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Tumor Necrosis Factor Expression during Mouse Hepatitis Virus-Induced Demyelinating Encephalomyelitis

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Neutralizing anti-tumor necrosis factor alpha (TNF-α) antibody treatment of mice infected with the neurotropic JHMV strain of mouse hepatitis virus showed no reduction of either virus-induced encephalomyelitis or central nervous system demyelination. TNF-α-positive cells were present in the central nervous system during infection; however, TNF-α could not be colocalized with JHMV-infected cells. In vitro, TNF-α mRNA rapidly accumulated following JHMV infection; however, no TNF-α was secreted because of inhibition of translation. Both live and UV-inactivated virus inhibited TNF-α secretion induced by lipopolysaccharide. These data show that TNF-α is not secreted from infected cells and indicate that it contributes to neither JHMV-induced acute encephalomyelitis nor primary demyelination.

Tumor necrosis factor alpha (TNF-α) is a pluripotent proinflammatory cytokine produced during inflammation primarily by macrophages (4, 42). It plays a role in regulating immune responsiveness, either alone or synergistically with other cytokines (3, 4, 16, 42), and has been implicated as a mediator of damage during both infection and autoimmune disease of the central nervous system (CNS). TNF-α-positive monocytes, microglia, and astrocytes are found in the CNS of patients with subacute sclerosing panencephalitis, progressive rubella panencephalitis, and multiple sclerosis (17, 18). In addition, it has been implicated in the regulation of parasitic encephalitis and experimental allergic encephalomyelitis (EAE) (13, 14, 20, 32). For example, TNF-α increases the severity of EAE (20), and T-cell-mediated transfer of EAE is inhibited by anti-TNF antibody (5, 32), possibly by preventing increased blood-brain barrier permeability (19, 33). Furthermore, in vitro incubation of spinal cord segments with TNF-α results in selective damage to the myelin sheath (30), and cultured oligodendroglia derived from neonates are susceptible to TNF-α-mediated cytotoxicity (34, 35). These data support the hypothesis that TNF-α plays a direct role in CNS diseases, especially those associated with demyelination (3, 16).

TNF-α exhibits functional dualities during viral infections. Viral infection (46) or purified viral proteins stimulate TNF-α release from macrophages (25), which, in turn, exhibits in vitro antiviral activity (46). TNF-α treatment during viral infections has resulted in dramatically different outcomes. Early treatment inhibits the clinical symptoms of lymphocytic choriomeningitis virus-induced leptomeningitis, while later treatment exacerbates both lymphocytic choriomeningitis virus and cytomegalovirus infections (7, 15). CNS infection by the neurotropic JHMV strain of mouse hepatitis virus results in acute encephalomyelitis accompanied by primary demyelination in both primates and rodents (9, 22, 23, 26), which appears to be due to preferential infection and destruction of oligodendroglia (23). There is, however, a clear requirement for an immune component since demyelination is prevented in immunosuppressed mice (43). After in vitro infection with JHMV, many host mRNAs are degraded; however, TNF-α mRNA increases (21). Similarly, the infection of mice with the OBLV60 variant of JHMV induces TNF-α mRNA in the CNS (27). JHMV replicates in recruited macrophages, astrocytes, and microglia within the CNS (37, 39), all of which can potentially secrete TNF-α (3, 4, 12, 24, 25). Therefore, during JHMV infection, cells of the immune infiltrate as well as resident CNS cells have the capacity to secrete cytokines, including TNF-α. Indeed, it has been suggested that the secretion of TNF-α during infection contributes to either JHMV-induced encephalomyelitis or demyelination (40).

Comparison of infections in both immunocompetent and nude mice indicated that TNF-α mRNA was expressed by both endogenous CNS cells as well as infiltrating mononuclear cells (27). Similar results were found when both BALB/c and C57BL/6 immunocompetent mice and mice immunosuppressed by 400-R whole-body irradiation were compared (data not shown). To determine if TNF-α influences either JHMV-induced encephalitis or demyelination, BALB/c and C57BL/6 mice (obtained from the Jackson Laboratory, Bar Harbor, Maine, and used at 6 to 7 weeks of age) were infected by intracranial injection of 10^4 PFU of JHMV antigenic variant 2.2-V-1 (11), kindly supplied by J. Fleming (University of Wisconsin). Concentrated serum-free neutralizing rat anti-TNF-α monoclonal antibody (MAb) MP6-XT22, kindly supplied by R. Coffman, DNAX Research Institute (Palo Alto, Calif.), was injected (1 mg per recipient) into the peritoneal cavity 2 days before and 2 days after infection. This MAb neutralizes both murine TNF-α and TNF-β, is effective in vivo (1, 29), and neutralizes 50% of murine TNF-α activity at approximately 150 ng/ml. No differences in either the course of clinical disease or virus titer within the CNS were found when anti-TNF-α MAb-treated and untreated control mice were compared (data not shown). For histopathological analysis, mice were sacrificed at 7 and 12 days postinfection by CO_2 asphyxiation. Brains were removed and fixed for 3 h in Clark's solution (75% ethanol and 25% glacial acetic acid). Paraffin-embedded sections were stained with either hematoxylin and eosin or luxol fast blue. No significant differences in inflammatory cell infiltration were found when the brains of anti-TNF-α MAb-treated C57BL/6 and BALB/c mice were compared with those

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of untreated control mice at 7 days postinfection (data not shown). Similarly, comparisons of untreated and anti-TNF-α MAb-treated BALB/c and C57BL/6 mice at 12 days postinfection showed no differences in the extent of either encephalitis or demyelination (Fig. 1), suggesting that the neutralization of TNF-α affects neither the cellular infiltration nor extent of virus-induced demyelination. Diffuse immunoreactivity specific for rat immunoglobulin was found in the white matter of anti-TNF-α MAb-treated mice at 7 days postinfection but was not found in untreated mice, confirming that the transferred anti-TNF-α MAb had crossed the blood-brain barrier during infection (data not shown). In addition, histological analysis of the CNS showed that TNF-α-positive microglia and astrocytes were widespread within the parenchyma of JHMV-infected mice. JHMV antigen and TNF-α were examined by immunoperoxidase staining (Vectastain-ABC kit; Vector Laboratories, Burlingame, Calif.) and by using anti-JHMV MAb, J.3.3, specific for the nucleocapsid protein (10) or anti-TNF-α MAb (Pharmingen, San Diego, Calif.). However, JHMV antigen and TNF-α could not be localized to the same cells, suggesting that infection activated the synthesis of TNF-α only in uninfected cells (data not shown).

To establish a correlation between increased TNF-α mRNA and the release of active cytokine, TNF-α secretion from JHMV-infected or lipopolysaccharide (LPS)-treated J774.1 cells and peritoneal exudate cell (PEC)-derived macrophages were compared. The J774.1 BALB/c-derived macrophage cell line was propagated as previously described (38). PEC were induced by intraperitoneal injection of 3.0 ml of thioglycolate broth (Difco, Detroit, Mich.) (39). Mice were sacrificed 72 h later, and PEC were harvested from the peritoneal cavity of each mouse. After overnight incubation at 37°C, nonadherent cells were removed by three washes with RPMI medium. Cells were infected with the DM isolate of JHMV (37) by using cell-associated virus prepared by infecting monolayers of DBT cells on 150-mm-diameter plates with JHMV at a multiplicity of infection of approximately 0.01. After overnight incubation, cells were removed and suspended in 4 ml of Dulbecco's phosphate-buffered saline (pH 7.4). A cell-free supernatant was prepared by sonication and subsequent clarification at 1,500 × *g* for 20 min. Alternatively, J774.1 cells were incubated in RPMI medium containing 5% fetal calf serum and 200 ng of lipopolysaccharide (LPS) (*Escherichia coli* 055:B5; Sigma Chemical Co., St. Louis, Mo.) per ml to induce TNF-α mRNA and secretion. PEC-derived macrophages were incubated in the same medium supplemented with 25 ng of LPS per ml. TNF-α cytotoxicity was measured by microassay with BC10ME target cells treated with 1 μg of actinomycin D per ml as previously described (44). Various concentrations of human recombinant TNF-α (Collaborative Biomedical Products, Bedford, Mass.) and UV-inactivated test supernatants were incubated with target cells for 18 h at 37°C. TNF-α was not detected in the medium derived from JHMV-infected cells at 6 h postinfection (Fig. 2). In addition, no TNF-α was detected as late as 12 h post-JHMV infection of J774.1 cells or as late as 24 h postinfection of PEC-derived macrophages (data not shown). As expected, LPS induced the secretion of TNF-α from all three cell types by 6 h (Fig. 2). The J774.1 cell line and macrophages from both strains of mice were infected with JHMV and incubated in the presence of LPS for 6 h to determine the effects of virus infection on the release of TNF-α. JHMV infection prior to LPS addition significantly reduced TNF-α release compared with that of cells treated with LPS only (Fig. 2). Infection of both J774.1 cells and macrophages from BALB/c mice prior to the addition of LPS reduced the TNF-α yield by ≥80%. By contrast, only a partial (≈60%) reduction in TNF-α yield from C57BL/6-derived macrophages was found (Fig. 2). To begin to understand the role of virus in suppressing TNF-α release, cells were infected either with UV-inactivated virus only or with UV-inactivated virus, followed by the addition of LPS. Infection with UV-inactivated virus alone did not result in the secretion of TNF-α (Fig. 2), demonstrating the absence of LPS in the virus preparation. Infection with UV-inactivated virus was almost as effective as infectious virus at suppressing LPS-induced TNF-α release from J774.1 cells and both BALB/c- and C57BL/6-derived macrophages (Fig. 2).

In parallel to the determination of TNF-α, RNA was extracted and relative mRNA expression was quantitated as previously described (41). Figure 2D compares the levels of TNF-α mRNA to the levels of tubulin mRNA in BALB/c-derived macrophages. Tubulin mRNA was chosen for comparison since it is less sensitive to viral infection than actin mRNA is (21); however, it is partially degraded by 6 h postinfection and therefore underestimates the level of TNF-α mRNA induced. Viral infection resulted in an eightfold increase in TNF-α mRNA in BALB/c-derived PECs and a sevenfold increase in C57BL/6-derived PECs, relative to tubulin mRNA, consistent with the previous demonstration of TNF-α mRNA induction in J774.1 cells (21). LPS induced a 14-fold increase in BALB/c-derived cells and a 25-fold increase in C57BL/6-derived cells, consistent with the higher level of LPS-induced TNF-α release from C57BL/6-derived cells (Fig. 2). Viral infection prior to LPS treatment induced 6- and 20-fold increases in TNF-α mRNA in BALB/c-derived cells and C57BL/6-derived cells, respectively. Therefore, prior infection with JHMV resulted in a minimal reduction in LPS-induced TNF-α mRNA. This reduction was not considered to be sufficient to account for the inhibition of TNF-α secretion, since there was at least a fourfold increase in TNF-α mRNA in both BALB/c- and C57BL/6-derived cells by 4 h post-LPS induction (data not shown). Infection of BALB/c-derived cells with UV-inactivated virus did not induce TNF-α mRNA; however, only a twofold increase in TNF-α mRNA was found following subsequent induction with LPS (Fig. 2). These data demonstrate that infection with live JHMV, but not UV-inactivated JHMV, induces the transcription of TNF-α mRNA and that JHMV infection results in a reduction in LPS-induced TNF-α mRNA which is insufficient to account for the absence of TNF-α secretion from infected cells.

These data suggest that JHMV prevents either TNF-α secretion or translation or the proteolytic processing of the 26-kDa TNF-α precursor to the 17-kDa secreted form (7). TNF-α synthesis in PEC-derived macrophages was examined at 6 h postinfection by Western blot (immunoblot) with a polyclonal rabbit anti-TNF-α antibody (Genzyme, Boston Mass.) reactive to both the 26- and 17-kDa TNF-α proteins. No immunoreactive species of the expected molecular weights were detected in mock-infected PEC derived from BALB/c or C57BL/6 mice (Fig. 3). Similarly, cells infected with JHMV showed no evidence of translation of either TNF-α species. By contrast, cells treated with LPS contained both the 26- and 17-kDa forms of TNF-α, consistent with the translation, processing, and secretion of bioactive TNF-α (Fig. 2). These data indicate that JHMV infection activates TNF-α transcription but inhibits TNF-α translation, consistent with previous data that demonstrated decreased overall protein synthesis in infected cells and preferential translation of mRNAs containing 5′ viral leader sequences (41).

Analysis of BALB/c-derived cells infected with UV-inactivated JHMV prior to LPS treatment reveals faint 26- and 17-kDa TNF-α bands. Similarly, no TNF-α was detected in C57BL/6-derived macrophages following absorption of UV-inactivated JHMV; however, both the 26- and 17-kDa bands were detected following LPS induction (Fig. 3), consistent with the inability of JHMV infection to completely inhibit TNF-α

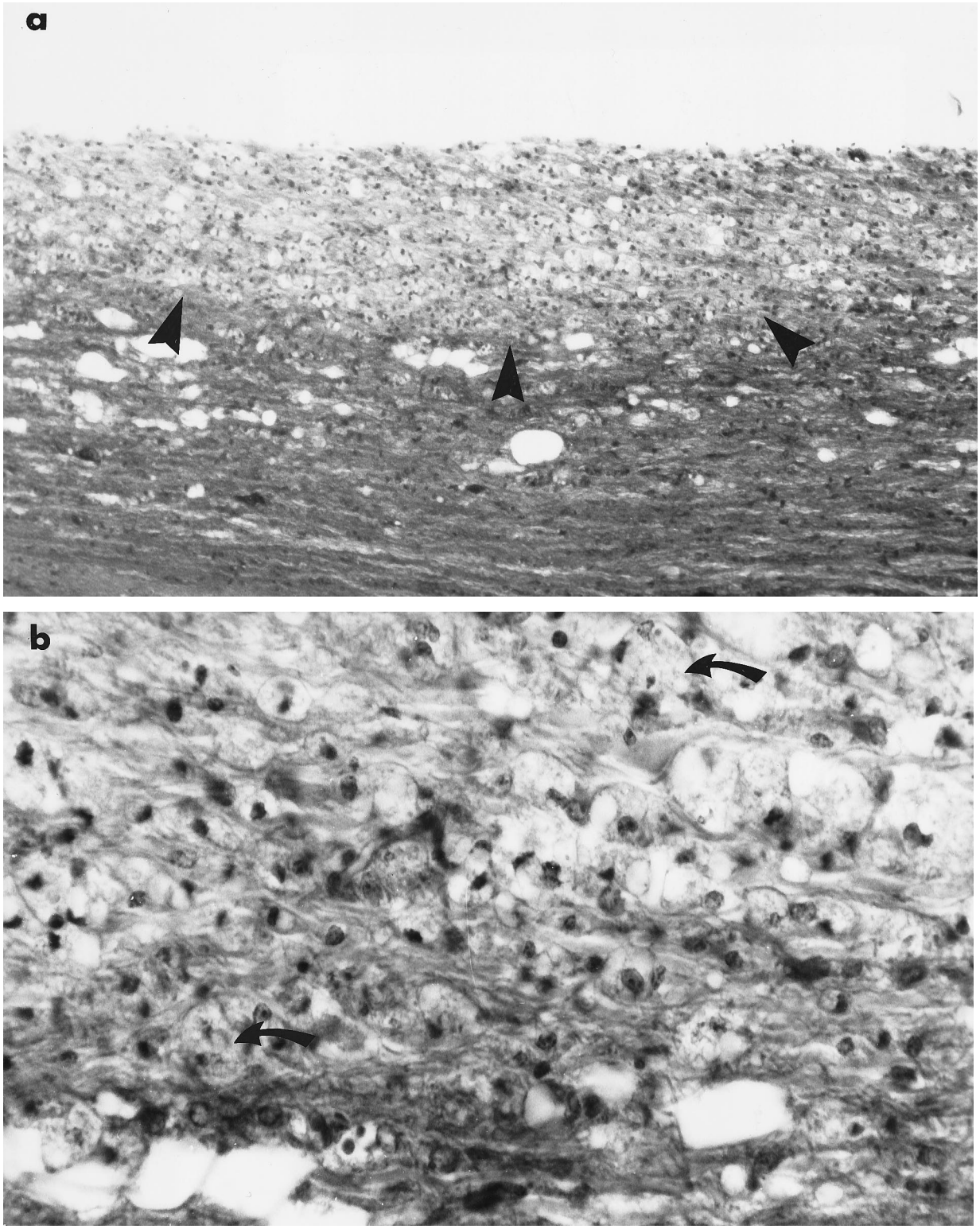

FIG. 1. Encephalomyelitis and demyelination in anti-TNF-α-treated JHMV-infected mice. Spinal cord tissues were stained with luxol fast blue. Untreated (a and b) and anti-TNF-α-treated (c and d) BALB/c mice at 12 days postinfection. Areas of demyelination are outlined by arrowheads. (b and d) The presence of numerous lipid-filled macrophages in the areas of demyelination in both untreated (b) and anti-TNF-α-treated animals (d) is shown by arrows. Magnification: ×115 (a and b); ×460 (b and d).

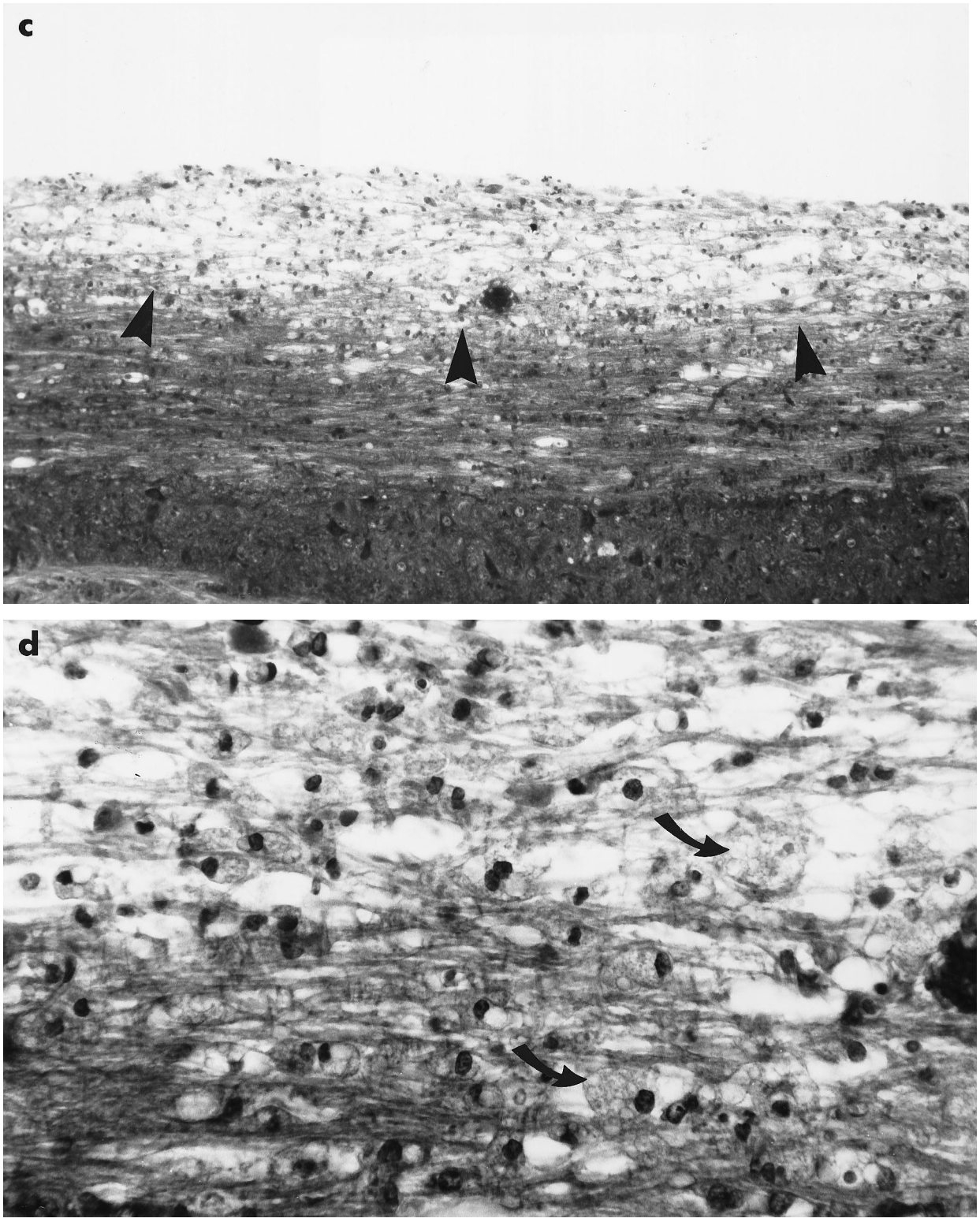

FIG. 1—*Continued.*

release (Fig. 2). Partial inhibition of TNF-α release is consistent with increased host cell mRNA degradation, translational inhibition of host proteins (which increases over time post-JHMV infection [41]), and rapid LPS-mediated induction of TNF-α.

Our interest in the potential role of TNF-α during JHMV infection was based on the finding that JHMV infection activates the transcription of TNF-α mRNA (21), unlike other host cell mRNAs. In addition, TNF-α mRNA is found in the CNS of JHMV-infected mice (reference 27 and unpublished

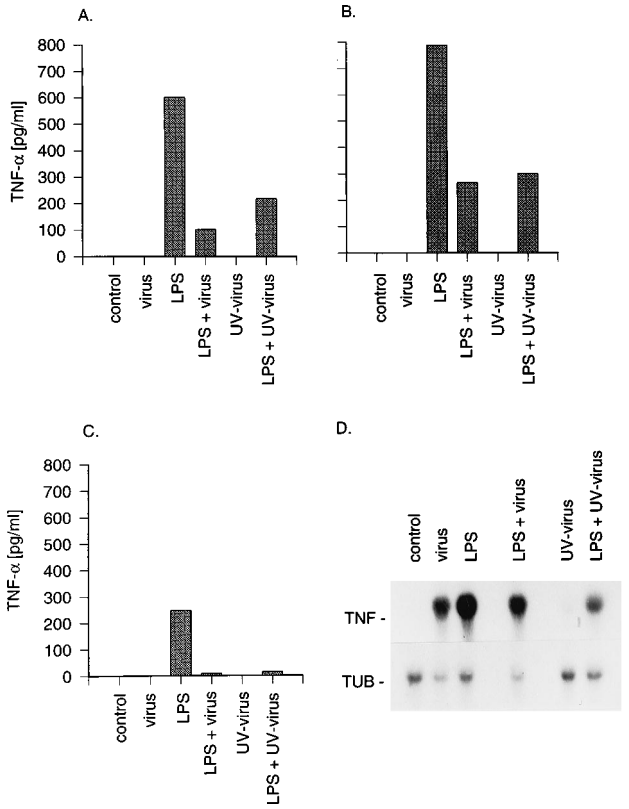

FIG. 2. TNF-α release from LPS-treated and JHMV-infected macrophages. TNF-α secretion from cells infected with JHMV (virus), cells treated with LPS, cells infected with JHMV and treated with LPS following virus absorption, cells treated with UV-inactivated JHMV (UV-virus), and cells treated with LPS following absorption of UV-inactivated JHMV (LPS + UV-virus). Samples were obtained at 6 h posttreatment from J774.1 cells (A), C57BL/6 PEC-derived macrophages (B), and BALB/c PEC-derived macrophages (C). (D) Northern (RNA) analysis of TNF-α and tubulin (TUB) mRNAs in BALB/c PEC-derived macrophages.

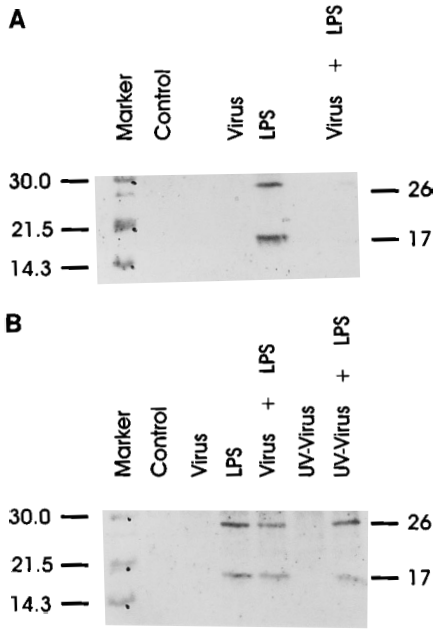

FIG. 3. Western blot analysis of the synthesis of TNF-α in PEC-derived macrophages. Samples were separated on 12.5% polyacrylamide gels and tested with rabbit anti-TNF-α antiserum. (A) BALB/c-derived macrophages; (B) C57BL/6-derived macrophages. Mock-infected controls, virus-infected cells, LPS (25 ng/ml)-treated cells, and cells infected with virus and treated with LPS were harvested at 6 h postinfection. Marker, molecular mass markers (in kilodaltons).

data). Indeed, histological analysis of the brains of JHMV-infected mice showed the presence of TNF-α positive cells (data not shown). The induction of TNF-α mRNA during JHMV infection, coupled with its ability to disrupt the blood-brain barrier and facilitate entry of EAE-inducing T cells (19, 32, 33), suggests a role in JHMV-induced encephalomyelitis. Furthermore, demyelination following JHMV infection can be blocked by immunosuppression (43). These data suggested that the release of TNF-α from CNS resident cells or from macrophages recruited into the CNS during infection facilitates the primary demyelination associated with this infection (23, 40). However, treatment of JHMV-infected mice with neutralizing anti-TNF-α MAb altered neither the extent of mononuclear cell infiltration nor virus-induced CNS demyelination. Consistent with our data, other studies have shown that anti-TNF-α antibody does not prevent delayed-type hypersensitivity (29) or LPS-induced uveitis (31), suggesting either that anti-TNF-α antibody has difficulty in accessing relatively immune privileged sites or it plays a minor role in delayed-type hypersensitivity, virus-induced encephalitis, and autoimmune uveitis. By contrast, anti-TNF-α antibody prevents both contact dermatitis and EAE (28, 32). Whether these data reflect differences in antibodies or their access to tissues or a more fundamental difference in the ability of TNF-α to modulate tissue-specific immune responses is not clear. The presence of extensive mononuclear infiltrates in anti-TNF-α MAb-treated mice is consistent with the early loss of blood-brain barrier integrity following JHMV infection (45). This suggests that anti-TNF-α antibody has access to the CNS parenchyma and that TNF-α does not participate in the loss of blood-brain barrier integrity during viral infection of the CNS. The data presented are consistent with previous suggestions that JHMV-induced demyelination is the result of cytolytic infection of oligodendroglia (23) followed by active myelin removal by mononuclear phagocytes (43). Furthermore, injection of TNF-α into neonatal mice results in reactive astrogliosis (2), while injection of TNF-α into the adult CNS results in transient inflammatory infiltration but no direct cytolytic effects (36, 47), suggesting that it is not directly cytotoxic to oligodendroglia in situ. This study is consistent with the concept that macrophage-mediated removal of myelin from the areas of viral destruction is required for histological evidence of demyelination (43), a process inhibited by TNF-α (6).

JHMV infection induces TNF-α mRNA without resulting in the secretion of TNF-α. This effect is not due to a defect in secretion or proteolytic processing but to inhibition of TNF-α translation. Recent evidence indicates that mouse hepatitis virus infection results in conversion from the translation of host cell proteins to the preferential translation of viral proteins, possibly mediated by interaction between the viral nucleocapsid protein and the conserved 5′ untranslated leader regions of viral mRNAs (41). Although purified viral proteins can induce the secretion of TNF-α (28), UV-inactivated JHMV did not induce TNF-α mRNA, suggesting that active viral replication is required. Inactivated virus was, however, nearly as efficient as infectious virus at inhibiting TNF-α release after LPS induction, suggesting that the control of TNF-α occurs at two levels during JHMV infection, with one requiring active viral replication and the other mediated by a structural component. It is intriguing that a viral structural component,

possibly released from infected cells, may have a negative influence on the secretion of TNF-α, thereby reducing its immunomodulation- and immunopathology-inducing potential within the CNS during JHMV infection. These data indicate that TNF-α secretion is inhibited in JHMV-infected cells; thus, it may provide one mechanism by which this virus is able to partially evade the host's immune response and establish a chronic infection with associated ongoing demyelination.

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