Activation of Cerebral Cytokine Gene Expression and Its Correlation with Onset of Reactive Astrocyte and Acute-Phase Response Gene Expression in Scrapie†

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The pathogenesis of scrapie, a transmissible subacute spongiform encephalopathy, is unclear. However, certain aspects of the known cellular and molecular neuropathology in scrapie led us to hypothesize that cytokines could mediate cerebral pathological changes in this neurodegenerative disease. Therefore, expression of multiple cytokine genes in the brain and peripheral organs of scrapie-infected mice was examined. Late in the course of scrapie, expression of tumor necrosis factor alpha (TNF- α), interleukin-1 α (IL-1 α), and IL-1 β mRNA was markedly increased in the brain but not the spleen, kidneys, or liver. In time course studies, scrapie-infected mice exhibited increased cerebral expression of the TNF- α , IL-1 α , and IL-1 β mRNAs by week 15 postinoculation—a time point that coincided with the onset of clinical symptoms. Thereafter, the levels of these cytokine transcripts increased progressively to the terminal stages of the disease (week 25). To determine the relationship of the increased cerebral expression of the cytokine mRNAs to the development of pathological changes in scrapie, we examined the expression of the glial fibrillary acidic protein gene (a marker for astrocytosis) and the murine acute-phase response gene homologous to the α_1 -antichymotrypsin gene (designated EB22/5.3). Markedly increased expression of both the glial fibrillary acidic protein and EB22/5.3 mRNAs was observed in the brain but not the peripheral organs of scrapie-infected mice. The increased expression of both these gene products also occurred at week 15 of infection and, thereafter, increased progressively to the terminal stages of the disease. Therefore, infection of mice with scrapie resulted in significant increases in the expression of the TNF- α , IL-1 α , and IL-1 β gene products, whose pattern correlated with the onset and development of molecular and clinical pathological changes. Since scrapie is known not to evoke an immune response, the present findings strongly suggest the existence of a localized cerebral host response to the agent during which proinflammatory cytokines could be key pathogenic mediators.

Scrapie is a transmissible subacute, progressive neurodegenerative disease occurring naturally in sheep and goats (12) and has been adapted to the laboratory mouse (6). The clinical features of ataxia, tremor, wasting, and death, combined with the striking spongiform encephalopathy characteristic of scrapie, make this disease a prototype for the slow transmissible neurodegenerative diseases of humans, exemplified by Creutzfeldt-Jakob disease and kuru (12). There is now considerable evidence that the transmissible agent for these spongiform encephalopathies is an abnormal isoform of the cellular prion protein PrP^c (termed PrP^{Sc} in scrapie) (30, 35). In contrast with PrP^c, PrP^{Sc} is protease resistant and accumulates in extracellular amyloid fibrils. Diedrich et al. (7) showed recently that whereas the predominant source of PrP^c in the brain is in neurons, PrP^{Sc} accumulates principally in astrocytes prior to the development of neuropathological changes, thus suggesting that this cell type might also be involved in the replication of the PrP^{Sc}.

It is unclear how PrP^{Sc} accumulation gives rise to the profound neurodegeneration characteristic of scrapie. However, it is noteworthy that although the etiology of Alzheimer's disease remains unknown, there are some similarities in the neuropathological changes between this disease and scrapie,

including reactive astrocytosis, amyloid formation, neuron loss, and vacuolation (32). Further similarities also exist between these two neurodegenerative diseases at the molecular level, with increased expression of the genes for apolipoprotein E and cathepsin D (8) and for glial fibrillary acid protein (GFAP), transferrin, and sulfated glycoprotein 2 (9). It has been proposed that the cellular and molecular correlates between scrapie and Alzheimer's disease reflect convergent pathological mechanisms (8). Therefore, elucidating the mechanisms of neurodegeneration in scrapie could be relevant to understanding the neurodegenerative processes that occur in Alzheimer's disease.

Cytokines are a broad group of soluble mediators possessing a spectrum of hormone-like actions both within (13, 29) and outside (18) the central nervous system. Localized production of cytokines within the brain is possible from astrocytes (25, 26) and microglia (15, 25), both of which have been shown to secrete interleukin-1 (IL-1), IL-6, transforming growth factor β , and tumor necrosis factor alpha (TNF- α). Intracerebral injection of either IL-1 (17) or gamma interferon (36) induces reactive astrocytosis, a common feature of many neurodegenerative diseases including scrapie (11). We have recently observed (4), in transgenic mice with cerebral overexpression of IL-6, degenerative changes in hippocampal neurons similar to those reported in scrapie (22) and reactive astrocytosis. Therefore, we hypothesized that cytokines may underlie some of the molecular and cellular neuropathological changes exhibited in scrapie. To test this possibility, we have analyzed scrapie-infected mice for the expression of multiple cytokine

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genes in the brain and other organs throughout the progression of disease. Furthermore, to define the relationship of any changes in cytokine gene expression to the development of pathological changes, we also examined the temporal expression of the genes for glial fibrillary acid protein (GFAP; a marker for reactive astrocytosis) and EB22/5.3 (an acute-phase response gene analogous to the human α_1 -antichymotrypsin [ACT] gene [23]).

MATERIALS AND METHODS

Mice and induction of scrapie. Male SWR/j mice were maintained under pathogen-free conditions in the closed breeding colony of the Scripps Research Institute and were used at 4 to 6 weeks of age. For induction of scrapie, we used the Chandler strain (31), obtained from Richard Race, Rocky Mountain Laboratories, Hamilton, Mont.; this strain was maintained by serial intracerebral passage of brain homogenate from terminally affected mice. Inocula were prepared from brain tissue homogenized in sterile phosphate-buffered saline solution at 10% (wt/vol) and clarified by centrifugation at $1,000 \times g$ for 10 min. Aliquots were stored at -70°C until used for injection. Mice were inoculated with either 30 μl of scrapie or normal brain homogenate (control animals) via midline intracranial injection. In the scrapie-infected mice, the onset of clinical symptoms (ataxia) occurred reproducibly between weeks 15 and 17 postinoculation and resulted in death by week 26. Mice inoculated with normal brain homogenate remained healthy over the same period.

Isolation of poly(A)⁺ RNA. At various times following inoculation, mice were killed and brain and peripheral organs were removed, snap frozen in liquid nitrogen, and stored at -85°C . Poly(A)⁺ RNA was prepared by a rapid isolation procedure (2) applied directly to tissue lysates as described previously (4, 5).

RNAse protection assay for cytokine mRNA. The RNAse protection assay for cytokine mRNA was performed as described previously (5, 21). The mIL-1 α , mIL-1 β , mIL-2, mIL-3, mIL-4, mIL-5, mIL-6, murine gamma interferon, mTNF- α , mTNF- β , and mL32 subclones in pGEM-4, described in a previous report (21), were linearized with *Eco*RI and used as templates for the T7 polymerase-directed synthesis of ^{32}P -labeled antisense RNA probe sets. The hybridization reactions [6×10^5 cpm per 5 μg of target poly(A)⁺ RNA], RNAse treatments, and isolation of "protected" RNA-RNA duplexes were as described previously (21). Final samples were dissolved in 80% formamide and dyes, denatured at 90°C for 2 min, and electrophoresed in standard 5% acrylamide-8 M urea sequencing gels. Dried gels were placed on XAR film (Kodak, Rochester, N.Y.) with intensifying screens and exposed for various periods at -70°C . Within each assay, control hybridization included the probe set plus an equimolar pool of synthetic sense RNAs (prepared as described previously [21]) to serve as sizing standards and to assess assay performance and the probe set run without target RNA to serve as a degradation control.

Northern (RNA) blot hybridization for GFAP and EB22/5.3. Poly(A)⁺ RNA (5 μg) was denatured, separated by electrophoresis in 1% agarose-2.2 M formaldehyde gels, transferred to nylon membranes, and hybridized overnight at 45°C with ^{32}P -labeled cDNA probes. Probes used were the following: a 2.7-kb *Xho*I-*Bam*HI fragment of rat GFAP cDNA (cDNA clone; GFA 15 obtained from R. Milner, Hershey Medical School, University of Pennsylvania, Hershey, Pa.), a 1.8-kb *Eco*RI fragment of the murine EB22/3 cDNA which hybridizes with mRNA products of the *Sp1-2* gene (23) (provided by J.

FIG. 1. Expression of cytokine mRNA in the brain and peripheral organs in scrapie. The figure shows a representative experiment in which SWR/j mice were injected intracerebrally with normal or scrapie-infected brain homogenate (10% [wt/vol]), poly(A)⁺ RNA was isolated from the indicated organs 23 weeks later, and 5 μg was analyzed by RPA. Samples derived from two control (lanes -) or two scrapie-infected (lanes +) mice are shown in this analysis. A complementary set of synthetic sense RNAs was also included (lane Ref.), with the size of the protected probe fragments spanning 333 nucleotides (TNF- β) to 89 nucleotides (L32). For target mRNA, the sizes of the protected probes are smaller than the corresponding sense protected probes, spanning 323 nucleotides (TNF- β) to 82 nucleotides (L32).

Inglis, Medical Research Council Human Genetics Unit, Western General Hospital, Edinburgh, United Kingdom), and a 0.26-kb fragment of the murine β -actin gene (34) generated by PCR (provided by M. Nerenberg, Scripps Research Institute). Following hybridization, membranes were washed, were placed on XAR film with intensifying screens and exposed for various times at -70°C .

RESULTS

Cytokine gene activation in the brain. The expression of multiple cytokine genes was assessed by RPA of poly(A)⁺ RNA prepared from the brain, kidneys, spleen, and liver of control or scrapie-infected mice at week 23 postinoculation (Fig. 1). At this time, the scrapie-infected animals were severely debilitated and were in the late stages of disease. Concurrently in these animals, but not in controls, there was a pronounced increase in TNF- α , IL-1 α , and IL-1 β mRNA expression in the brain. Quantitatively the levels of IL-1 α mRNA were predominant over those for TNF- α and IL-1 β mRNAs were lower. The expression of TNF- β mRNA and the absence of the IL-4, IL-5, IFN- γ , IL-2, IL-6, and IL-3 mRNAs in the brain were not altered by scrapie infection. In contrast with the brain, the expression of the TNF- α , IL-1 α , IL-1 β , or other cytokine mRNAs in the kidneys, spleen, and liver was not altered by scrapie infection.

To determine the kinetics of the cerebral IL-1 α/β and TNF- α gene response in scrapie, we performed a detailed time course experiment (Fig. 2). Increased levels of mRNA for all three cytokines were first detectable by week 15 postinocula-

FIG. 2. Time course of cytokine mRNA expression in the brain following scrapie infection. Control or scrapie-infected mice were inoculated as described in the legend to Fig. 1, poly(A)⁺ RNA was isolated at the indicated times, and 5 μ g was analyzed by RPA. In this representative analysis, samples prepared from one control and two scrapie-infected mice were analyzed for each time point.

tion and increased progressively until end-stage disease at week 25. The pattern of expression during this period did not change, with IL-1 α mRNA levels predominating over those of TNF- α and IL-1 β . Expression of TNF- β mRNA, which was constitutively expressed in the brain, did not alter with the development of scrapie.

Induction of molecular neuropathological changes. To establish whether the activation of cytokine gene expression in scrapie correlated with the induction of molecular pathological changes, we analyzed the expression of GFAP and EB22/5.3 mRNAs by Northern blot hybridization (Fig. 3 and 4). Compared with controls, expression of mRNA for both these genes was markedly increased in the brain of scrapie-infected mice at week 23 postinoculation (Fig. 3). Moreover, these scrapie-dependent changes were tissue specific as evidenced by the lack of increase in EB22/5.3 gene expression in the kidneys, spleen, and liver. As expected for an acute-phase response gene, the highest levels of EB22/5.3 mRNA were detectable in liver from both control and scrapie-infected mice.

The kinetics of the GFAP and EB22/5.3 gene response in

the brain from scrapie-infected mice was examined (Fig. 4). A small increase in the levels of GFAP and EB22/5.3 mRNAs was discernible at week 15 postinoculation, became more pronounced by week 17 postinoculation, and increased progressively with the development of end-stage disease.

DISCUSSION

The pathogenesis of scrapie is poorly understood. However, on the basis of some of the known molecular and cellular neuropathological manifestations of this neurodegenerative disease, we reasoned that cytokines may be important pathogenic factors. The findings in the present study showed not only that there is pronounced activation of cerebral TNF- α , IL-1 α and IL-1 β gene product expression in scrapie but also that the increased expression of these cytokine mRNAs correlates with the progression of the clinical features and molecular neuropathological changes. Furthermore, the increased expression of the TNF- α , IL-1 α , and IL-1 β mRNAs in scrapie was specific for the brain and did not occur in the peripheral organs represented by the spleen, liver, and kidneys.

The tissue specificity of this cytokine gene response, even though the scrapie agent is known to replicate in the periphery (particularly in the spleen [12]), suggests the existence of a highly localized cerebral host response to the infection. This view is supported further by our observation that EB22/5.3 gene expression was also increased in the brain but not in the peripheral organs of scrapie-infected mice. The EB22/5.3 gene is an acute-phase response gene encoding a protein homologous to human ACT (23). The expression of this gene is activated by the host immunoinflammatory response and would therefore be expected to increase, particularly in the liver, if there were a generalized host response to scrapie. Scrapie in the mouse, as with other spongiform encephalopathies, is not associated with observable immunoinflammatory cell infiltration in the brain (12). Further evidence that this disease does not elicit a significant immune response is highlighted by the observation that scrapie-infected immunodeficient mice show a similar disease course to that of their immunologically competent littermates (28). Therefore, we

FIG. 3. Expression of GFAP and EB22/5.3 mRNA in the brain and peripheral organs in scrapie. Poly(A)⁺ RNA (5 μ g per lane) prepared from control (lanes -) or scrapie-infected (+) mice was analyzed by Northern blot hybridization. Blots were hybridized sequentially (after stripping) with ³²P-labeled cDNA probes for the indicated mRNA targets. The β -actin probe served as a control for RNA loading. A representative experiment showing sample analysis from two control and two scrapie-infected mice is presented.

FIG. 4. Time course of GFAP and EB22/5.3 mRNA expression in the brain following scrapie infection. Poly(A)⁺ RNA (5 µg per lane) from control and scrapie-infected mice was isolated from the brain at the indicated times and analyzed by Northern blot hybridization. Samples analyzed were prepared from one control and two scrapie-infected mice at each time point.

conclude that resident central nervous system cells are primarily responsible for the increased cytokine gene expression. We have attempted to use *in situ* hybridization to identify the cells expressing the TNF- α , IL-1 α , and IL-1 β RNA in the brain of scrapie-infected animals, but have not been successful. However, likely sources are reactive astrocytes or microglia, both of which produce these cytokines (15, 25, 26).

The relationship between the accumulation of PrP^{Sc} and the activation of cytokine gene expression in scrapie was not examined in the present study. However, from previous studies of hamsters (3, 24) and mice (7), it is known that the prion protein accumulates prior to the development of astrocytosis, indicating that PrP^{Sc} may be causally involved in the development of neuropathology. Whether PrP^{Sc} directly activates astrocytes remains unknown. In any case, accumulation of PrP^{Sc} is also likely to precede activation of IL-1 α/β and TNF- α gene expression in scrapie, and the possibility that the prion protein is responsible for this activation should be considered.

Our results showing a correlation between the cerebral expression of the cytokine genes and the clinical and molecular progression of the disease suggest that TNF- α , IL-1 α , and IL-1 β may be involved in the pathogenesis of the neurodegenerative processes that occur in scrapie. Following scrapie inoculation, the earliest detectable increase in the cerebral expression of the TNF- α , IL-1 α , and IL-1 β mRNA gene products was at approximately week 15, which is the time when the initial clinical feature of hindlimb ataxia also became apparent. In addition, at this time we observed the first detectable increases in GFAP and EB22/5.3 mRNA expression, indicating the onset of pathological changes in the brain. The increased expression of the GFAP gene is consistent with the development of reactive astrocytosis in scrapie (9, 11). Astrocytes have a crucial role in the development and support of neurons (10, 19). Therefore, the long-term changes in these cells that occur in scrapie could significantly affect neuronal function and viability. Expression of the EB22/5.3 gene has not been previously described in this neurodegenerative disease. Because of its close homology with human ACT, EB22/5.3 is likely to be a serine protease inhibitor (23). Increased expression of serine protease inhibitors is observed in the brain in a

variety of inflammatory and degenerative disorders (1). The function of the EB22/5.3 gene product in scrapie is not known but, by analogy with ACT (1), could be diverse, including the inhibition of potentially damaging serine proteases released locally by astrocytes and possibly by degenerating neurons as well as the formation of scrapie amyloid plaques.

The correlation between the increased expression of the TNF- α , IL-1 α , and IL-1 β mRNAs and the onset of pathological changes in scrapie is suggestive of a causal relationship, but this is yet unproven. Recent studies indicate that cerebral overexpression in transgenic mice of another cytokine, IL-6, gives rise to a neurodegenerative disorder (4). In addition to a pronounced reactive astrocytosis in these mice, morphological changes seen in hippocampal neurons, including vacuolization and decreased dendritic arborization, resemble the neuronal pathological changes described in scrapie (22). Although cerebral IL-6 gene expression was not significantly elevated in scrapie, this cytokine has many overlapping actions with IL-1 and TNF- α . In general terms, TNF- α and IL-1 α/β are known to have diverse actions within the central nervous system (see references 18 and 29 for reviews). More specifically, in scrapie some of the known cellular and molecular changes are compatible with actions mediated by these cytokines. As discussed above, reactive astrocytosis concomitant with increased GFAP expression represents a prominent response in scrapie. IL-1 (16) and TNF- α (33) are known to be mitogenic for astrocytes. In addition, intracerebral injection of IL-1 in rats promotes reactive astrocytosis (17). The increased expression in the brain of the acute-phase response gene, EB22/5.3, during scrapie infection may also reflect a response to IL-1 and/or TNF- α . Cytokines including IL-1 and TNF- α are potent regulators of hepatic acute-phase-response gene expression (20). Additionally, IL-1 and TNF- α have been shown to induce the production of ACT (the human homolog of the EB22/5.3 gene product) from HepG2 cells (27). Therefore, although our findings do not establish whether the increased cerebral expression of the IL-1 α/β and TNF- α genes represents a causative or coincidental response in scrapie, their potent and diverse cellular actions indicate that these mediators could play a significant role in the pathogenesis of this disease.

Finally, the findings in the present study add further support to the concept of convergent pathological mechanisms in scrapie and Alzheimer's disease (8). Cerebral expression of both IL-1 (14) and ACT (1) is increased in Alzheimer's disease and, as we have shown here, in scrapie. Therefore, these gene products can be added to those for apolipoprotein E and cathepsin D (8) and for GFAP, sulfated glycoprotein 2, and transferrin (9), which also have increased cerebral expression in scrapie and Alzheimer's disease. This molecular profile of increased expression of cytokines, acute-phase response products, protease inhibitors, and proteases strongly implicates the involvement of localized chronic nonimmune inflammatory processes in the pathogenesis of these neurodegenerative diseases. Therefore, therapeutic strategies targeted at abating these inflammatory processes may be beneficial in ameliorating the progressive neuronal impairment and loss that are currently inevitable in both scrapie and Alzheimer's disease.

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