In this study, we provide the first evidence implicating the newly identified Fas/Apo 1 (CD95) receptor/Fas ligand (Fas-L) cell death pathway in MS lesion pathogenesis. We show that Fas-L is widely present on glial cells in many chronic MS plaques and that white matter cells commonly carry receptors for this cytotoxin, rendering them sensitive to attack as judged by DNA fragmentation studies and Fas-L co-localization.

**Materials and Methods**

**Autopsy Specimens**

The study was performed on chronic MS plaques (n = 15) and control brains obtained from the Rocky Mountain Multiple Sclerosis Center (Englewood, CO), the MS human neurospecimen bank (West Los Angeles, CA) and from material collected over the past 23 yr at St. Vincent’s Hospital (New York, NY). Two paraffin-embedded acute MS plaques obtained from Dr. John Prineas (Newark, NJ) were also studied. The average post-mortem interval for MS paraffin-embedded material was 17.8 h and 5 were obtained within 7 h of death. The paraffin-embedded non-MS control brains included two ALS patients whose autopsies were performed within 3–6 h of death, HIV/AIDS encephalitis-2, Alzheimer’s disease-1, cerebrovascular disease-1, Parkinson’s disease-1, progressive multifocal leucoencephalopathy (PML)-1, acute hemorrhagic leucoencephalitis (AHLE)-1, glioblastoma multiforme-1.
tumor-1, and a child’s brain with chronic measles virus infection, subacute sclerosing panencephalitis (SSPE)-1.

Immunohistochemistry for Fas Ligand, Fas Receptor, and In Situ TUNEL Detection of Nuclear DNA Fragmentation.

Fas-L Detection. 3-μm paraffin embedded sections were attached to slides precoated with vectabond adhesive and dewaxed. Endogenous peroxidase activity was blocked by washing with 3% hydrogen peroxide for 5 min and antigenic sites were exposed by immersion in target unmasking fluid (TUF; Signet Labs, Dedham, MA) preheated to 95°C for 10 min. The slides were removed from the water bath and allowed to remain in the heated TUF solution for an additional 10 min. The slides were washed with PBS for 5 min × 3 and incubated overnight at 4°C with polyclonal anti-human Fas-L antibody at 0.5 μg/ml (Q20-Santa Cruz Biotechnology, CA). The slides were again washed with PBS for 5 min × 3 and antibody-positive cells were visualized with diaminobenzidine after reaction with the Vector ABC detection kit following the manufacturer’s instructions. Slides on which the primary antibody was omitted were used to control for non-specific binding. As a further test of specificity, lymph node tissue sections were tested with Fas-L antisera, and virtually all cells were completely negative with positive cells being restricted to small intensely stained clusters within the nodal parenchyma. The staining pattern obtained with Fas receptor antibody was also largely negative, except for a thin rim of positive cells which were localized to the lymph node subcapsular region. As an additional negative control, tissue sections from one of the non-inflammatory neurologic control brains which had no positive staining cells was routinely included in each experiment.

Fas/APO 1 (CD95) Receptor Detection. The slides for Fas/APO1 receptor detection were processed exactly as described for Fas-L except that a mouse monoclonal anti-human Fas receptor antibody at 10 μg/ml was used as the primary antibody (Oncor, Inc., Bethesda, MD).

In Situ TUNEL Detection. We used a modification of the TUNEL procedure (7) in which cells containing fragmented DNA are labeled with digoxigenin-11-dUTP and subsequently detected by an immunoperoxidase localization system. The in situ experiments were performed using kits purchased from Oncor (ApopTag kit) with minor modifications to the manufacturer’s directions and no counterstain was usually employed. When testing 3-μm-thick paraffin-embedded sections, we greatly curtailed the suggested deproteinization times with proteinase K (0–10 min) and reduced the terminal deoxynucleotidyl transferase (TdT) incubation time from the suggested 60 min to a range of 3–60 min. FITC-fluorescent TUNEL labeling was used in several co-localization experiments and these sections were evaluated by confocal microscopy. For these experiments, the Fas-L second label was visualized with a Cy3 anti-rabbit detection system.

Double Staining Immunohistochemistry. The ABC system was used for glial cell marker immunocytochemistry following the Fas-L detection procedure and visualization of the Fas reaction products with diaminobenzidine. Monoclonal primary antibody against astrocyte-specific glial fibrillary acidic protein (GFAP); dilution 1:300, undiluted Human NK marker (HNK-1) (Becton-Dickinson, Mountain View, CA) against oligodendroglia, and CD-3 pan T cell marker; dilution 1:1,200 (all from Dako Corp., Carpinteria, CA) was applied overnight at 4°C. The slides were rinsed and incubated for 30 min with biotinylated secondary antibody and the procedure repeated with peroxidase-conjugated avidin-biotin complex (Elite Peroxidase ABC kit; Vector Labs., Inc., Burlingame, CA). Reaction product on double-labeled cells was visualized with Vector SG chromagen. The monocYTE lineage-specific RCA-1 biotinylated lectin probe; dilution 1:1500 (Vector Labs.) was reacted for 1 h and reactions in the absence of primary antibody were used to control for nonspecific binding.

Fas ligand-labeled cells were quantified by two observers by light microscopy and video projection using a 24-mm eyepiece disk reticle (5 × 5 mm in 1-mm subdivisions) calibrated with a stage micrometer. 10 fields (0.1 mm²) from areas with maximal signal were quantified at 20× magnification. The number of Fas-L-bearing cells in the MS group was compared to the two control groups by the Kruskal-Wallis one way analysis of variance and the Mann Whitney U test. For double labeled cells, an average of 138 cells labeled for each glial-specific marker were scored for presence of the second (Fas ligand) label and expressed as a percentage of the total number counted.

Results

Quantification of Fas-L in MS Plaques. We first investigated whether or not Fas-L could be detected in sections from chronic plaques of MS white matter and from non-MS control brains by immunohistochemistry. Fig. 1 shows that of 16 chronic MS plaques tested 12 lesions had many more cells reacting with Fas-L antibody than control brains from neurologic conditions without inflammation where 4 of 5 brains were completely negative. The most reactive areas of the MS lesions on average had 190 Fas-L-positive cells in an area of 1 mm² and the two acute plaques had much higher values. In contrast to the non-inflammatory control brains, 4 of 6 brains from neuropathologic conditions associ-
Figure 2. Identity of Fas-L-positive cells in chronic MS lesions. (Top left), Fas-L (brown stain) co-labeled with RCA-1 lectin for macrophage/microglia lineage cells (blue stain). A large double labeled macrophage is visible on the left. Three smaller round cells which morphologically appear to be Fas-L-positive oligodendrocytes are also present. Magnification ×400. (Bottom left) Co-localization Fas-L experiment using an astrocyte marker - glial fibrillary acidic protein (GFAP) (blue stain). Double labeled astrocytes are present as well as numerous non-GFAP-positive cells which are heavily labeled by Fas-L antibody (brown stain). Magnification ×200. (Right) Co-localization with oligodendroglia-specific marker HNK-1 (blue stain). Many Fas-L HNK-1-positive oligodendroglia are present within the MS brain. Magnification ×200.

Related with inflammation clearly also had clusters of Fas-L-positive cells within their white matter lesions. Three of the four positive inflammatory brains were from conditions where multiple foci of primary demyelination are common. Two were associated with chronic CNS viral infections (SSPE and PML) and the third patient had acute hemorrhagic encephalitis, a primary demyelinating disease. Nerve cells served as an additional internal negative control for antibody specificity since they were consistently Fas-L negative in all brains tested.

Identification of Fas Ligand-positive Cells in MS Brain. The identity of Fas-L-positive cells in chronic MS lesions was determined by co-localization staining with markers specific for monocyctic lineage (macrophage/microglia [lectin/RCA 1]), astrocytes (GFAP), and oligodendroglia (HNK 1). Fig. 2 shows photomicrographs obtained when Fas-L-positive MS brain was dual labeled with the cell markers described above. The figure illustrates that several different cell types within MS lesions bear the Fas ligand. A few ligand-positive cells were reactive with lectin indicating they were of monocyctic lineage (upper left), but Fas-L-positive resident glial cells of either astrocytic or oligodendroglial origin were much more common (Fig. 2, lower left and right). The population profile of Fas-L-positive cells by cell type was quantified in chronic MS plaques and these results are shown in Fig. 3. The data confirms that the pattern of Fas-L-reactive cells in chronic lesions was complex composed mostly of ligand-positive resident glial cells with a small overlay of Fas-L-reactive macrophages. While many CD3-positive T cells were readily detectable in a SSPE control brain known to contain extensive T cell infiltrates, CD3 reactive cells were rarely found in chronic MS MS plaques and were detected in only two chronic MS brains.

The complex pattern characteristic of MS lesions was clearly not present in two of the inflammatory non-MS control brains where increased numbers of heavily labeled Fas-L cells were also present. The labeled cells were mainly of macrophage origin (>90%) in the white matter lesions from the child with chronic measles infection (SSPE) and the labeled cells in the adult PML brain could be readily distinguished from the MS pattern because Fas-L staining was largely restricted to white matter cells with viral inclusions (>90%). The pattern of Fas-L-positive cells in acute hemorrhagic encephalomyelitis, a primary demyelinating disease, was complex as seen in MS. These findings in three other conditions with demyelinating lesions suggest that the potential for involvement of the Fas death pathway is not restricted to MS and may be the preferred pathway of killing for other inflammatory diseases with white matter pathology.

Fas/Apo 1 (CD95) Receptor Expression in Diseased Brain. We next investigated to what extent Fas/Apo 1 (CD95) receptor was expressed in MS brains and non-MS controls as judged by immunohistochemistry because the demonstration of Fas ligand without its cognate receptor being present in the diseased area of the brains would be difficult to interpret. Fig. 4 shows that remarkably large numbers of strongly reactive Fas receptor-positive cells including some with glial cell morphologic characteristics were present within an acute white matter MS lesion, and the edematous plaque also contained numerous phagocytic macrophages which had engulfed Fas-positive cells. The presence of phagocytosed Fas-positive corpses in the MS lesion suggests that the Fas system is likely involved in the MS white matter pathologic process. Smaller numbers of strongly labeled Fas re-
Figure 4. Fas/Apo 1 (CD95) receptor antibody-positive cells in acute MS white matter plaque (brown stain). Many intensely stained receptor-positive individual cells and phagocytic macrophages containing several ingested Fas receptor-positive cells are present within the lesion (arrow). Magnification X400.

Figure 5. Confocal microscopy of Fas-L-positive MS plaques which have been previously FITC-labeled by the TUNEL procedure for fragmented DNA and then labeled for Fas ligand. (Left) Numerous Fas-L-positive cells have been detected by Cy3 (red label) and two dying cells are visible which were detected by the TUNEL assay (yellow signal). The fluorescent yellow staining cells (arrow) show good co-localization with cytoplasmic Fas ligand. Magnification X630. (Right) Higher magnification of another co-localization experiment showing two more TUNEL-positive cells with Fas-L-positive cytoplasm.

Fas-L Co-localizes with TUNEL-positive Cells in MS Brain. To examine the possibility that the Fas pathway contributes to cell killing in MS white matter, we first employed an FITC-immunofluorescent in situ TUNEL assay which detects low molecular weight fragmented DNA within dying cells. Slides from several TUNEL-positive chronic MS lesions were subsequently dual labeled with the Fas-L antisera which was visualized with a Cy3 detection system. The slides were evaluated by laser scanning confocal microscopy and as shown in Fig. 5, numerous Cy3-positive red staining Fas-L cells were found. The figure also illustrates that the yellow FITC-TUNEL-positive dying cells showed good co-localization with presence of Fas-L. The converse experiment in which TUNEL-positive lesions were dual labeled for Fas receptor similarly showed that the dying cells also displayed the Fas/Apo 1 (CD95) receptor (data not shown). These findings indicate that attack of receptor-positive cells by Fas-L is possible in MS brain and that Fas/Fas-L mediated killing could be an important factor in the destruction of MS white matter.

Discussion

Evidence is rapidly accumulating that the Fas/Apo 1 (CD95)/Fas-L system is an important pathway responsible for the induction of cell death by apoptosis in homeostasis and disease states within several diverse tissues. Fas receptor (CD95) is a cell surface glycoprotein member of the tumor necrosis factor/nerve growth factor receptor superfamily and is widely expressed throughout the body (8, 9). Fas receptor can be readily induced by cytokines such as TNF-α and interferon-γ in some tissues including glial cells where it is not constitutively expressed (13, 14). A cytoplasmic region called the “death domain” is present on the Fas receptor which shows strong homology with a similar domain on the TNF-R (15). This highly conserved domain is necessary and sufficient for transduction of the apoptotic signal (16).

The evidence to date indicates that Fas ligand, the other member of the pair, is much more restricted in its distribution and is constitutively expressed mainly in T cell-rich organs (17, 18), stroma cells of the eye, and the testes (19, 20). Fas-L expression can be upregulated in cytotoxic T cells, NK cells, and the TH1 subset of T helper cells. These activated T cells can generate both membrane bound and soluble forms of Fas-L (sFas-L) which in certain leukemia/lymphoma patients achieves measurable serum levels (21). The patients with high levels of sFas-L are neutropenic and some have hepatic damage (22). The Fas pathway is thought to be important in controlling the rapid turnover of mature neutrophils by apoptosis (23). Several additional lines of evidence point to a role for accelerated Fas mediated destruction of liver tissue infected with hepatitis B or hepatitis C (24, 25). The Fas receptor/Fas-L system also plays a critical role in the destruction of T pleo...
homeostatic role in regulation of normal T cell deletion in the periphery as demonstrated in vivo by the enlarged lymphoid organs and enhanced lymphoproliferation present in lpr/lpr mutant mice which are defective in Fas receptor (26–29).

In this report, we present evidence that significant numbers of glial cells within MS plaques bear the cytotoxic effector molecule Fas-L. Of equal importance, ~30% of the positive cells belong to an HNK-1-positive oligodendroglial population. This glial cell is responsible for the production and support of myelin within the central nervous system. We also showed immunohistochemical evidence of widespread Fas receptor upregulation in the same MS lesions, indicating that both Fas death system components are available for interaction and possible Fas mediated cell destruction. The co-localization of TUNEL-positive dying cells with Fas-L, and the presence of numerous Fas-receptor-positive corpses within lesional macrophages support the notion that Fas receptor carrying cells are indeed a target of Fas-L attack in MS lesions.

The precise pathway for Fas signaling in brain white matter has not yet been studied. However, a recent report on Fas mediated apoptosis in mouse w4 transformed cells (which constitutively express mouse Fas receptor) found that Fas activation initially stimulates ICE-like proteases, and then CPP32-like proteases downstream to induce the morphologic changes and DNA fragmentation pattern typical of apoptosis (30). This in vitro Fas-mediated process could be readily blocked by specific inhibitors of ICE and CPP32 family proteases indicating that if a role for Fas involvement and its pathway can be defined in MS, it may be possible to block the Fas-L–induced destruction by inhibition of the intracellular protease families mentioned above, solubilized Fas receptor, or other pharmacologic means.

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