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Heightened expression of tumor necrosis factor α, interleukin 1α, and glial fibrillary acidic protein in experimental Creutzfeldt–Jakob disease in mice

(demyelination/reverse transcription-coupled PCR/cytokine)

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ABSTRACT **The ultrastructural pathology of myelinated axons in mice infected experimentally with the Fujisaki strain of Creutzfeldt–Jakob disease (CJD) virus is characterized by myelin sheath vacuolation that closely resembles that induced in murine spinal cord organotypic cultures by tumor necrosis factor α (TNF-α), a cytokine produced by astrocytes and macrophages. To clarify the role of TNF-α in experimental CJD, we investigated the expression of TNF-α in brain tissues from CJD virus-infected mice at weekly intervals after inoculation by reverse transcription-coupled PCR, Northern and Western blot analyses, and immunocytochemical staining. Neuropathological findings by electron microscopy, as well as expression of interleukin 1α and glial fibrillary acidic protein, were concurrently monitored. As determined by reverse transcription-coupled PCR, the expression of TNF-α, interleukin 1α, and glial fibrillary acidic protein was increased by approximately 200-fold in the brains of CJD virus-inoculated mice during the course of disease. By contrast, β-actin expression remained unchanged. Progressively increased expression of TNF-α in CJD virus-infected brain tissues was verified by Northern and Western blot analyses, and astrocytes in areas with striking myelin sheath vacuolation were intensely stained with an antibody against murine TNF-α. The collective findings of TNF-α overexpression during the course of clinical disease suggest that TNF-α may mediate the myelin sheath vacuolation observed in experimental CJD.**

Creutzfeldt–Jakob disease (CJD), a rapidly progressive transmissible spongiform encephalopathy, occasionally occurs as a panencephalopathic form characterized by severe white matter damage (1), resulting from active degradation of myelin by macrophages and astrocytes (2). The vacuolation of myelinated axons in experimental CJD (2), scrapie (3, 4), and kuru (5) closely resembles that induced by tumor necrosis factor α (TNF-α) in murine spinal cord organotypic cultures (6) and that found in demyelinating diseases, such as experimental allergic encephalitis (EAE) (7–11) and multiple sclerosis (MS) (12, 13). Moreover, our recent studies indicate similar pathological lesions in the optic nerves of mice after intraocular inoculation with recombinant murine TNF-α (14).

TNF-α and interleukin 1α (IL-1α) are synthesized in microglia and astrocytes (15–20), and immunopositivity for these cytokines has been detected in neurons (20). Both cytokines stimulate astrogliosis (21–23), one of the principal neuropathological hallmarks of the subacute spongiform encephalitidies. Recently, TNF-α and IL-1α transcripts have been found elevated in brains of scrapie-infected mice (24). To determine whether TNF-α is involved in the pathogenesis of myelin dilatation in experimental CJD, we examined brain tissues from CJD virus-infected mice, at weekly intervals after inoculation, for TNF-α expression by reverse transcription-coupled polymerase chain reaction (RT–PCR), Northern and Western blot analyses, and immunocytochemistry. Comparisons were made with neuropathological findings and with mRNA expression of IL-1α and glial fibrillary acidic protein (GFAP).

MATERIALS AND METHODS

CJD Virus. The Fujisaki strain of CJD virus, isolated from the brain of a 56-year-old Japanese man with progressive dementia (25) and passaged three times in mice in this laboratory, is characterized by an incubation period of 16–18 weeks after intracerebral inoculation in mice and by severe destruction of white and gray matter in the brain (2). Weanling, 4-week-old NIH-Swiss mice (Animal Production Area, Frederick Cancer Research and Development Center, Frederick, MD), lightly anesthetized with methoxyflurane, were injected intracerebrally (into the left hemisphere) with 0.03 ml of a 10% clarified brain suspension prepared from mice terminally ill with the Fujisaki strain of CJD virus (infectivity titer, 3.1×10^4 LD_{50} per 0.03 ml, by the intracerebral route). Control mice were inoculated with 0.03 ml of a 10% clarified normal NIH-Swiss mouse brain suspension. All procedures were conducted in accordance with guidelines of the National Institutes of Health Animal Care and Use Committee. Mice were killed at weekly intervals, from 1 to 22 weeks after inoculation. Brains were rapidly frozen in liquid nitrogen and stored at −80°C until use.

RT–PCR. Total cellular RNA, extracted using RNAzol (Tel-Test, Friendswood, TX) from approximately 100 mg of brain (frontal lobe of right cerebral hemisphere) collected at weekly intervals, was diluted in diethylpyrocarbonate-treated water to 400 μg/ml. RT–PCR was performed under identical conditions for amplification of TNF-α (product size: 264 bp; 5′-GAATGGGTGTTCATCCATTCT-3′/5′-ACATTCGAGGCTCCAGTGAATTCG-3′), IL-1α (product size, 491 bp; 5′-AAGATGTCCAACTTCACCTTCAAGGAGAGCCG-3′/5′-AGGTCGGTCTCACTACCTGTGATGAGTTTTGG-3′) (CLONTECH), GFAP (product size, 394 bp; 5′-CACAGGACCTCGGCACCCTG-3′/5′-GGAGGAGCTCTGCGTTGCGG-3′) (26), and β-actin (product size, 348 bp; 5′-TGG-

Abbreviations: TNF-α, tumor necrosis factor α; CJD, Creutzfeldt–Jakob disease; RT–PCR, reverse transcription-coupled polymerase chain reaction; IL-1α, interleukin 1α; GFAP, glial fibrillary acidic protein; EAE, experimental allergic encephalitis; MS, multiple sclerosis.

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AATCCTGTGGCATCCATGAAA-3′/5′-TAAAACGCA-GCTCAGTAACAGTCCG-3′) (27). Oligonucleotide primers were used at a concentration of 0.1 μM in a reaction mixture of 50 μl containing 0.4 μg of sample RNA, 1.25 units of *Thermus aquaticus* DNA polymerase (Perkin–Elmer and Roche Molecular Systems, Branchburg, NJ), 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, all four dNTPs (each at 0.2 mM), 20 units of RNasin, and 4 units of avian myeloblastosis virus reverse transcriptase (Promega). After incubation for 1 hr at 42°C, samples were heated for 60 sec at 96°C and then cycled 35 times (45 sec at 96°C, 45 sec at 55°C, 60 sec at 72°C) in a DNA thermal cycler (Perkin–Elmer model 480). RT–PCR for TNF-α, IL-1α, and GFAP was also performed on serial dilutions (1:10, 1:100, 1:200, 1:400, 1:800, and 1:1600) of brain RNA extracted from CJD virus-infected and control mice 22 weeks after inoculation. Amplified products were size fractionated by electrophoresis on 2% agarose gels containing ethidium bromide at 0.5 μg/ml.

Northern Blot Analysis. Ten micrograms of total cellular RNA, extracted from brains of CJD virus-infected and control mice, were electrophoresed on formaldehyde/0.8% agarose gels, then denatured, neutralized, and transferred to Nytran membranes (Schleicher & Schuell). Membranes were hybridized at 42°C overnight with random-primed ^{32}P-labeled cDNA specific for murine TNF-α in 5× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 50% formamide, 5× Denhardt's solution, 0.1% $NaDodSO_4$, and heat-denatured salmon sperm DNA at 100 μg/ml. Membranes were washed to final stringency of 0.1% SSC/0.1% $NaDodSO_4$ at 55°C for 45 min and were exposed to Kodak X-Omat film with intesifying screens at -80°C for 18–48 hr. The intensities of the radiolabeled bands were quantitated and compared using COLLAGE version 4.0 (Fotodyne, New Berlin, WI) and SIGMAGEL (Jandel, San Rafael, CA).

Western Blot Analysis. Individual cerebral hemispheres from CJD virus-infected and control mice were homogenized using Tenbroeck grinders placed on ice, and the resultant homogenates, prepared in chilled phosphate-buffered saline (PBS, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride and 1 mM EDTA, were centrifuged at 10,000 rpm in a Sorvall GSA rotor at 4°C for 10 min. Supernatants were kept on ice and protein concentrations were determined using the Bio-Rad protein assay kit. Aliquots (25 μg of protein) of CJD and control mouse brain homogenates were separated on $NaDodSO_4$/10% polyacrylamide gels, and proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked with 5% nonfat dairy milk, prepared in 50 mM Tris·HCl (pH 7.5) and 150 mM NaCl, for 2 hr at room temperature, then incubated overnight with rabbit antiserum prepared against murine TNF-α (Genzyme) at a dilution of 1:250 in 5% nonfat dairy milk. Membranes were washed and incubated at room temperature for 2 hr with alkaline phosphatase-conjugated affinity-purified goat anti-rabbit IgG [$F(ab')_2$ fragment]. Color was developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The intensities of the immunoreactive bands were analyzed using the imaging software noted above.

Immunohistochemistry. Formalin-fixed paraffin-embedded sections of brains, harvested at weekly intervals after inoculation, were stained by the avidin-biotin technique (Vector Laboratories), using polyclonal antisera (rabbit) prepared against murine TNF-α (Genzyme) and bovine GFAP (Dako). TNF-α and GFAP antibodies were used at dilutions of 1:400 and 1:100, respectively. Sections were incubated with the primary antibody overnight at 4°C and with the secondary antibody for 1 hr at room temperature. Color was developed using 0.05% diaminobenzidine (Sigma) with 0.01% hydrogen peroxide for approximately 10 min, and sections were counterstained with Mayer's hematoxylin. To ascertain the specificity of immunostaining, the primary antibody was omitted from the staining protocol or replaced with irrelevant antibodies.

Electron Microscopy. For ultrastructural studies, two or three CJD virus-inoculated and control mice were killed at weekly intervals by intracardiac perfusion with 180 ml of 1% paraformaldehyde and 1.5% glutaraldehyde prepared in PBS. Mice were kept at 4°C for 2 hr, and then brains were removed and rinsed in cold fixative overnight. Samples of the right parietal cortex, adjacent corpus callosum, and white matter of the internal capsule, all measuring approximately 1 mm^3, were rinsed in PBS, postfixed in 1% osmium tetroxide, dehydrated through a graded series of ethanols and propylene oxide, and embedded in Embed 812 (Electron Microscopy Sciences, Ft. Washington, PA). Ultrathin sections were stained with lead citrate and uranyl acetate, and coded specimens were examined using a Hitachi 11E or Philips 300 transmission electron microscope at 75 kv or 60 kv, respectively.

RESULTS

RT–PCR. As determined by the intensity of ethidium bromide-stained RT–PCR-amplified bands, TNF-α and IL-1α expression were significantly increased in brains of CJD virus-infected mice, beginning 15 week after inoculation (Fig. 1). Similarly, GFAP expression was more pronounced toward the terminal stage of experimental CJD (Fig. 1). Comparative RT–PCR on serially diluted RNA samples showed that these three transcripts were approximately 200-fold more abundant in CJD virus-infected brains at 22 weeks after inoculation, as evidenced by the intensity of the ethidium bromide-stained bands (Fig. 2). By contrast, β-actin expression in brain during the time course was identical in infected and uninfected mice and did not correlate with disease progression.

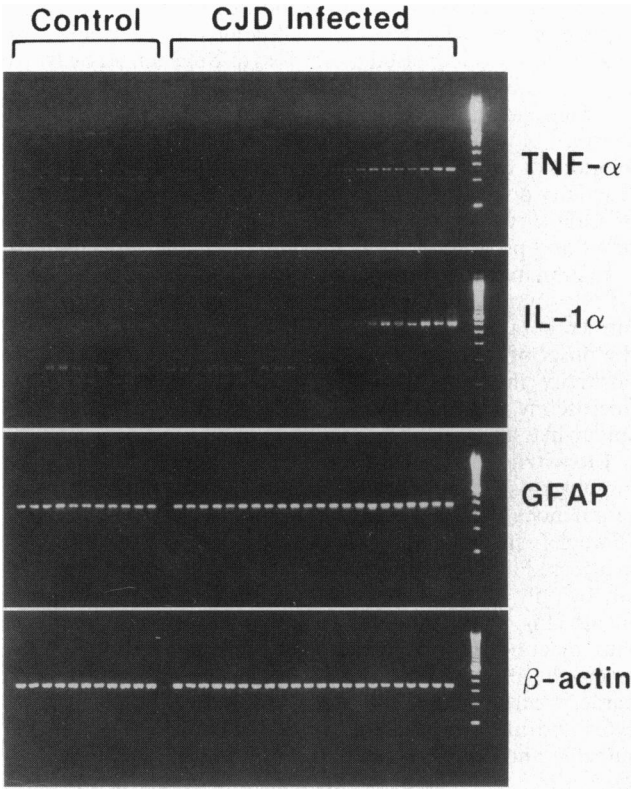

FIG. 1. Ethidium bromide-stained RT–PCR products of TNF-α, IL-1α, GFAP, and β-actin. Lanes: 1–11, brains from control mice at 2-week intervals from 2 to 22 weeks after inoculation; 12–33, brains from CJD virus-infected animals, at weekly intervals from 1 to 22 weeks after inoculation; 34, 100-bp ladder.

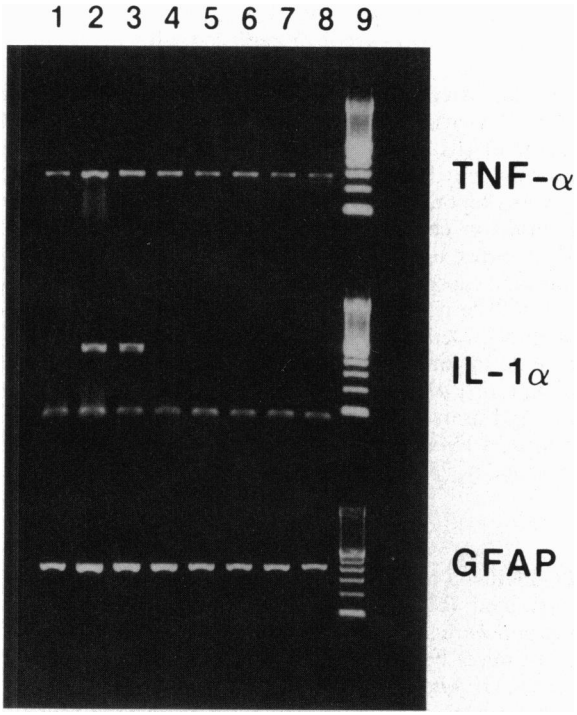

FIG. 2. Ethidium bromide-stained RT–PCR products of TNF-α, IL-1α, and GFAP. Lanes: 1, control brain at 22 weeks after inoculation; 2–8, CJD virus-infected brain at 22 weeks after inoculation. Dilutions were as follows. Lanes: 2, undiluted RNA; 3–8, total RNA at dilutions of 1:10, 1:100, 1:200, 1:400, 1:800, and 1:1600, respectively.

Northern and Western Blot Analyses. RT–PCR results for TNF-α were corroborated by Northern and Western blot analyses. Compared with control mouse brains, TNF-α mRNA expression in CJD virus-infected brains at 22 weeks after inoculation was increased by 1.5-fold as determined by Northern blot analysis. Moreover, as measured by densitometric scanning and quantitation of TNF-α-immunoreactive bands on Western blot, the TNF-α protein level was increased by 20% in brains of CJD virus-infected mice (Fig. 3). Increased TNF-α reactivity on Western blots, which was observed in three sets of CJD virus-inoculated mouse brains, correlated with the onset and progression of clinical disease.

Immunohistochemistry. Marked astrocytosis, visualized by GFAP immunostaining, was first noted 14–16 weeks after intracerebral inoculation with CJD virus; this coincided with the onset of clinical disease, as well as with TNF-α immunopositivity in astrocytes and myelin dilatation by electron microscopy. Immunopositivity for TNF-α was found only in spider-like astrocytes in CJD-infected mouse brains.

Ultrastructural Neuropathology. Vacuoles greatly distending myelin sheaths, initially observed 13 weeks after intracerebral inoculation with CJD virus, became more widespread at 16 weeks with the onset of clinical disease. Typically, the axons in affected fibers were shrunken and were adherent, occasionally by only a thin "neck," to the inner surface of the myelin sheath (Fig. 4). Astrocytes contained abundant glial filaments, thus meeting the criterion for hypertrophic astrocytes, and macrophages contained lipid droplets, myelin figures and paracrystalline "lyre"-like bodies. Occasionally both astrocytes and macrophages contained myelinated vacuoles, presumably undergoing digestion.

DISCUSSION

Vacuolation of myelinated fibers is now known to be an important feature of the ultrastructural pathology of the panencephalopathic form of CJD (1, 28–30). Myelin sheath

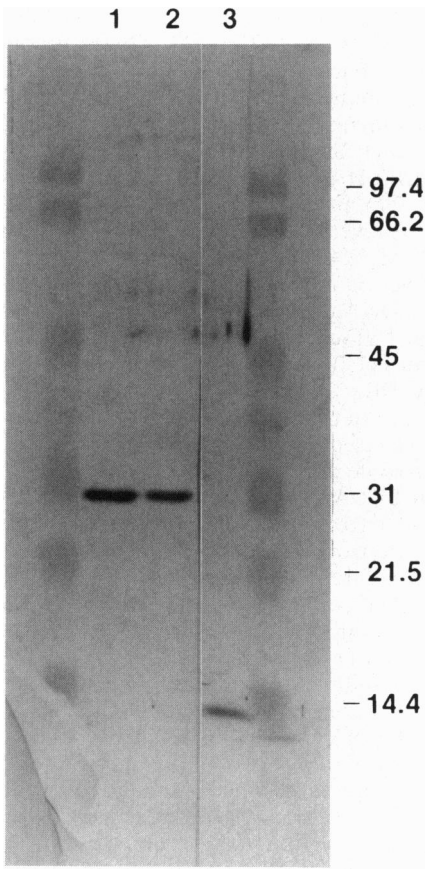

FIG. 3. Increased expression of TNF-α, as determined by Western blot analysis, in brain homogenates of mice killed 18 weeks after intracerebral inoculation with the Fujisaki strain of CJD virus. Lanes: 1, CJD virus-infected brain; 2, control brain; 3, recombinant murine TNF-α.

dilatation ("ballooning"), nearly identical to that observed in experimental CJD, has also been reported in scrapie (3, 31, 32) and kuru (5, 31, 32) and is frequently found in MS (12, 13) and in animal models of EAE (7–11). In addition, the formation of intramyelin vacuoles has been observed in rhesus monkeys with vitamin B_{12} deficiency (33), in rats after widespread axonal damage (34), in mice infected with Semliki Forest virus (35), in "quaking" mutant mice (36), and in mice intoxicated with cuprizone (37) or sodium cyanate (38). The separation of myelin lamellae at either the major dense or intraperiod lines contributes to the formation of vacuoles in the above-mentioned conditions. Myelin stripping results from macrophages introducing their cytoplasmic processes between myelin lamellae, and both macrophages and astrocytes participate in digestion of myelin (2).

The pathogenesis of myelin dilatation in CJD and demyelinating diseases, such as MS, is unknown. Myelin sheath vacuolation, ultrastructurally indistinguishable from those presented here, has been produced in mouse spinal cord cultures treated with recombinant human TNF-α (6). Myelin ballooning was accompanied by oligodendrocyte degeneration and astrocytic hypertrophy, and cytotoxic activity of TNF-α and lymphotoxin was reported (6). Similar findings in EAE have prompted the hypothesis that TNF-α, a cytokine released from astrocytes (17, 18) and activated microglia/macrophages (19), is directly involved in myelin breakdown in demyelinating disorders, presumably by interacting with sodium channels on the axolemma (23). This hypothesis has been further substantiated by immunohistochemical detection of TNF-α in astrocytes in brain tissues of patients with MS (39, 40) and acquired immunodeficiency syndrome (41), as well as by blocking

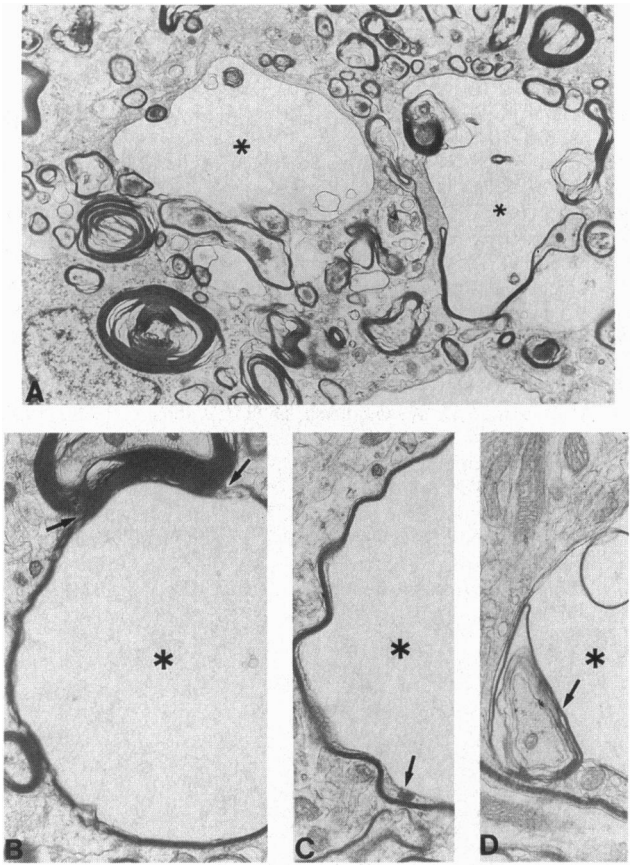

FIG. 4. Myelin sheath dilatation in the parietal cortex of an NIH-Swiss mouse 18 weeks after intracerebral inoculation with the Fujisaki strain of CJD virus. Lead citrate and uranyl acetate staining. Vacuoles are indicated by asterisk. Arrows indicate splitting of myelin lamellae in *B* and *D* and a shrunken axon in *C*. (*A*, ×3000; *B–D*, ×10,000.)

passive transfer of EAE by anti-TNF-α neutralizing antibodies (42) or by pentoxyifilline, a TNF-α inhibitor (43). Furthermore, the injection of recombinant murine TNF-α into the vitreous of the mouse eye produces lesions in the optic nerve that are indistinguishable from those observed in the panencephalopathic type of CJD (14).

CJD and the other subacute spongiform virus encephalopathies are regarded as polioencephalopathies or disorders of the gray matter (44). The Fujisaki strain of CJD virus is atypical in that it produces widespread myelin and axonal pathology (2). However, myelin pallor and focal accumulation of the products of myelin degradation are constant features of experimental kuru, CJD, scrapie, and bovine spongiform encephalopathy (4, 45–48), but to a lesser degree. We believe there is a common mechanism for axonal and myelin pathology in all the subacute spongiform encephalopathies. The ultrastructural pathology of myelinated fibers and the detection of TNF-α in astrocytes in areas of myelin vacuolation in mice infected with the Fujisaki strain of CJD virus indicate that myelin vacuolation in the subacute spongiform encephalopathies may be a cytokine-mediated phenomenon. The heightened expression of TNF-α mRNA (24) and TNF-α immunoreactivity in astrocytes (49) in brain tissues of scrapie-infected mice is consistent with these findings. Thus, accumulation of the infectious amyloid of CJD may initiate the following cascade of events: induction of the release of TNF-α, IL-1α, and probably other cytokines produces secondary astrocytosis and microglial infiltration, leading in turn to further release of cytokines (50) and ultimately to white matter damage.

In conclusion, upregulation of TNF-α and IL-1α in CJD-affected mouse brains correlated with the onset and progression of clinical disease. Thus, these cytokines appear to serve as molecular mediators of white matter degeneration in experimental CJD. That said, overexpression of TNF-α in neurodegenerative diseases as diverse as CJD, AIDS vacuolar myelopathy, and MS suggests that proinflamatory lymphokines may act as end-stage mediators of axon and myelin damage, irrespective of its cause.

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