie and suggest that this cell type might also be involved in the replication of the scrapie agent.**

Infection by the scrapie agent, and by other unconventional agents, is accompanied by conversion of a 33- to 37-kDa cellular protein referred to as Cp33–37 or PrP^C (cellular isoform of the prion protein) to an altered form (Sp33–37 or PrP^{Sc}) that is more resistant to proteolysis and forms fibrils characteristic of all transmissible spongiform encephalopathies (1, 2). Aggregated PrP^{Sc} also copurifies with infectivity and is a major component of the amyloid deposits in diseases induced by unconventional agents (3–6).

The type of cell in the central nervous system in which the conversion of PrP^C to PrP^{Sc} takes place has not been defined but has been generally assumed to be the neuron, as the normal cellular isoform is found predominantly in this cell type (7–11). In our studies of the molecular basis for pathological changes in scrapie, we have used differential hybridization screening of cDNA libraries constructed from mRNAs isolated from the brains of infected animals to identify genes whose expression increases during infection (12). Subsequent sequencing and homology searches have revealed three genes [encoding glial fibrillary acidic protein (GFAP; refs. 13 and 14), apolipoprotein E, and cathepsin D (J.F.D., H. Minnigan, R.I.C., J. N. Whitaker, R. Race, T. Rustan, W. Frey II, and A.T.H., unpublished work)] that are activated in scrapie. Because increased expression in all three cases occurs in astrocytes, we became curious about the role the astrocyte might play in amyloid formation in scrapie, and therefore reexamined infected tissues with more sensitive techniques for detecting amyloid and its major component in scrapie, PrP^{Sc}. By examining tissue sections from animals early in the course of infection, we found that PrP^{Sc} accumulates in astrocytes rather than neurons. In this report we describe this discovery and the relationship of PrP^{Sc} accumulation in astrocytes to astrocytosis, amyloid deposition, vacuolation, and neuronal death.

MATERIALS AND METHODS

Infection of Mice and Histological Procedures. For most experiments C57BL/6J mice were inoculated intracerebrally with brain homogenate of mice infected with the 22L strain of scrapie (five animals) or with normal mouse brain homogenate (five animals). Some animals were stereotactically injected in the cerebellum (four control and four infected animals). At intervals of 4 weeks, the mice were perfused with phosphate-buffered saline (PBS) and then neutral 10% formalin. The brains were removed, fixed by immersion for 12–16 hr in neutral 10% formalin, and embedded in paraffin. Sagittal sections of 6–10 μm were cut and either adhered or covalently linked (15) to Denhardt-coated glass slides.

Immunocytochemical Procedures. Sections were deparaffinized in xylene, placed in 100% ethanol, and then placed in 1% H_2O_2 in methanol for 10 min, to block endogenous peroxidase activity. The sections were rehydrated through graded ethanol solutions, rinsed in PBS, incubated 20 min with normal goat serum diluted 1:40 in PBS, and then incubated overnight at 4°C with antiserum to the 27- to 30-kDa protease resistant fragment (PrP27–30) of the 33- to 37-kDa PrP^{Sc} (4) diluted 1:250 in normal goat serum. Two additional antisera directed against PrP33–37 of the mouse ME7 or hamster 263K strains of the scrapie agent were tested and gave results similar to those obtained with the anti-PrP27–30 serum. After reaction with the primary antiserum, sections were incubated sequentially at room temperature with biotinylated secondary antibody, avidin–biotinylated horseradish peroxidase complex, and diaminobenzidine substrate (Vector Laboratories). Following immunostaining, sections were counterstained with cresyl violet. The specificity of staining was confirmed by substitution of preimmune serum for the primary antiserum; no peroxidase reaction products were observed. For localization of GFAP, sections were immunostained as described above except the primary serum was rabbit anti-GFAP (gift from L. Eng, Stanford University) diluted 1:1000.

Procedures to Enhance Detection of PrP^{Sc} and Demonstrate Amyloid. To unmask epitopes of the aggregated abnormal isoform of the scrapie protein, sections either were digested with proteinase K (5 μg/ml) for 7 min at room temperature prior to reaction with the primary antiserum or were pretreated with 96% formic acid for 5 or 15 min, respectively, in the double- or single-label immunocytochemical experiments. Following formic acid pretreatment, anti-PrP27–30 was used at a 1:500 dilution. For the double-label analyses, sections were first immunostained for PrP^{Sc} following a 5-min formic acid pretreatment and then incubated overnight at 4°C with guinea pig anti-GFAP (Advanced Immunochemical Services, Los Angeles) diluted 1:100. The sections were

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Abbreviations: PrP, prion protein; GFAP, glial fibrillary acidic protein; pi, post-inoculation.

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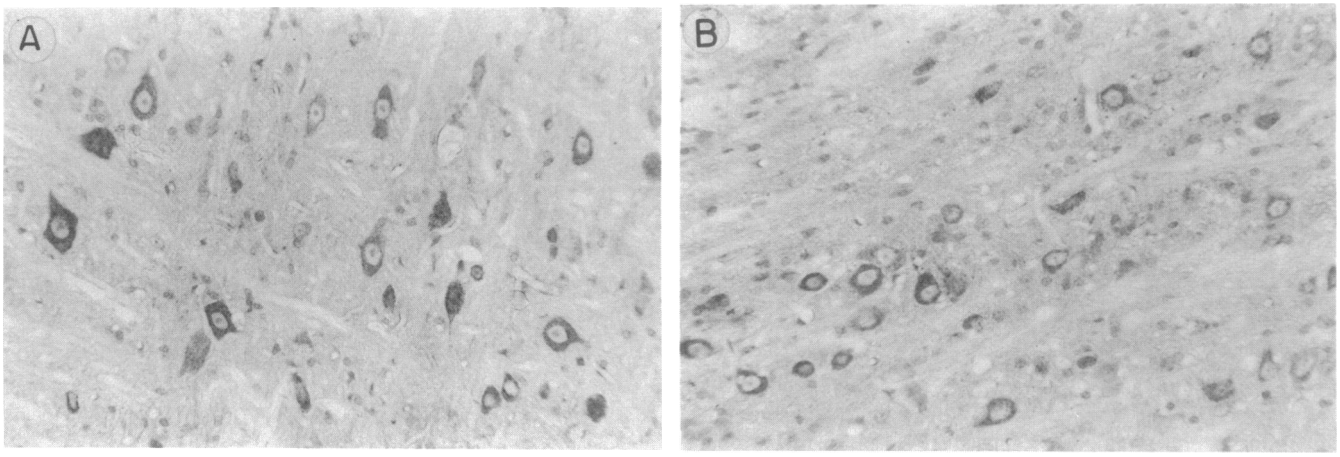

FIG. 1. Distribution of PrP^C in the brainstem of a control mouse (*A*) and a scrapie-infected mouse (*B*). Sections were reacted with anti-PrP27–30 serum without pretreatment. PrP^C (dark reaction product) can be seen in the cell body of many neurons in both the infected and the uninfected animal. No change in the distribution of PrP^C occurred in the course of infection. Vacuoles are present within the neuropil of the scrapie-infected mouse (*B*). (×240.)

rinsed in PBS, incubated 1 hr with alkaline phosphatase-conjugated anti-guinea pig IgG (Sigma) diluted 1:1000, rinsed in PBS, incubated with the alkaline phosphatase substrate (Vector Red; Vector Laboratories) for 1 hr, and counterstained with cresyl violet.

For visualization of amyloid, tissue sections were deparaffinized in xylene and hydrated using graded ethanol solutions. They were then stained for 10 min in Mayer's hematoxylin, rinsed in three changes of distilled water, and treated with alkali (80% ethanol saturated with NaCl to which 0.01

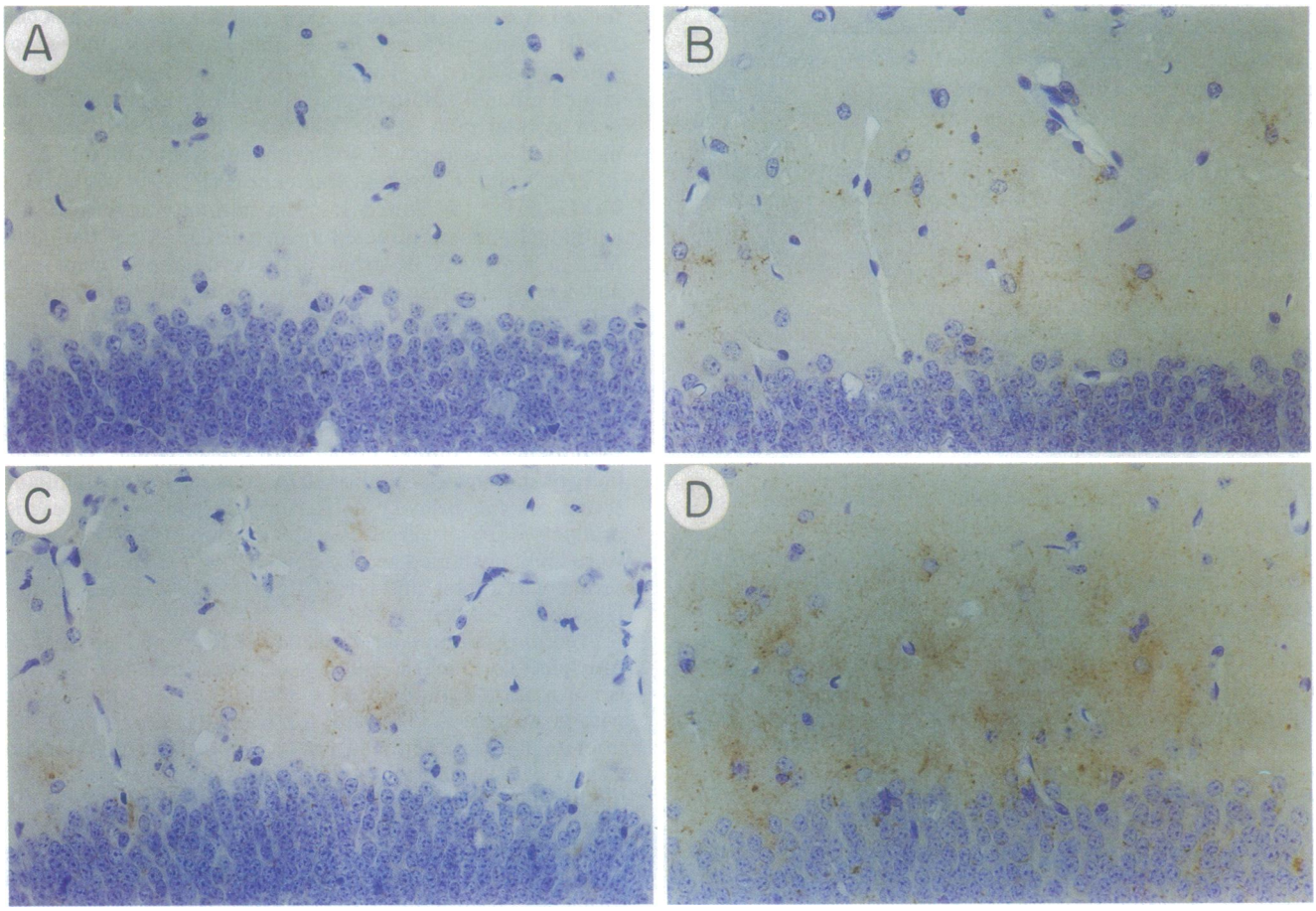

FIG. 2. Distribution of PrP^{Sc} in the dentate gyrus of the hippocampus of a control mouse (*A*) and scrapie-infected mice (*B–D*). Sections were reacted with anti-PrP27–30 serum following formic acid pretreatment to reveal PrP^{Sc}. Antigen–antibody reaction is represented by a brown precipitate against the purple-blue counterstain. The section from a control animal at 16 weeks post-inoculation (pi) shows no PrP^{Sc} (*A*), but the sections from infected animals at various times during disease show the change in distribution of PrP^{Sc}. At 8 weeks pi (*B*) PrP^{Sc} is evident in astrocytes; by 12 weeks pi (*C*) immunoreactive PrP^{Sc} forms a pericellular "blush" around the astrocytes and subsequently is seen as a diffuse stain throughout the neuropil in areas of astrocytosis at 16 weeks pi (*D*). (×280.)

volume of 1% NaOH was added immediately before use) for 20 min. The sections were then stained for 20 min in alkaline Congo red (80% ethanol saturated with NaCl and Congo red to which 0.01 volume of 1% NaOH was added immediately before use), rapidly dehydrated through three changes of absolute ethanol, cleared in xylene, and mounted in Permount (16, 17).

RESULTS

Localization of the Normal and Abnormal Isoforms of the Scrapie-Associated Protein and Amyloid. When sections from the 18 mice were immunostained without pretreatment or proteinase K digestion, we reproducibly found, as have others (8–11), that the scrapie-associated protein was present predominantly in neurons of control and scrapie-infected animals (Fig. 1). We classified this immunoreactive species as PrP^C because it was found in uninfected control animals, did not accumulate in the brains of infected mice, and did not require pretreatment to expose otherwise inaccessible epitopes of the aggregated PrP^{Sc}. In proteinase K-digested sections from infected animals, but not from control animals, there was immunoreactive material in the neuropil in areas of astrocytosis as well as in neurons. We agree with others (8) that the immunoreactive protein in the neuropil is probably aggregated PrP^{Sc} whose epitopes have been partially exposed by the mild protease treatment.

When we used formic acid pretreatment, the most sensitive method available to expose antibody-binding sites in amyloid (18), we observed a very different distribution of the scrapie-associated protein. We could no longer detect the protein in control animals (Fig. 2*A*), but in infected animals there was abundant immunoreactive protein in astrocytes and in the surrounding neuropil (Figs. 2 and 3). These results suggest that formic acid pretreatment of formalin-fixed tissues markedly reduces PrP^C reactivity but reveals more of the aggregated PrP^{Sc}, so classified because it was not present in uninfected animals, accumulated in infected animals, and was not detectable without formic acid pretreatment.

To unequivocally establish the localization of PrP^{Sc} in astrocytes, we used double immunolabeling to demonstrate colocalization of PrP^{Sc} and the astrocyte-specific marker GFAP. By decreasing the length of exposure to formic acid and by using a high concentration of anti-GFAP, we detected PrP^{Sc} and GFAP in astrocytes of infected animals (Fig. 3) but not in the controls, again compatible with the reduction of PrP^C immunoreactivity with even a brief exposure to formic acid under our fixation conditions.

Since PrP^{Sc} is a major component of amyloid in scrapie, we also assessed the distribution of the latter. When sections are treated with Congo red and viewed in polarized light, the stained amyloid exhibits a characteristic birefringence. The amyloid fibrils demonstrated in this way were clearly associated with astrocytes in scrapie-infected animals but were not seen in control animals (Fig. 4).

Temporal Studies. In sections pretreated with formic acid, we first detected PrP^{Sc} in astrocytes 8 weeks after inoculation (Fig. 2*B*). Thereafter the quantity of PrP^{Sc} increased with advancing infection, with deposits, in addition to those within astrocyte cell bodies, evident as pericellular "blushes" at 12 weeks pi (Fig. 2*C*) and then as diffuse accumulations throughout the neuropil at 16 weeks pi (Fig. 2*D*). In the terminal phases of infection, the localization of PrP^{Sc} in the neuropil is in accord with other studies that have focused on the late stages of disease (8, 9, 19). The accumulation of PrP^{Sc} within astrocytes during scrapie precedes astrocytosis, first evident 12 weeks pi, and the detection of amyloid by Congo red staining at 16 weeks pi.

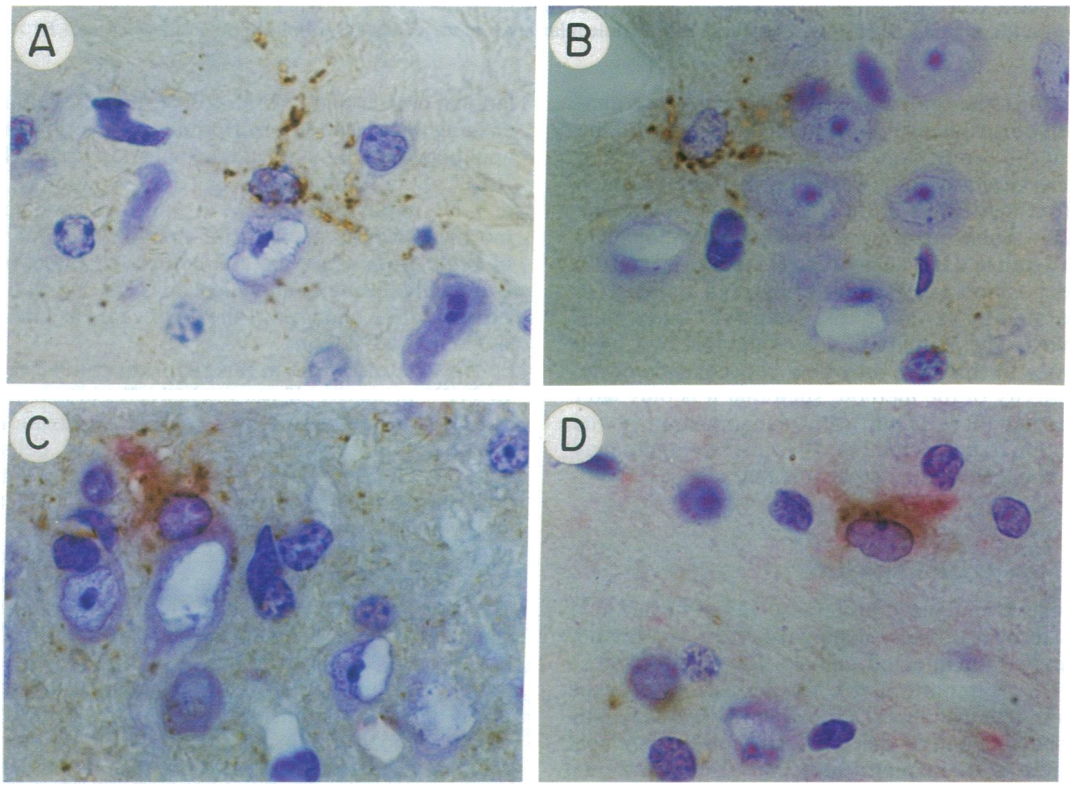

FIG. 3. (*A* and *B*) PrP^{Sc} (brown reaction product) closely associated with histologically identifiable astrocytes in the brains of scrapie-infected mice at 8 weeks pi. (*C* and *D*) Colocalization of PrP^{Sc} (brown reaction product) and GFAP (red reaction product) in similar astrocytes of scrapie-infected mice at 16 weeks pi. Sections were immunostained after a 15-min (*A* and *B*) or 5-min (*C* and *D*) pretreatment with formic acid. (×875.)

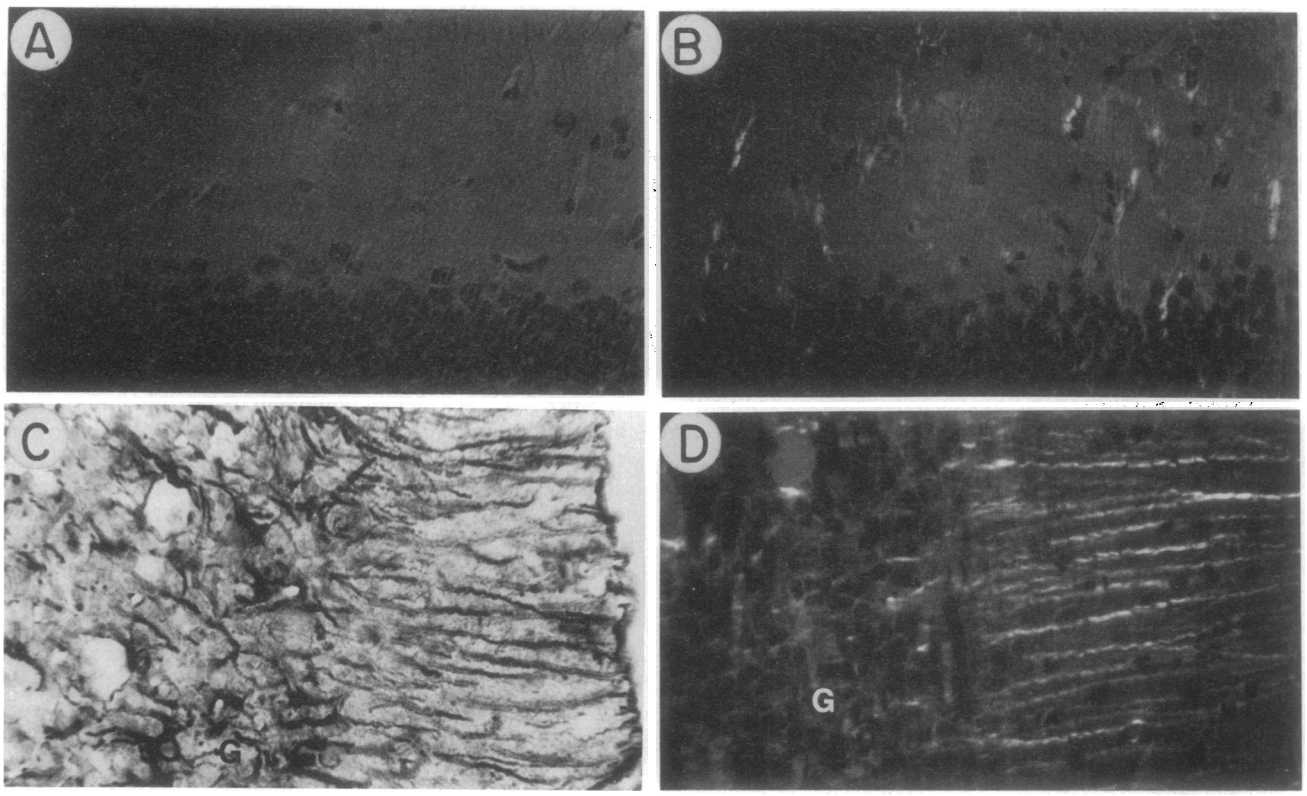

FIG. 4. Close association of astrocytes and amyloid in scrapie-infected mice. (*A* and *B*) Sections from a control (*A*) and a scrapie-infected (*B*) mouse (20 weeks pi) were stained with Congo red and viewed with polarized light to reveal amyloid, which is evident in the photographs as white birefringent material against the dark background. Amyloid fibrils are seen extending from histologically identifiable astrocytes in the scrapie section, but not in the control. (*C*) Bright-field micrograph of a section from a scrapie-infected mouse (20 weeks pi) reacted with antiserum directed against GFAP. Immunoreactive GFAP (seen here as a dark precipitate) is present in the Bergmann glial fibers coursing through the molecular layer of the cerebellum and in astrocyte fibers surrounding vacuoles in the granular layer (G). (*D*) Dark-field micrograph of a section from a scrapie-infected mouse (20 weeks pi) stained with Congo red. Amyloid fibrils extend through the molecular layer of the cerebellum and encircle vacuoles in the granular layer (G). The fibrils in the molecular layer have a twisted or spiral appearance. (*A* and *B*, ×240; *C* and *D*, ×420.)

To further define the temporal order of events, we analyzed animals that had been stereotactically injected with the scrapie agent in the cerebellum. We again detected PrP^{Sc} in astrocytes in the cerebrum at 8 weeks pi, the time at which infectivity is first detectable in the cerebrum as well as the cerebellum (20). Subsequently, astrogliosis develops (12 weeks pi) with death and loss of neurons (14 weeks pi; ref. 20) and amyloid deposition assessed by Congo red staining (16 weeks pi) separated by several weeks from PrP^{Sc} accumulation in astrocytes. The inability to detect amyloid by Congo red staining prior to this time may be a consequence of the relative insensitivity of the method, such that it is only later in the disease that there is sufficient accumulation of the aggregated protein to be detectable; alternatively, there may be qualitative changes in the aggregates that alter dye binding.

DISCUSSION

In these studies we have used proteinase K and formic acid pretreatments to operationally distinguish PrP^{C} and the abnormal isoform PrP^{Sc} in infected animals. The results of these studies establish an important role for the astrocyte in the formation of amyloid in scrapie, a role that this cell may play in other neurodegenerative diseases, since the β-amyloid precursor protein of Alzheimer disease has also been shown to be produced by astrocytes in response to neuronal injury (21). In our current reconstruction of the molecular pathogenesis of scrapie, we envision replication of the agent as the inducing stimulus to programmed alterations in gene expression that occur primarily in astrocytes, with the posttranslational conversion of PrP^{C} to PrP^{Sc} as the initial event. The ensuing increased transcription of several genes (GFAP, apolipoprotein E, and cathepsin D; J.F.D., H. Minnigan, R.I.C., J. N. Whitaker, R. Race, T. Rustan, W. Frey II, and A.T.H., unpublished work) in astrocytes and accompanying morphological transformation are succeeded by progression of PrP^{Sc} from astrocyte cell bodies to pericellular sites and eventually throughout the neuropil (Fig. 2 *B–D*). Quantitative or qualitative changes in the aggregates at late stages result in Congo red staining.

The demonstration in this report of cellular accumulations of PrP^{Sc} primarily, if not exclusively, in astrocytes and the recent documentation by *in situ* hybridization of mRNA encoding this protein in astrocytes and cells other than neurons (7, 22, 23) are certainly compatible with the notion that the scrapie agent in fact replicates in astrocytes and induces the conversion of PrP^{C} to PrP^{Sc}. PrP^{Sc}, in turn, may be a glial growth factor responsible for astrocytosis and activation of gene expression in astrocytes (24). We cannot exclude, however, an alternative interpretation in which replication of the agent in neurons or other cell types converts PrP^{C} to PrP^{Sc}, and PrP^{Sc} is rapidly assimilated by the astrocyte. Also unresolved is the enigmatic nature of the agent itself and its relationship to PrP^{Sc}. PrP^{Sc} may be the agent itself, its coat protein, or a pathological by-product of infection. In any case, we show here that it is the astrocyte that responds to the initiating stimulus of replication in accumulating PrP^{Sc}, setting a process in motion that will result in amyloid deposition as well as the other characteristic neuropathological changes of the transmissible spongiform encephalopathies.

We thank Joy Laiti and Tim Leonard for preparation of the manuscript and figures and the National Institutes of Health for support.

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