

TUMOR NECROSIS FACTOR IDENTIFIED  
IN MULTIPLE SCLEROSIS BRAIN

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Multiple sclerosis (MS) is a disease characterized by focal perivascular inflammatory cell infiltrates and demyelination of the white matter in the central nervous system (CNS). At the plaque site, there may be a depletion of oligodendroglial cells, proliferation of astrocytes, and an accumulation of positive macrophages (1-3). In a previous study, we found the cells in the MS lesion to be IL-2-R<sup>+</sup> cells, also staining for the cytokines IL-1, IL-2, and prostaglandin E (PGE) (4). These results suggested that an active immune response was taking place in the MS brain lesion. In this report, we further characterize the cell populations in the MS plaque, and find TNF<sup>+</sup> cells present. Using single and double immunohistochemistry techniques, we identified the TNF<sup>+</sup> cells as astrocytes as well as macrophages.

Materials and Methods

*Tissue Samples.* Six cases of MS were studied. The postmortem autopsy tissues came from patients diagnosed as having either the progressive or relapsing-remitting form of the disease. Tissues were obtained from the peri-ventricular white matter of frontal, temporal, or occipital lobes regions. The MS lesions included in this study were chronic, active plaques based on the morphological criteria (5). Six normal control brain tissues were obtained at autopsy from adult patients who died of nonbrain-related disease and demonstrated no visible plaques. Other neurologic diseases studied included six cases of Alzheimer's disease and one case of subacute sclerosing panencephalitis (SSPE). The tissues used in this study were frozen within 10 h of death.

*Tissue Processing.* Autopsy specimens were snap frozen in liquid nitrogen and then stored at -80°C. Cryostat sections, 8-10 μm thick, were cut, air dried overnight, and fixed in acetone (reagent grade) for 5 min at 25°C.

*Antibodies Used.* Both rabbit polyclonal TNF (kindly supplied by Genetech, S. San Francisco, CA) and mouse monoclonal TNF (kindly supplied by Cetur Corp., Emeryville, CA) were titrated on PHA-activated PBMC to determine the optimal concentrations. The anti-macrophage antibodies, CD-11C (monoclonal IgG1; Becton Dickinson & Co., Mountain View, CA) and HAM56 (monoclonal IgM; Enzo Biochem, Inc., New York, NY), and anti-HLA-Dr (Becton Dickinson & Co.) are routinely used in this laboratory and were tested on a wide variety of tissues to determine cell specificity and optimal antibody concentration. Both CD-11C and HAM 56 stain monocytes, macrophages, microglia, and resident tissue macrophages (e.g., Kupffer cells, alveolar macrophages). HAM 56 also stains endothelial cells. Polyclonal

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rabbit antiglial fibrillary acidic protein was obtained from Dakopatts, Carpinteria, CA. Irrelevant antibodies were used as controls for monoclonal and polyclonal antibodies. Negligible background was observed.

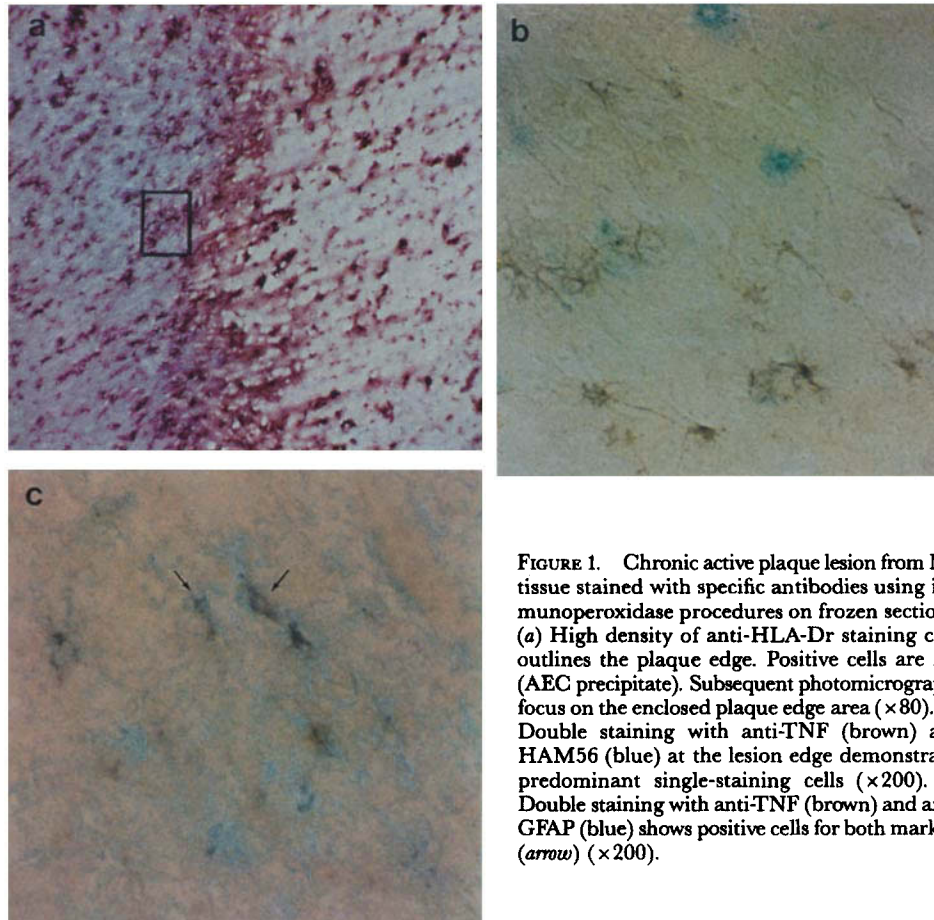
**Immunoperoxidase Single-staining Procedure.** This method has been described extensively (6). Briefly, frozen sections were incubated with primary antibody for 30 min at 25°C for polyclonal TNF and 37°C for monoclonal TNF in a humidified chamber. Subsequently, the biotin-labeled secondary antibody was used, either biotin-conjugated horse anti-mouse IgG, goat anti-mouse IgM, or goat anti-rabbit Ig (Vector Laboratories, Inc., Burlingame, CA) depending on the nature of the primary antibody used. Tissue sections were then incubated in a complex of StreptAvidin horseradish peroxidase (Biogenex, San Ramon, CA) for 20 min and the colored substrate amino-ethyl carbazole (AEC) was applied. The slides were then counterstained with Mayer's hematoxylin and coverslipped.

**Double-staining Procedure.** The double-staining method is a modification of a technique previously reported (4). Frozen sections were treated with the primary antibody for 30 min. Subsequently, the slides were incubated with biotinylated horse anti-mouse affinity-purified antibody (Vector Laboratories, Inc.) at 1:100 for 30 min. The slides were then exposed to the StreptAvidin horseradish peroxidase for 20 min. After this incubation, the slides were exposed to diaminobenzidine (DAB) (dark brown precipitate). At this point, the second primary mAb was applied to the section for 30 min. Subsequently, biotinylated goat anti-rabbit Ig (Vector Laboratories, Inc.) was added to the slide for 30 min followed by a 20-min incubation with B-galactosidase-conjugated StreptAvidin (Biogenex). The substrate, consisting of 20 mg/ml 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside in *N,N* dimethyl formamide, 10 mM NaPO<sub>4</sub>, pH 7.2, 0.15 M NaCl, 1.0 mM MgCl<sub>2</sub>, was added for 15 min at 37°C. The slides were then washed in tap water for 10 min and mounted in glycerol/gelatin. No nuclear counterstain was used to avoid difficulty in interpreting three-color tissue preparations. Control slides included the omission of the second primary antibody; we observed no blue staining in these controls, indicating that the second secondary reagent did not bind to any of the previously applied reagents in the first part of the staining procedure. Other controls included double staining with anti-T cell and antimacrophage antibodies to identify background double staining. All controls were performed initially on human tonsil and then on MS brain tissue, based on the knowledge that brain tissue may have unique background staining characteristics.

**Analysis of Staining Pattern.** For MS tissues, the areas examined were in the center of the plaque, the edge of the plaque, and the adjacent white matter. In SSPE tissues, the lesions and white matter adjacent to the demyelinated area were evaluated. In normal brain, white matter and cortex was examined; in Alzheimer's disease, hippocampus was analyzed. Double-staining results were calculated as follows: a 1-mm<sup>2</sup> grid was placed on three to five different fields at the outer edge of the plaque, chosen because this area had the greatest number of TNF<sup>+</sup> cells. The cells within the grid, ranging from 10 to 50 cells/mm<sup>2</sup>, were identified as blue, brown, or double staining. A total of 200 stained cells were counted for each group; the percent positive was calculated as the number of single or double-staining cells divided by the total number of staining cells.

## Results

The MS lesions examined included chronic active plaques and adjacent white matter. To identify and localize the MS plaque edge, cryostat sections were stained with anti-HLA-Dr. Class II antigens have been shown to be most dense at the edge of chronic active plaque lesions (Fig. 1 *a*). Six MS specimens were then stained with monoclonal and polyclonal anti-TNF. The results demonstrate that TNF<sup>+</sup> cells were most numerous distributed at the lesion edge, with very few positive cells within the plaque. The majority of TNF<sup>+</sup> cells were morphologically identified as reactive fibrous astrocytes based on their characteristic spherical or ellipsoidal nuclei, with numerous fine cytoplasmic processes. Adjacent sections stained with anti-GFAP demonstrated morphologically characteristic reactive astrocytes at the lesion edge.



**FIGURE 1.** Chronic active plaque lesion from MS tissue stained with specific antibodies using immunoperoxidase procedures on frozen sections. (a) High density of anti-HLA-Dr staining cells outlines the plaque edge. Positive cells are red (AEC precipitate). Subsequent photomicrographs focus on the enclosed plaque edge area ( $\times 80$ ). (b) Double staining with anti-TNF (brown) and HAM56 (blue) at the lesion edge demonstrates predominant single-staining cells ( $\times 200$ ). (c) Double staining with anti-TNF (brown) and anti-GFAP (blue) shows positive cells for both markers (*arrow*) ( $\times 200$ ).

In the one case of SSPE, which exhibited severely gliotic white matter with high numbers of perivascular leukocytes,  $\text{TNF}^+$  cells with astrocyte characteristics were also present in the diseased area. Six cases of Alzheimer's disease and six normal control brain specimens did not exhibit anti-TNF staining.

To determine whether the TNF staining was associated with the macrophages present at the lesion site, two sets of double-staining studies with anti-TNF and antimacrophage antibodies were performed. In double staining with monoclonal anti-TNF and antimacrophage HAM56, the majority of cells exhibit single staining, indicating that the majority of  $\text{TNF}^+$  cells were not macrophages (Table I). On a second MS specimen, similar results were obtained with an alternative double-staining combination, using polyclonal anti-TNF and monoclonal anti-CD11C (Table I). Fig. 1 *B* shows the results of double staining at the lesion edge.

Double-staining experiments were performed using anti-GFAP and anti-TNF to confirm the TNF association with astrocytes. In one experiment, shown in Fig. 1 *c*,  $\text{GFAP}^+$  cells (blue) comarked with TNF (brown), indicating double-staining cells.

TABLE I  
*Double Staining for TNF and Macrophage Markers*

Exp.		Percent positive		
		TNF	Macrophage	Double staining
I	TNF + macrophage	11	88	1
II	Macrophage + TNF	25	68	7

The great majority of positive cells were GFAP, as expected. Counting single- and double-staining cells was very difficult and not clear because astrocytes have numerous processes throughout the different layers of tissue.

### Discussion

The presence of TNF<sup>+</sup> cells at the MS lesion site, strongly suggests that an active immune response with participating stimulated leukocytes and glial cells is taking place. Recently, a report by Merrill et al. (7) has shown that in MS patients, the levels of TNF and IL-1 are increased in the blood and spinal fluid. These studies suggest that TNF may be relevant in MS in disease development. TNF has been reported to have a wide range of functions, including the induction of IL-1 secretion (8) and enhancement of monocyte cytotoxicity (9). Studies have also shown that TNF can function synergistically with IL-1 and IFN- $\gamma$  (10, 11) to augment monocyte/macrophage cytotoxicity. Recent reports have demonstrated that TNF may participate in destruction of oligodendroglial cells and the degeneration of the myelin sheath (12, 13). Thus, TNF is implicated directly in demyelination or indirectly by activating a cell-mediated cytotoxicity process.

Double-staining studies indicate that TNF is predominantly associated with astrocytes in the MS lesion. Although TNF is produced by activated macrophages, recent studies have suggested that activated astrocytes (12) and microglial cells (14) can also synthesize and secrete this cytokine. Astrocytes have many functional properties similar to those of macrophages, such as class II antigen expression (15), antigen presentation (16), PGE, and IL-1 secretion (17, 18). Thus, the astrocytes in the MS lesion, which are likely to be activated based on class II antigen expression, may also be responsible for the production of TNF. These immunocytochemical studies showing TNF binding to astrocytes do not identify the cellular source of this cytokine. Thus, macrophages may indeed be producing TNF, while the astrocytes may be the target cell for binding and internalization of this factor. Further experiments using molecular probes to determine the cellular source of TNF are underway.

TNF<sup>+</sup> cells were also detected in the virally induced disease SSPE, but not in Alzheimer's disease or normal CNS tissue. SSPE brain often exhibits inflammatory cell infiltration, which correlates with the site of demyelination. Our studies have shown that in SSPE, class II antigen expression on astrocytes as well as macrophages was prevalent (data not shown). This cell marker pattern was clearly reminiscent of the MS lesion. Since TNF<sup>+</sup> cells were also found in SSPE, we hypothesize that TNF may be universally involved in inflammatory cell-related demyelination.

In summary, this study demonstrates that TNF is present at the MS lesion site and is associated with the astrocytes at the plaque edge. This cytokine is also found in another neurological disease that demonstrates inflammatory cell infiltration. These data suggest that TNF may be produced during an active immune response in the CNS and is involved in demyelination and disease progression.

### Summary

Frozen brain specimens from patients with multiple sclerosis (MS) and other neurologic diseases were analyzed using immunocytochemical techniques for the presence of TNF. In brain lesions in MS, and subacute sclerosing panencephalitis, TNF<sup>+</sup> cells were demonstrated. At the lesion site in MS, TNF<sup>+</sup> staining is associated with both astrocytes and macrophages. These observations were not made in Alzheimer's disease or normal brain tissue. The presence of TNF in MS lesions suggests a significant role for cytokines and the immune response in disease progression.

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