CHEMOKINE RECEPTOR 5 ANTAGONIST D-ALA-PEPTIDE T-AMIDE REDuces MICROGLIA AND ASTROCYTcy ACTIVATION WITHIN THE HIPPOCAMPUS IN A NEUROINFLAMMATORY RAT MODEL OF ALZHEIMER’S DISEASE

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Abstract—Chronic neuroinflammation plays a prominent role in the progression of Alzheimer’s disease. Reactive microglia and astrocytes are observed within the hippocampus during the early stages of the disease. Epidemiological findings suggest that anti-inflammatory therapies may slow the onset of Alzheimer’s disease. Chemokine receptor 5 (CCR5) up-regulation may influence the recruitment and accumulation of glia near senile plaques; activated microglia express CCR5 and reactive astrocytes express chemokines. We have previously shown that neuroinflammation induced by chronic infusion of lipopolysaccharide into the 4th ventricle reproduces many of the behavioral, neurochemical, electrophysiological and neuro-pathological changes associated with Alzheimer’s disease. The current study investigated the ability of D-Ala-peptide T-amide (DAPTA), a chemokine receptor 5 chemokine receptor antagonist of monocyte chemotaxis, to influence the consequences of chronic infusion of lipopolysaccharide. DAPTA (0.01 mg/kg, s.c., for 14 days) dramatically reduced the number of activated microglia and astrocytes, as compared with lipopolysaccharide-infused rats treated with vehicle. DAPTA treatment also reduced the number of immunoreactive cells expressing nuclear factor κ binding protein, a prominent component of the proinflammatory cytokine signaling pathway. The present study suggests that DAPTA and other CCR5 antagonists may attenuate critical aspects of the neuroinflammation associated with Alzheimer’s disease. © 2005 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: anti-inflammatory, lipopolysaccharide, microglia, Alzheimer’s disease.

Chemokines belong to a large family of chemotactic proteins that regulate leukocyte chemotaxis to sites of inflammation via interaction with chemokine receptors (Mueller and Strange, 2004). Chemokines and their receptors are present in the CNS where they play a critical role in neuronal development by guiding cellular migration and neuronal survival during development as well as in normal neurogenesis and pathological neuroinflammation (Xia et al., 1998). The chemokine receptor chemokine receptor 5 (CCR5) is up-regulated on reactive microglia associated with senile plaques in Alzheimer’s disease (AD); stimulation of these receptors may drive the conversion of diffuse plaques into compact neuritic plaques and contribute to an amplification of the neuroinflammation processes (Xia et al., 1998). AD is associated with a condition of chronic neuroinflammation (Aikyama et al., 2000). Increased microglia activation occurs in brain regions that ultimately demonstrate the greatest concentration of senile plaques and brain atrophy in AD patients (Cagnin et al., 2001). Therefore, chemokine receptors may be a favorable target for drug development because of their potential role in chronic inflammation.

We tested the effect of d-Ala-peptide T-amide (DAPTA), a specific CCR5 chemokine receptor antagonist (Redwine et al., 1999; Polianova et al., in press) in an animal model of chronic neuroinflammation. Chronic infusion of the proinflammogen lipopolysaccharide (LPS) into the 4th ventricle of young rats reproduces many of the behavioral, neurochemical, electrophysiological and neuropathological changes associated with AD (Hauss-Wegrzy niak et al., 1998, 2000, 2002; Rosi et al., 2003, 2004, 2005). DAPTA blocks monocyte chemotaxis (Redwine et al., 1999) and improves memory and cognitive abilities in people with acquired immunodeficiency syndrome (AIDS, Wetterberg et al., 1987; Heseltine et al., 1998) a disease that is also characterized by gial activation and widespread brain inflammation. The results suggest that chemokine receptors may be an important therapeutic target for chronic neuroinflammation associated with neurodegenerative disease.

EXPERIMENTAL PROCEDURES

Subjects

Eighteen male, three month old, F-344 (Harlan Sprague–Dawley, Indianapolis, IN, USA) were assigned to three groups: 1) artificial cerebrospinal fluid (aCSF)-infused (n = 6); 2) LPS-infused, vehicle-treated (n = 6); 3) LPS-infused, DAPTA-treated (0.01 mg/kg/day, s.c.; n = 6). LPS (1.0 µg/µl) or was chronically infused (0.25 µl/h for 14 days) through a cannula implanted into the 4th ventricle of the brain that was attached to an osmotic minipump as previously
described (Hauss-Wegrzynek et al., 1998, 2000; 2002; Rosi et al., 2003, 2004, 2005). DAPTA was freshly dissolved in the vehicle (0.01 mg/ml in distilled water) and administered every morning for 14 days beginning the day after the surgery.

Materials
LPS (E. coli, serotype 055:B5) was obtained from Sigma Chem. (St. Louis, MO, USA). DAPTA, d-ASPNTN-NH₂, Pert et al., 1986) was obtained from Bachem (Torrence, CA, USA) and was >95% pure as determined by tandem mass spectroscopy. Solutions were prepared fresh daily from powder.

Histological procedures
Two weeks after surgery each rat was anesthetized with isoflurane and prepared for histological analysis by in situ perfusion of the brain with cold saline containing 1 U/ml heparin, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain was removed and the location of the cannula in the 4th ventricle was confirmed. The brains were then post-fixed 1 h in the same fixative and then stored (4 °C) in phosphate buffer.

Immunohistochemistry
Single fluorescence staining was run for activated microglia, astrocytes and NFkB. Free-floating, serial coronal sections (30 μm) were preparing using a vibratome, six sections from each animal were selected from the medial portion of the dorsal hippocampus (ca 3.0–3.5 mm posterior to Bregma) and washed in phosphate-buffer saline (PBS) (pH 7.4). The mouse monoclonal antibody OX-6 (final dilution 1:400, Pharmingen, San Diego, CA, USA), directed against class II major histocompatibility complex (MHC II) antigen, was used to visualize activated microglial cells. A glial fibrillary acidic protein (GFAP) was visualized using anti-rabbit polyclonal antibodies raised against the amino terminal domain of GFAP p65. NFkB is a heterodimer composed of p50 and p65 subunits. The p65 subunit has a nuclear translocation signal sequence which is masked by a specific inhibitor of NFkB holds in the cytosol. Release of the inhibitor is initiated when specific kinases phosphorylate the inhibitor and the released p65 subunit is then translocated to the nucleus. Considering that the proinflammatory transcriptional factor NFkB induces transcription of proinflammatory cytokines (Baeuerle and Henkel, 1994) and chemokines (Ueda et al., 1997); and since DAPTA is an inhibitor of NFkB, the hippocampus was divided into two regions of interest (DG and CA3). The hippocampus was then post-fixed 1 h in the same fixative and then stored (4 °C) in phosphate buffered saline.

Brain inflammation was determined using standard immunohistochemistry for NFkB and the presence of the MHC II antigen. Immunofluorescence staining for MHC II was performed as described above; following several washes in PBS the slides were quenched and blocked, as described above and then incubated either with anti-NeuN antibody (1:1000, Chemicon, San Diego, USA) or with the OX-6 monoclonal antibody (1:400, Pharmingen). Before applying the biotinylated monoclonal secondary rat-adsorbed antibody (Vector) for 1 h, the tissue was incubated with Avidin Biotin Blocking Kit (Vector) for 30 min to block cross reaction with the primary antibody. After additional treatment with an Avidin Biotin amplification system (Vector), the staining was then visualized with Cy3 TSA fluorescence system (PerkinElmer) and the nuclei counterstained with SYTOX Green (1:10,000, Molecular Probes). No staining was detected in the absence of the primary or secondary antibodies. For co-localization with the rabbit polyclonal GFAP antibody the goat polyclonal antibody against NFkB p65 was used in order to avoid any cross-reaction with the rabbit GFAP.

RESULTS
Chronic infusion of LPS into the 4th ventricle of young rats for 2 weeks was well tolerated by all rats. Initially after surgery, all LPS-infused rats lost a few grams of weight. Within a few days, however, most rats had regained weight and continued to gain weight normally for the duration of the study (Rosi et al., 2005).

DAPTA treatment dramatically reduced activated microglia during LPS-infusion
Brain inflammation was determined using standard immunocytochemical biomarkers for activated microglia, i.e. the presence of the MHC II antigen. Immunofluorescence staining for MHC II found numerous and highly activated microglia distributed throughout the DG and CA3 hippocampal areas following chronic infusion of LPS into the 4th ventricle (Fig. 1A). CA1 areas did not show immunopositive microglial cells, consistent with previous study (Rosi et al., 2005). Activated microglia were characterized by a contraction of their highly ramified processes that appeared bushy in morphology (Fig. 1B). In contrast, rats infused with aCSF had few mildly activated microglia scat-
tered throughout the brain (not shown) similar to our previous reports (Rosi et al., 2003, 2004, 2005). Daily injections of DAPTA (0.01 mg/ml/kg, s.c.) reduced the number of activated microglia within the DG and CA3 hippocampal areas (Fig. 1C). Most importantly, these microglia were characterized by a morphology that suggest they were less activated than those from LPS-infused rats (Fig. 1B), i.e. the MCH II positive cells showed long and thin ramified processes typical of the resting state (Fig. 1D).

The quantitative cell count analysis demonstrated that DAPTA treatment was associated with a statistically significant ($P<0.001$, by paired t-tests) reduction in the number of activated microglia within the DG and CA3 hippocampal areas (Fig. 1C). Most importantly, these microglia were characterized by a morphology that suggest they were less activated than those from LPS-infused rats (Fig. 1B), i.e. the MCH II positive cells showed long and thin ramified processes typical of the resting state (Fig. 1D).

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**Reduced fluorescence immunostaining for NFkB**

Chronic LPS infusion increased NFkB immunostaining throughout the hippocampus (Fig. 2C). Following treatment with DAPTA, NFkB immunofluorescence staining was dramatically reduced (Fig. 2F). Double immunofluorescence staining demonstrated that the NFkB was associated primarily with hypertrophic astrocytes (Fig. 3A–C). LPS infused animals had numerous NFkB immunopositive cells within the hilar region of the dentate gyrus (Fig. 2C); the enhanced staining was found prominently within the cytoplasm and the nuclei. Reduction of NFkB staining was observed following treatment with the chemokine receptor antagonist DAPTA (Fig. 2F).

Co-localization of NFkB with the NeuN selective neuronal marker revealed only scattered double-labeled cells within the hippocampus (Fig. 3D). In addition, while activated microglia were found near NFkB positive cells OX-6 did not co-localization with NFkB (Fig. 3E–F). Double immunohistochemistry for NFkB and GFAP demonstrated considerable co-localization (Fig. 3A–C). The NFkB immunopositive staining was found in the cytoplasm within the cell body and inside the astrocyte nuclei (see Fig. 3A, B). The remaining NFkB positive cells that were not co-local-
DISCUSSION

Chronic infusion of LPS into the 4th ventricle produced an extensive inflammatory reaction throughout the brain, particularly within the hippocampus and temporal lobe regions. The inflammatory response was characterized by a significant increase in the number of reactive microglial cells, a marked hypertrophy of numerous astrocytes and an elevation in the NFkB staining. The effects of LPS infusion upon microglial and astrocyte activation were consistent with our previous reports (Hauss-Wegrzyniak et al., 1998, 2000; Rosi et al., 2003, 2004, 2005). Previous findings show that DAPTA completely prevents NBM lesion-induced cortical atrophy in aged rats (Socci et al., 1996). Here we demonstrate that daily peripheral administration of DAPTA dramatically attenuated the inflammatory response induced by LPS as demonstrated by a decrease in both the number and reactive state of microglial and astrocytes.

The effects of LPS are mediated primarily through the release of cytokines from activated microglial cells (Wenk and Hauss-Wegrzyniak, 2003). DAPTA targets the CCR5 chemokine receptor expressed by activated microglia and this may block the release of the pro-inflammatory cytokine tumor necrosis factor-α and interleukin-1 (Ruff et al., 2003). In turn, pro-inflammatory stimuli can activate NFkB expression (Sparacio et al., 1992), which can further induce specific genes that regulate the expression of inflammation and acute phase genes leading to the continued elevation of inflammatory proteins. This cycle may exist for many years in the brains of AD patients and contribute to the degeneration and vulnerability of selected brain regions (Akiyama et al., 2000; Cagnin et al., 2001; Griffin et al., 1998; Wenk and Hauss-Wegrzyniak, 2001, 2003). In the present study, chronic infusion of LPS elevated intracellular expression of NFkB immunoreactivity within astrocytes but not within neurons or microglia. DAPTA treatment significantly reduced the level of NFkB expression within astrocytes in the hippocampus. Astrocytosis is a typical morphological feature of the AD brain and chemokines are released from activated astrocytes that are near senile plaques (Griffin et al., 1998; Akiyama et al., 2000).

Fig. 2. Immunohistochemical staining for either GFAP-positive astrocytes (A, B, D, E) and NFkB (C, F) (both in red) within the CA3 (A, D) and DG (B, C, E, F) area of the hippocampus of LPS-infused, saline-treated (top row) or LPS-infused, DAPTA-treated, rats (bottom row). Nuclei are counterstained green. To view this figure in color, please see the version of this paper published online. Scale bars=100 μm.
We speculate that the beneficial effects of DAPTA treatment in the current study were due to its antagonism at the CCR5 receptor which attenuates the cascade of biochemical and immunological changes that were induced by the chronic infusion of LPS. The AD brain is characterized by chronic neuroinflammation (Akiyama et al., 2000) and long term treatments with anti-inflammatory drugs lessen the risk of AD (Aisen and Davis, 1994). Previous studies using this animal model of chronic brain inflammation have demonstrated that treatment with non-steroidal anti-inflammatory drugs reduces the level of activation of microglia but not astrocytes (Hauss-Wegrzyniak et al., 1998 and unpublished findings); in contrast to the actions of these anti-inflammatory drugs, DAPTA was effective against both important inflammatory cells in the brain. This dual action of DAPTA may also explain its cognitive benefits in patients with AIDS, a disease characterized by microglial and astrocyte activation, as well as enhanced inflammatory cytokines. Our results suggest that DAPTA, currently in phase II trials for HIV disease, should be tested for efficacy in AD, and that other chemokine receptor antagonists may reduce the consequences of chronic neuroinflammation that appear to drive the pathology associated with AD.

Acknowledgments—Supported by the U.S. Public Health Service, Contract grant number AG10546 and the Alzheimer’s Association, IIRG-01-2654 to G.L.W.

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Fig. 3. Double immunohistochemistry for NFκB (red) and GFAP (blue) on sections from LPS-infused rats showed considerable co-localization (A–C). The NFκB immunopositive staining was found in the cytoplasm within the soma as well as inside the astrocyte nuclei (A, B). Co-localization of NFκB with the NeuN selective neuronal marker revealed only scattered double-labeled cells within the hippocampus (D). Activated microglia (OX-6 immunoreactive) were not co-localized with NFκB (E, F). The remaining NFκB positive cells that did not co-localize with GFAP were characteristic of vascular endothelial cells; arrows in D, E. To view this figure in color, please see the version of this paper published online. Scale bars = 100 μm.

(Accepted 15 April 2005)
(Available online 27 June 2005)