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Myelin basic protein peptide 45–89 induces the release of nitric oxide from microglial cells

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Continuous (24 h) exposure of mixed oligodendrocyte/microglial cells to peptides 45–89 derived from citrullinated C8 isoforms of myelin basic protein (MBP) induces cell death. In contrast, MBP-C8 at the same molecular concentration is not toxic to oligodendrocyte/microglial cells as detected by the MTT test and trypan blue exclusion method. The loss of oligodendrocyte/microglial cells resulted in the release of cytochrome c from mitochondria, suggesting MBP 45–89-induced apoptosis. On the other hand, peptides 45–89 stimulated the secretion of nitric oxide from microglial cells only via induction of iNOS. The addition of peptide 45–89 to the microglial cells led to a decrease of the level of the inhibitory protein IkB, indicating that activation of the transcription factor NF-kB is involved in these processes. We propose that the immunodominant peptide 45–89 induces damage of oligodendrocytes by activation of microglial cells and subsequent generation of nitric oxide, and that this may be the first step in the initiation of autoimmunity.

1. INTRODUCTION

Abnormal activation of microglial cells has been implicated in various neurodegenerative and autoimmune diseases. Myelin basic protein (MBP)[1] accounts for approximately 30% of central nervous system myelin proteins (Morell et al., 1989), and is responsible for adhesion of the cytoplasmic surfaces of the compact multilayered myelin sheath in the central nervous system. Several peptide fragments of MBP are formed in the brain during demyelinating diseases. MBP contains three mitogenic regions (MBP 1–44, MBP 88–151, MBP 152–167) which in conjugation with microglial secreted factors may stimulate astrogliosis after demyelination *in vivo* (South et al., 2000). MBP 1–44 and MBP 152–167 associate with ganglioside GM1 and bFGF receptor respectively to stimulate Schwann cell proliferation (Tzeng et al., 1999). Myelin basic protein itself induces death of mature oligodendrocytes *in vitro* and produces demyelination *in vivo* by a rapid and dramatic rise of intracellular calcium, whereas MBP peptides (1–44 or 88–151) cause only a modest and transitory elevation of intracellular calcium ions, and protects oligodendrocytes from death (Tzeng et al., 1995, Althaus et al., 2000).

MBP peptide 45–89 (MBP 45–89), which contains an immunodominant epitope, is detected in body fluids after the degradation of MBP (Whitaker , 1998). This peptide is released more rapidly from the posttranslationally citrullinated isomer MBP C8 (also denoted MBP Cit 6 in the literature) than from the unmodified (MBP C1) isomer and has unusual stability: the presence of secondary structure seems to protect it from further enzymatic digestion, and consequently it circulates in extracellular fluids for rather a long time (Cao et al., 1999). The influence of this peptide on the activity of glial cells is not known at present.

In this study, we demonstrate that MBP peptide 45–89 significantly increased the production of NO by microglia and reduced the viability of oligodendrocytes/microglial primary cell cultures. Western blot analysis was used to show that the viability of these cells was correlated with the decrease of IkB, the inhibitor of NF-kB, and the induction of iNOS in the microglia.

2. MATERIALS AND METHODS

Preparation of MBP and MBP 45–89 peptide. MBP was isolated and purified from bovine brain white matter according to the method of Chou et al. [3],

[1] A list of abbreviations is given at the end.

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followed by HPLC. Briefly, the acid-soluble material was dissolved in urea-glycine buffer, pH 9.6 and applied to a CM-52 cellulose cation exchange column, equilibrated in urea-glycine buffer, pH 10.5. Following application of the sample, urea-glycine buffer (pH 10.5) flow was continued until the first big peak had completely eluted. This unbound fraction is the least cationic isomer, i.e. MBP C8. For the tryptic digestion MBP was suspended in 1 ml of 0.1 ammonium hydrogen carbonate, pH 8.0, containing 10 mM calcium acetate. A solution of trypsin (0.3 mg/ml in 0.1 M hydrochloric acid) was added to the protein suspension and the mixture was incubated at 37 °C for 20–24 h (Martenson et al. 1981). MBP 45–89 peptide were purified by HPLC as described by Cao et al. (1999).

Cell cultures. Primary mixed glial cultures containing astrocytes, microglia and oligodendrocytes were prepared from whole brains of 2 day old Wistar rats. The initial mixture of dissociated glial cells was seeded in 75 cm^2 flasks (2–3 brains/flask) coated with poly-D-lysine. The cultures were maintained in DMEM with 10% fetal calf serum and cultured for 48 h. This step provides a means of separating adherent cells, such as microglia and astrocytes, from nonadherent cells, such as oligodendrocytes. The nonadherent oligodendrocyte fraction was plated into poly-D-lysine-coated 25 cm^2 flasks. For isolation of microglial cells, confluent cultures between days 12 and 14 were agitated on a rotary shaker at 37 °C at 220 rpm for 1 h. The floating cells were collected and incubated in tissue culture plates. After 1 h the cultures were washed. The adherent cells were identified as microglia. The astrocytes, i.e. the cells remaining adherent after the removal of microglia, were removed from the flasks by incubation with 0.25% trypsin for 10 min.

Cytotoxicity assays. To assess cell viability, a modified 3-(4,5-dimethylthiasol-2yl)-2,5 diphenyltetrazolium bromide (MTT) assay, a sensitive first indicator of oxidative damage (Liu et al., 1997), was carried out as described by Liu et al. Briefly, 10^6 glial cells were treated with 0.5 mg/ml MTT followed by incubation at 37 ^{0}C for 2 h in a CO_2 incubator. After brief centrifugation, supernatants were carefully removed and the formazan reaction product was dissolved in acidic isopropanol. The formazan product was quantified spectrophotometrically by its absorbance at 570 nm. Data are presented as a percentage of the absorbance obtained in untreated cultures, after subtraction of blank values obtained by incubation of MTT in medium alone. Trypan blue exclusion assays were carried out after incubation (5 min before counting) of cells with 0.04% trypan blue (Sigma). Dead cells were stained blue. Three fields per coverslip were counted for each experimental condition, and between 100 and 200 cells were counted on average per field. Statistical significance was tested using the ANOVA computer program.

Assay for NO. Synthesis of NO was determined by assaying the culture supernatants for nitrite (Pahan et al., 2000). Briefly, 400 µl of culture supernatant was allowed to react with 200 µl of Griess reagent (Green et al., 1982) and incubated at room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture medium served as the blank in all experiments.

Western blot analysis. Following 24 h of incubation in the presence or absence of MBP 45–89 peptide, cells were removed from the dishes, washed with PBS and homogenized in 50 mM Tris-HCl, pH 7.4, containing protease inhibitors (1 mM PMSF, 5 µg/ml aprotinin, 5 µg/ml pepstatin A, and 5 µg/ml leupeptin). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane. Membranes were treated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, USA) and immunoreactivity was detected by enhanced chemiluminescence autoradiography (ECL kit, Amersham) in accordance with the manufacturer's instructions. The following primary antibodies (Santa Cruz Biotechnology) were used: against IkB, against iNOS, and against cytochrome c. Protein concentrations were determined using a BCA protein assay kit (Pierce).

Detection of cytochrome c release. Approximately 10^7 cells were trypsinized and collected by centrifugation. The cell pellets were washed with PBS and resuspended in 125 µl buffer containing 250 mM sucrose, 20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF). The cells were then homogenized and the mitochondria-enriched fraction was pelleted by centrifugation at 15 000 rpm for 15 minutes. The supernatant was subjected to Western blot analysis with a monoclonal antibody against cytochrome c.

3. RESULTS

MBP 45–89-induces cell apoptosis in mixed primary oligodendrocyte/microglial cell cultures

After continuous 24 h exposure of mixed oligodendrocyte/microglial cells to 0.5 µM MBP 45–

89 only about 65 % of the cells were still alive. In contrast, MBP C8 at the same concentration is not toxic to oligodendrocyte/microglial cells, as detected by the MTT test and the trypan blue exclusion method (Fig. 1A). Interestingly, the decrease of the viability of cells takes place only in mixed oligodendrocyte/microglial cultures, but not in pure cultures of astrocytes, oligodendrocytes or microglia separately (data not shown).

To examine whether the MBP 45–89-induced cell death has apoptotic features, the release of mitochondrial cytochrome c into the cytosol was determined. We isolated the cytosolic fractions from oligodendrocyte/microglial cells treated or not with MBP 45–89 and detected the released cytochrome c by Western blot analysis. The exposure of oligodendrocyte/microglial cells to 0.5 μM MBP 45–89 resulted in the release of cytochrome c from mitochondria (Fig. 1B). Thus, MBP 45–89 appears to induce apoptosis mainly via the mitochondrial pathway.

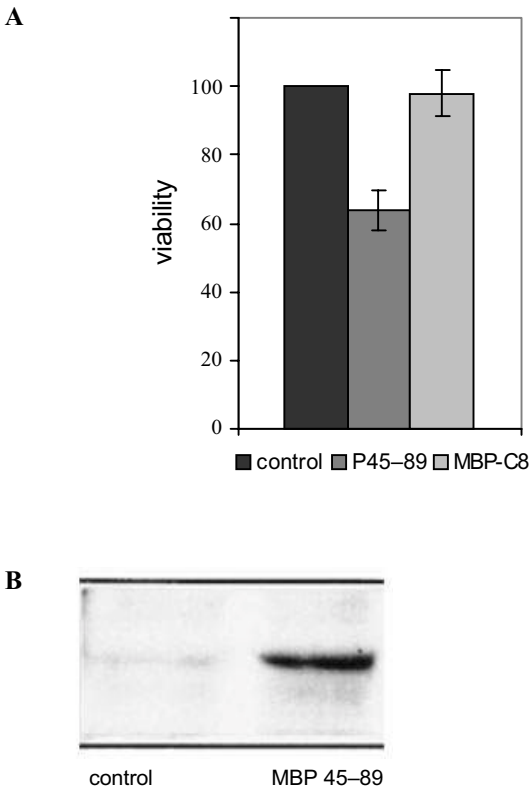

Figure 1. MBP 45–89 reduces cell viability and stimulates release of cytochrome c from mitochondria in mixed oligodendrocyte/microglial cell cultures.
A: Oligodendrocyte/microglial cell viability after exposure to 0.5 μM MBP 45–89 or 0.5 μM MBP C8 for 24 h assessed by the MTT test. All data were normalized to control values (untreated cells) of 100. Data are presented as the means ± SEM of three independent experiments.
B: Western blot analysis of cytoplasmic fractions. Cells were treated with 0.5 μM MBP 45–89 for 24 h and then the cytoplasmic fraction was analyzed for cytochrome c by an immunoblotting technique using an anti-cytochrome c antibody.

MBP 45–89 induces production of NO and activation of NF-kB in microglial cell cultures

Nitric oxide, produced by inducible NO synthase (iNOS), may play a central rôle in inflammatory demyelinating disease of the central nervous system. NO derived from the activation of iNOS in astrocytes and microglia under the influence of proinflammatory cytokines is presumed to contribute to oligodendrocyte degeneration in demyelinating diseases (Merill et al., 1993; Pahan et al., 2000). Because microglia rather than astrocytes are implicated in demyelinating pathology (Tran et al., 1997), in the next series of experiments we examined the effect of MBP 45–89 on the production of NO and activation of NF-kB in microglial cell culture.

Fig. 2A shows that continuous exposure for 24 h of primary microglial cells to MBP 45–89 markedly increased production of NO. To understand the mechanism of this stimulation, we examined the effect of MBP 45–89 on the level of iNOS. Western blot analysis with antibodies against iNOS clearly shows that MBP 45–89-mediated nitrite production is engendered by the induction of iNOS (Fig 2B). As

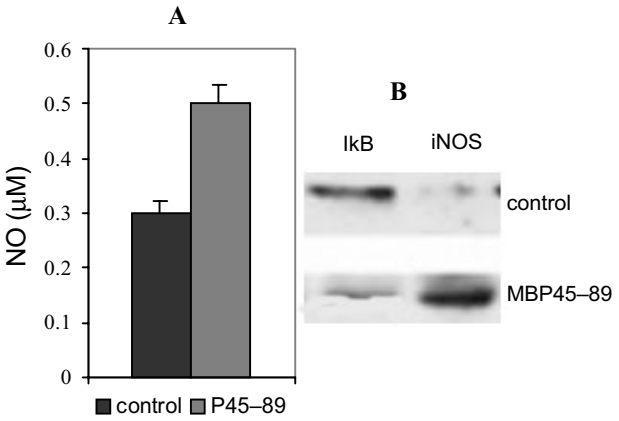

Figure 2. MBP 45–89 stimulates production of nitric oxide, degradation of IkB and induction of iNOS in microglia primary culture cells.
A: Production of NO in primary microglia. Cells were treated with 0.5 μM MBP 45–89 for 24 h and then the yield of nitrite was measured in the supernatant. Data are means ± SEM of three independent experiments.
B: Western blot analysis of cell homogenate. Cells were treated with 0.5 μM MBP 45–89 for 24 h and then the homogenate was analyzed for IkB and iNOS proteins by an immunoblotting technique using anti-IkB and anti-iNOS antibodies.

the activation of NF-kB is known to be necessary for the transcription of iNOS gene in glial cells (Colasanti and Persichini, 2000), we examined the effect of MBP 45–89 on the activation of NF-kB. For this purpose, we determined the cytoplasmic levels of the inhibitory IkB protein after treatment of microglial cells with MBP 45–89. Western blot analysis with antibody against IkB showed that MBP 45–89 decreased the level of inhibitory protein (Fig. 2B).

All together, these results indicate that MBP 45–89 induces the degradation of IkB, the subsequent nuclear translocation of NF-kB, and induction of the iNOS gene. The resulting elevated concentration of nitric oxide causes the death of oligodendrocytes.

5. DISCUSSION

Several peptide fragments of MBP are formed in the brain during demyelinating diseases, which, together with proinflammatory cytokines, can influence proliferation or damage of glial cells (Tzeng et al., 1999; South et al., 2000). Myelin basic protein induces cell death of mature oligodendrocytes *in vitro* and produces demyelination *in vivo* accompanied by a rapid and dramatic rise of intracellular calcium, whereas MBP peptides (1–44 or 88–151) caused only a modest and transitory elevation of intracellular calcium ions (Tzeng et al., 1995, Althaus et al., 2000). This knowledge is now extended by our finding that the immunodominant peptide 45–89 induces damage of oligodendrocytes by activation of microglial cells and subsequent generation of nitric oxide.

NF-kB is the major transcriptional factor in iNOS transcriptional induction occurring in glial cells (Colasanti and Persichini, 2000). NF-kB is activated in microglia during experimental autoimmune encephalomyelitis(EAE).Pyrrolidine dithiocarbamate, an inhibitor of NF-kB activation, attenuates the clinical symptoms of EAE (Pahan, 2000). Astrocytes and microglia express inducible nitric oxide synthase in mice with EAE, and microglial NO is more cytopathic than astrocyte-derived nitric oxide (Tran et al., 1997). Our present results show that the most stable immunodominant peptide from citrullinated MBP C8 (45–89) induces activation of NF-kB and transcription of iNOS, resulting in excessive release of NO.

The molecular mechanism of action of MBP 45–89 on microglial cells is presently unknown. MBP is known to contain three mitogenic regions (MBP 1–44, MBP 88–151, and MBP 152–167), which in conjugation with microglial secreted factors may stimulate astrogliosis *in vivo*, after demyelination has taken place (South et al., 2000). MBP 1–44 and MBP 152–167 associate with ganglioside GM1 and bFGF receptor respectively to stimulate Schwann cell proliferation (Tzeng et al., 1999). Taking into account that $p21^{ras}$ is involved in the induction of nitric oxide synthase and activation of NF-kB in glial cells (Pahan et al., 2000), and that Ras-pathway inhibitors suppress EAE (Karussis et al., 2001) it is possible to infer that the Ras-signalling pathway participates in the action of MBP 45–89 on microglial cells.

The source and nature of the antigen presented initially to the immunocompetent cells remains unclear. Cao et al. (1999) supposed that deimination might be viewed as a first step in making available encephalitogenic peptides in MS and immunogenic peptides in rheumatoid arthritis. They postulate that the generation of peptide 45–89 is directly related to deimination of arginine to citrulline in MBP. On the other hand Matute et al. (2001) have proposed that some (presently unknown) myelin components, which are initially liberated from oligodendrocytes after glutamate-induced excytotoxicity, might activate microglia, which then release proinflammatory cytokines (TNF-α, IL-12), produce reactive oxygen species, and generate in turn fatal feedbacks that kill oligodendrocytes. It is thus probable that the stable immunodominant peptide 45–89 from citrullinated MBP C8, which via the NF-kB pathway induces transcriptional activity of microglial iNOS gene and produces toxic amounts of nitric oxide, directly initiates autoimmunity.

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ABBREVIATIONS

DMEM: Dulbecco's modified Eagle's medium
EAE: experimental autoimmune encephalomyelitis
HPLC: high-pressure liquid chromatography
NF-kB: a nuclear factor kB
IkB: inhibitor of NF-kB
iNOS: inducible NO synthase
MBP: myelin basic protein
PBS: phosphate buffered saline
PMSF: phenylmethylsulphonyl fluoride

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