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Mast Cell Activation Causes Delayed Neurodegeneration in Mixed Hippocampal Cultures via the Nitric Oxide Pathway

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Abstract: Mast cells are pleiotropic bone marrow-derived cells found in mucosal and connective tissues and in close apposition to neurons, where they play important roles in tissue inflammation and in neuroimmune interactions. Connective tissue mast cells, with which intracranial mast cells share many characteristics, contain cytokines that can cause inflammation. Here, we report that myelin basic protein, a major suspected immunogen in multiple sclerosis, as well as an antigenic stimulus, provokes mast cells to trigger a delayed cytotoxicity for neurons in mixed neuron–glia cultures from hippocampus. Neurotoxicity required a prolonged period (12 h) of mast cell incubation, and appeared to depend largely on elaboration of the free radical nitric oxide by astrocytes. Activation of astrocytes was mediated, in part, by mast cell-secreted tumor necrosis factor-α. Myelin basic protein and 17β-estradiol had a synergistic action on the induction of mast cell-associated neuronal injury. The cognate mast cell line RBL-2H3, when subjected to an antigenic stimulus, released tumor necrosis factor-α which, together with exogenous interleukin-1β (or interferon-γ), induced astroglia to produce neurotoxic quantities of nitric oxide. A small but significant proportion of mast cell-derived neurotoxicity under the above conditions occurred independently of glial nitric oxide synthase induction. Further, palmitoylethanolamide, which has been reported to reduce mast cell activation by a local autacoid mechanism, decreased neuron loss resulting from mast cell stimulation in the mixed cultures but not that caused by direct cytokine induction of astrocytic nitric oxide synthase. These results support the notion that brain mast cells could participate in the pathophysiology of chronic neurodegenerative and inflammatory diseases of the nervous system, and suggest that down-modulation of mast cell activation in such conditions could be of therapeutic benefit. **Key Words:** Mast cells—Inflammation—Degranulation—Myelin basic protein—Nitric oxide—Neurotoxicity—Astrocytes—Palmitoylethanolamide.
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Tissue mast cells are multifunctional bone marrow-derived immune cells and have been implicated in immediate allergic reactions and in inflammation (Galli, 1993). Mast cells are present in connective tissues of various organs, in serosal cavities, in mucosal epithelia, and in the nervous system (Bienenstock et al., 1987; Bernstein and Lawrence, 1990). Mast cells are located in close proximity to neurons in the peripheral and central nervous systems (Newson et al., 1983; Bienenstock et al., 1987; Johnson and Krenger, 1992; Marshall and Waserman, 1995), suggesting a functional role in normal and aberrant neurodegenerative states. They are able to synthesize and release nerve growth factor (Leon et al., 1994*a*), and are responsive to this neurotrophin (Horigome et al., 1994) and to other neurotrophic factors (Purcell and Atterwill, 1995). Mast cells, probably originating from the neural crest (Lambracht-Hall et al., 1990), have also been identified intracranially (Ibrahim, 1974), where they show a strict perivascular location (Lambracht-Hall et al., 1990) and where they secrete vasoactive amines upon stimulation (Dimitriadou et al., 1990). Connective tissue mast cells share many characteristics with brain mast cells (Purcell and Atterwill, 1995).

The role of mast cells in the pathogenesis of neurological diseases is poorly understood. Mast cells have been identified in multiple sclerosis plaques (Olsson, 1974), and may degranulate in rats with experimental autoimmune encephalomyelitis (Theoharides et al., 1991). Mast cells can contain an extraordinary variety of chemical mediators and neuroactive substances, including histamine, serotonin, peptides, kinins, leukotrienes, cytokines, and proteolytic enzymes (Kido et al., 1985; Gordon et al., 1990; Galli, 1993; White, 1993), which may orchestrate inflammation. Release of mast cell mediators by immunological and nonimmunological stimuli can directly modify blood–nerve barrier permeability (Harvey et al., 1994), compro-

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Abbreviations used: DIV, days in vitro; DMEM, Dulbecco's modified Eagle's medium; DNP, dinitrophenol; DNP-HSA, dinitrophenylated human serum albumin; FCS, fetal calf serum; IL-1β, interleukin-1β; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MBP, myelin basic protein; NO$^{\bullet}$, nitric oxide; NOS, nitric oxide synthase; TNF-α, tumor necrosis factor-α.

mise myelin integrity (Johnson et al., 1988; Dietsch and Hinrichs, 1991), and injure oligodendrocytes (Selmaj and Raine, 1988). Further, indirect mechanisms of injury can be envisaged. Exposure of rat microglial and astroglial cells in vitro (Murphy et al., 1993) or cultured human astrocytes (Lee et al., 1993) to combinations of cytokines results in transcriptional induction of a nitric oxide synthase (NOS). There is evidence for such induction in the CNS in vivo associated with infection, ischemia, and traumatic injury (Koprowski et al., 1993; Endoh et al., 1994; Van Dam et al., 1995; Vizzard et al., 1995). Nitric oxide ($NO^{\bullet}$) and its progenitors are potentially toxic molecules, and may mediate cytotoxicity through formation of iron–NO complexes of respiratory enzymes, oxidation of protein sulfhydryls, and nitrosylation of nucleic acids and DNA strand breakage (Radi et al., 1991; Wink et al., 1991). $NO^{\bullet}$ elaborated by microglia (Boje and Arora, 1992; Chao et al., 1992) and astroglia (Dawson et al., 1994; Skaper et al., 1995) can be toxic to neurons, as well as to oligodendrocytes (Merrill et al., 1993). Increased $NO^{\bullet}$ production reportedly occurs in multiple sclerosis, as well (Johnson et al., 1995).

The present study was designed to examine the effects of mast cell activation on hippocampal neuron survival in an in vitro coculture system, and the contribution of astrocytes and the nitridergic pathway to the injury process. Rat peritoneal mast cells and the cognate cell line RBL-2H3 were subjected to two established degranulating stimuli, namely, aggregation of high-affinity IgE receptors (FcεR1) (Galli, 1993) and myelin basic protein (MBP) (Johnson et al., 1988; Theoharides et al., 1993), the latter representing a potential major target of the autoimmune process in multiple sclerosis (Trotter et al., 1987). Further, palmitoylethanolamide, recently demonstrated to downmodulate mast cell activation in vitro (Facci et al., 1995) and in vivo (Aloe et al., 1993; Mazzari et al., 1994), was investigated for its ability to moderate neuronal injury caused by stimulated mast cells.

MATERIALS AND METHODS

Cell cultures

Primary astroglial cells were derived from neonatal BALB/c mouse hippocampus, essentially as described for rat (Skaper et al., 1990). Confluent cultures in 75-cm^2 flasks [10–12 days in vitro (DIV)] were shaken overnight at 250 rpm and the medium discarded. Cells were subsequently passaged twice, upon reaching confluence. These procedures were done to deplete astrocyte cultures of contaminating microglia (Simmons and Murphy, 1992; Skaper et al., 1995). Following the last passage, astrocytes (>99% pure by glial fibrillary acidic protein immunoreactivity) were detached by trypsinization and replated in 16-mm diameter 24-well plates (Falcon), 1.5×10^5 cells per well, in Dulbecco's modified Eagle's medium (DMEM) supplemented to contain 2 m*M* L-glutamine, 100 IU/ml penicillin, and 10% (vol/vol) fetal calf serum (FCS). These cultures were used 7 days later.

Cell dissociates of embryonic day 17–18 BALB/c mouse hippocampus were prepared as described previously for rat (Skaper et al., 1990). Dispersed hippocampal cells were plated (1×10^5 cells per well) onto established, semiconfluent monolayers of astrocytes in 24-well plates, as described above, in astrocyte medium supplemented with 20 m*M* KCl and 1 m*M* sodium pyruvate. Rodent hippocampal cells comprise predominantly pyramidal-like neurons at this developmental stage (Banker and Cowan, 1977). Cultures were used between 8 and 10 DIV.

Mast cells were collected from the peritoneal cavities of male Wistar rats (250–300 g; Charles River, Calco, Italy) and were purified over a bovine serum albumin gradient (Leon et al., 1994*a*). Purity was 90–95%, as judged by toluidine blue and safranin staining (Leon et al., 1994*a*).

Rat basophilic leukemia cells of the secreting subline 2H3 (RBL-2H3) were kindly provided by Dr. Pietro Ghezzi, Instituto "Mario Negri" (Milan, Italy). RBL-2H3 cells were maintained as stationary cultures at 37°C in Eagle's minimal essential medium supplemented to contain 2 m*M* L-glutamine, 100 IU/ml penicillin, and 20% FCS. Cells were passaged twice weekly. All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air. Comparisons were made on sister cultures derived from single platings.

Exposure of mixed neuron–glia cultures to mast cells

Purified mast cells were resuspended in DMEM plus glutamine/penicillin and seeded (5×10^4 cells per insert) into Transwell cell culture inserts (3-μm pore size, 9-mm diameter; Falcon) and placed into wells containing 8–10-day-old hippocampal neurons cultured over astrocytes. In the case of RBL-2H3, cells were detached and seeded (2×10^5 cells per insert) into Transwell inserts in DMEM plus glutamine/penicillin, followed by transfer to the wells of mixed neuron–glia cultures. Immediately prior to insert introduction, medium in the wells of neuron–glia cocultures was replaced with DMEM plus glutamine/penicillin containing 20 m*M* KCl, 1 m*M* pyruvate, 100 μg/ml transferrin, 5 μg/ml insulin, and 30 n*M* sodium selenite. Each experimental condition was carried out in triplicate.

Mast cell activation was achieved in one of two ways. In the first, medium in the inserts was exchanged for fresh medium containing 5–100 μ*M* MBP. In the second, mast cells were challenged with an immunogenic stimulus. This was done by sequentially incubating the mast cells with a mouse monoclonal IgE (0.3 μg/ml) specific for dinitrophenol (DNP) haptens followed by dinitrophenylated human serum albumin (DNP-HSA) (Facci et al., 1995). The mast cell-containing inserts were removed 1 or 12 h after initiating the stimulus.

Neuronal injury

Overall neuronal cell injury was estimated by examination of cultures under phase-contrast microscopy, usually 60 h after removal of activated mast cells, at which time the process of cell death was largely complete. Viable pyramidal-like neurons, i.e., those that had a soma that was phase-bright and round-to-oval in shape, with smooth, intact neurites, were clearly visible above the underlying carpet of flat, polygonal-shaped astrocytes. Neurons were considered nonviable when they exhibited neurite fragmentation and "beading," and when their soma exhibited swelling and vacuolation (Mattson et al., 1989; Pike et al., 1993). Control

experiments showed that the loss of viable neurons assessed morphologically by phase-contrast microscopy was proportional to the number of neurons damaged, as estimated by trypan blue staining (Pike et al., 1993).

Neuronal damage was quantitatively assessed in most experiments by measuring the number of cells that stained positively for the neuron-specific marker neurofilament protein, using the monoclonal antibody RT97 (1:500) and peroxidase-conjugated goat anti-mouse IgG (1:450). Cells were developed by means of the ABC kit (Vector Laboratories) and diaminobenzidine with $NiCl_2$ enhancement (Skaper et al., 1990). Numbers of RT97-positive cells were counted over two diametral strips per well, and converted to total immunopositive cells per well. These values closely paralleled those obtained when unstained fixed cultures were counted by optical microscopy. In some cases, neuronal vitality was also quantified by measurement of lactate dehydrogenase released by damaged or destroyed cells into the extracellular fluid at the end of the incubation period (Sigma Diagnostics LDH kit no. 500).

Data were analyzed by one-way ANOVA with Student–Newman–Keuls post hoc test for differences between groups. Differences were considered statistically significant at $p < 0.05$.

Materials

Culture medium and supplements, bovine MBP, monoclonal anti-DNP IgE (clone SPE-7), DNP-HSA (30–40 mol of DNP per mole of albumin), DNP-lysine, N^{ω}-nitro-L-arginine methyl ester-HCl (L-NAME), and N^{ω}-nitro-L-arginine were purchased from Sigma (St. Louis, MO, U.S.A.); FCS from BIOSPA (Wedel, Germany); and recombinant mouse tumor necrosis factor-α (TNF-α; 4×10^7 U/ml) and polyclonal rabbit anti-mouse TNF-α antiserum from Genzyme (Cambridge, MA, U.S.A.). Palmitoylethanolamide was synthesized by standard chemical procedures, with purity >99.5% as assessed by HPLC. Recombinant human interleukin-1β (IL-1β) was generously provided by Dr. Aldo Tagliabue (Dompè S.p.A., L'Aquila, Italy). Monoclonal antibody RT97 was a kind gift from Prof. Frank S. Walsh (Department of Experimental Pathology, UMDS, Guy's Hospital, London, U.K.).

RESULTS

Mast cell activation causes delayed neuronal injury

Incubation of peritoneal mast cells with MBP leads to degranulation (Johnson et al., 1988; Theoharides et al., 1993) and a concentration-dependent release of serotonin (Theoharides et al., 1993). When the peritoneal mast cells used here were loaded overnight with [^{3}H]serotonin and then stimulated with 100 μM MBP for 10 min (37°C), net serotonin release was 33.0 ± 2.8% (n = 3); 5 μM MBP gave no release over basal. These values are consistent with those reported for peritoneal mast cells from Sprague–Dawley rats (Theoharides et al., 1993).

To determine whether MBP-stimulated mast cells could provoke neuronal injury, mixed neuron–glia hippocampal cultures were incubated with purified peritoneal mast cells, utilizing a cell culture insert system (Falcon) to provide for a molecule-permeant (but not cell-permeant) partition between the cell populations. Because mast cell activation in response to a given stimulus will commonly vary among preparations, a fixed number of mast cells consistently yielding maximal responses was used throughout. Mast cells were treated with 100 μM MBP for 12 h, after which the insert was removed. A delayed degeneration of neurofilament-immunopositive cells displaying a pyramidal-like morphology was seen, which was largely complete 60 h later (Fig. 1A and B). In contrast, the underlying astrocyte monolayer showed no obvious signs of injury or trypan blue staining.

Neuronal degeneration induced by MBP-treated mast cells was concentration dependent between 5 and 100 μM (Fig. 2), resulting in up to a 74% reduction in neuron number; the EC_{50} value was generally around 20 μM MBP. Addition of 100 μM MBP to neuron–glia cultures without mast cells did not produce neurotoxicity. Although without effect by itself, 17β-estradiol (50 μM) significantly enhanced the loss of neurofilament-immunopositive cells caused by incubation with mast cells and 20 μM MBP, approaching that achieved using 100 μM MBP (Fig. 2). A synergistic action of 17β-estradiol and MBP on mast cell secretion and brain myelin changes was reported in an earlier study (Theoharides et al., 1993). Removal of the culture inserts containing MBP (100 μM)-treated mast cells after only 1 h did not lead to a measurable reduction in the number of neurons, suggesting that the immediate release of mast cell mediator(s) on its own is insufficient to account for this injury. In some experiments, neuronal survival was assessed by counting pyramidal-like cells in unstained fixed cultures with phase-contrast microscopy, or by measuring release of lactate dehydrogenase into the culture medium. These methods yielded values that were in good agreement with each other and with those obtained from neurofilament immunostaining (data not shown).

Antigen-induced crosslinking of IgE bound to its receptors on the surface of mast cells provides another strong activating signal. Peritoneal mast cells, introduced into mixed neuron–glia cultures, were incubated first with anti-DNP IgE and then with DNP-HSA to crosslink IgE receptors. The mast cell-containing inserts were then removed 12 h after first adding the DNP-HSA. A 46% reduction in the neuronal population was observed 60 h after insert removal (Table 1). Addition of an excess of the monovalent, competitive ligand DNP-lysine prevents binding of the polyvalent ligand and mast cell degranulation (Kanner and Metzger, 1983), as well as neuronal death (Table 1). As when stimulated with MBP, mast cells activated with antigen needed to be present for a prolonged period of time in order to elicit a cytotoxic action for hippocampal neurons (Table 1).

NO$^{\cdot}$ mediates the delayed neuronal death induced by mast cells and is astrocyte dependent

Proinflammatory cytokines are able to induce NOS in astrocytes (Lee et al., 1993; Murphy et al., 1993).

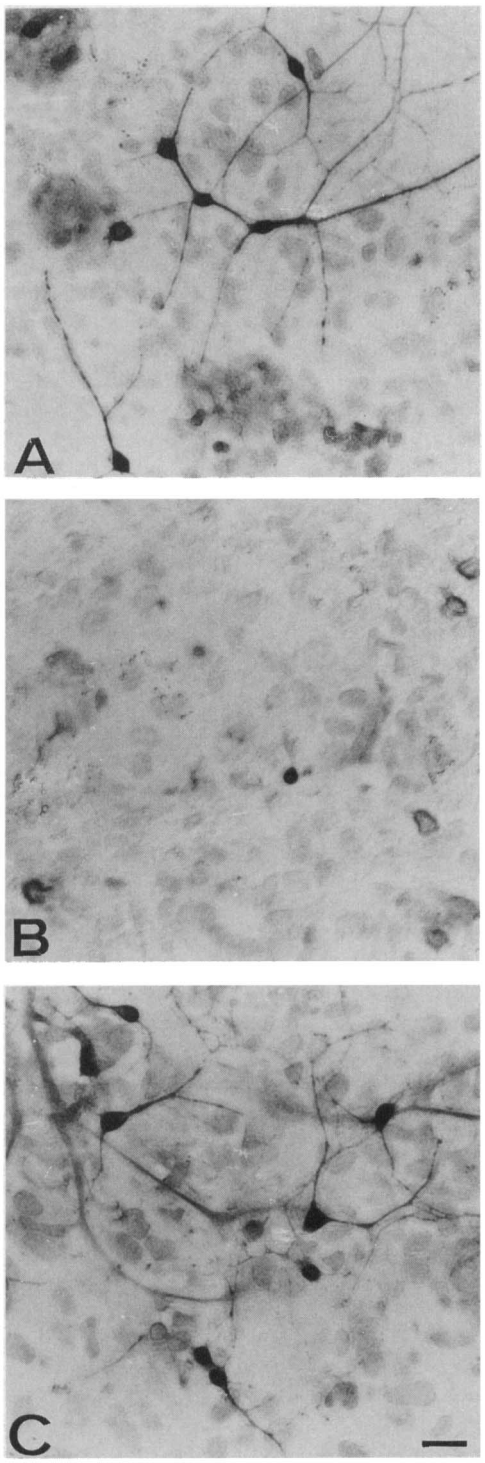

FIG. 1. Morphological appearance of hippocampal neurons following exposure to MBP-treated peritoneal mast cells. Dissociated cultures of embryonic day 18 hippocampus were plated over a semiconfluent monolayer of hippocampal astrocytes. At 8 DIV, the mixed culture was exposed for 12 h to 5×10^4 mast cells treated with 100 μM MBP, using the culture well insert system. Hippocampal cells were fixed 60 h after insert removal and processed for neurofilament protein immunocytochemistry. **A:** Control (untreated mast cells); **B:** inclusion of MBP-treated mast cells; **C:** L-NAME (1 mM)-treated hippocampal cells together with MBP-treated mast cells. Scale bar = 25 μm.

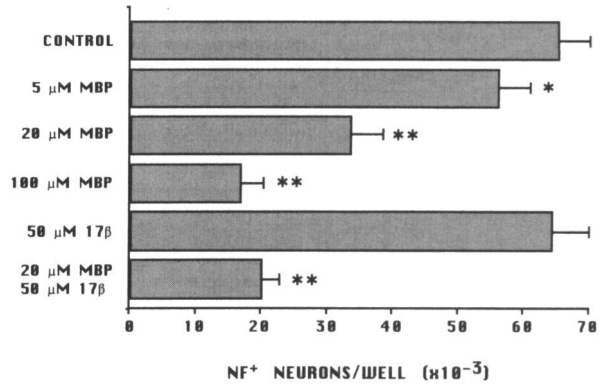

FIG. 2. Concentration-dependent activation by MBP of mast cells and delayed hippocampal neuron injury: synergism with 17β-estradiol. Mixed neuron–glia cultures were exposed for 12 h to 5×10^4 mast cells treated with the indicated concentrations of MBP and/or 17β-estradiol (17β). Numbers of neurofilament-positive (NF^+) cells were obtained 60 h after withdrawal of the mast cell-containing inserts. Values are means ± SD (three to four experiments). $^*p < 0.05$ or $^{**}p < 0.001$ compared with control.

$NO^\bullet$ derived from such activated astroglia can kill neurons (Dawson et al., 1994; Skaper et al., 1995). We therefore explored a possible role for astrocytic NOS in mediating neuronal injury occurring in the presence of degranulating mast cells. Addition of the NOS inhibitor L-NAME (1 mM) to the mixed neuron–glia cultures, together with MBP- or antigen-treated mast cells for 12 h, raised neuronal survival from 24 to 82% and from 52 to 81%, respectively (Fig. 1C, Table 2). A similar neuroprotective effect of oxyhemoglobin was seen, presumably due to its ability to bind and sequester extracellular $NO^\bullet$. When neutralizing antibodies to TNF-α were used instead of L-NAME, a more modest improvement in neuron survival was obtained (Table 2), suggesting that mast cell-released TNF-α may have participated to some extent in the induction of NOS. The combination of L-NAME plus anti-TNF-α was no more effective than L-NAME alone. It is interesting that a small percentage of mast cell-derived neurotoxicity occurred independently of glial NOS induction.

To evaluate more directly an astrocyte contribution to the neuronal injury triggered by mast cell activation, the antimitotic cytosine β-D-arabinofuranoside (5 μM) was added to the mixed neuron–glia cultures 24–48 h after plating. One week later, the number of glial fibrillary acidic protein-immunoreactive cells had been reduced by >85%. Introduction of MBP (100 μM)-treated mast cells into these glia-depleted cultures now produced a neuronal loss of 17 ± 2%, down from 75 ± 9% in astrocyte-rich cultures ($p < 0.001$, three experiments), thus implicating astroglia in the injury process. Cytokines also produced an astrocyte-dependent delayed neurotoxicity in mixed neuron–glia cultures, in the absence of mast cells, as will be described below.

TABLE 1. *Effect of IgE receptor-mediated activation of mast cells on hippocampal neuron survival*

Culture condition	NF^+ neurons/well
No antigen	65,037 ± 6,624
+Antigen (1 h)	64,906 ± 8,646
+Antigen (12 h)	
−DNP-lysine	35,012 ± 4,075[a]
+DNP-lysine (10 μM)	60,580 ± 5,275

Mixed hippocampal neuron–glia cultures were incubated for 1 or 12 h with inserts containing 5×10^4 mast cells activated by anti-DNP IgE/DNP-HSA ("antigen"). Hippocampal cells were processed for neurofilament immunoreactivity 60 h from the time of insert removal, and neurofilament-immunopositive (NF^+) cells were counted. Values are means ± SD (four experiments).

[a] $p < 0.001$, compared with all other groups.

Antigen-stimulated RBL-2H3 cells cause delayed neurotoxicity by activation of the NO· pathway in astrocytes

The cognate mast cell line RBL-2H3 expresses high-affinity IgE receptors (Oliver et al., 1988), and is widely used to study the signaling pathways leading to exocytotic release of inflammatory mediators from antigen-stimulated mast cells (Oliver et al., 1988; Ozawa et al., 1993; Apgar, 1994). Because the mast cell preparations unavoidably contained small numbers of contaminating peritoneal cells, we also tested the effects of activated RBL-2H3 cells on neuron vitality. When the mixed neuron–glia cultures were incubated for 12 h with antigen (anti-DNP IgE/DNP-HSA)-activated RBL-2H3 cells, a modest, delayed neurodegeneration was observed. This neuronal loss became more marked when IL-1β (10 ng/ml) was included in the incubation, such that 14% ($p < 0.05$) and 68% ($p < 0.001$) of the neurons, respectively, had been lost 60 h after removal of the RBL-2H3 cell-containing inserts (Table 3). A delayed neurotoxicity could also be achieved by substituting interferon-γ for IL-1β (data not shown). The neuronal injury caused by antigen-activated RBL-2H3 cells plus added IL-1β was prevented in large part (neuron loss reduced to 20–22%) by TNF-α antibodies or L-NAME, suggesting that RBL-2H3 cell-derived TNF-α acted in concert with exogenous IL-1β to induce astroglial NOS (Table 3). In glia-depleted (cytosine β-D-arabinofuranoside-treated) hippocampal cultures, cell loss caused by antigen-stimulated RBL-2H3 cells plus added IL-1β was only 23%, similar to the 14% cell loss obtained with antigen-stimulated RBL-2H3 cells (no added IL-1β) (Table 3). This latter portion of the overall neuronal injury was seen in the presence of L-NAME and anti-TNF-α antibodies, and in astrocyte-poor cultures.

TABLE 2. *Delayed neuronal death triggered by activated mast cells is mediated by the NO· pathway*

Addition	% of neuron survival
MBP activation	
None	23.8 ± 7.6
+L-NAME	81.8 ± 7.0[a]
+Anti-TNF-α	45.3 ± 8.2[a]
+L-NAME/+anti-TNF-α	82.7 ± 1.4[a]
+Oxyhemoglobin	73.5 ± 5.0[a]
Antigen activation	
None	52.5 ± 7.2
+L-NAME	80.7 ± 3.1[a]

Mixed hippocampal neuron–glia cultures were incubated for 12 h with inserts containing 5×10^4 mast cells treated with either 100 μM MBP or anti-DNP IgE/DNP-HSA ("antigen"). L-NAME (1 mM), oxyhemoglobin (200 μM), or anti-TNF-α antiserum (1 μg/ml) was added to the hippocampal cells at this time. Hippocampal cell cultures were fixed 60 h after insert removal. Neurofilament-immunopositive neurons were quantified as described in Materials and Methods. Values are means ± SD (three to four experiments).

[a] $p < 0.001$, compared with the respective control ("none").

TABLE 3. *IgE receptor-mediated activation of RBL-2H3 cells triggers delayed neurotoxicity dependent on the NO· pathway and astrocytes*

Culture condition	NF^+ neurons/well
Control	62,936 ± 5,044
Antigen	54,192 ± 3,306[a]
+L-NAME/anti-TNF-α	52,600 ± 3,710[a]
Astrocyte-depleted	54,111 ± 3,184[a]
Antigen + IL-1β	19,926 ± 2,133
+L-NAME	49,333 ± 2,866[b]
+Anti-TNF-α	49,444 ± 1,790[b]
+L-NAME/anti-TNF-α	51,853 ± 3,863[b]
Astrocyte-depleted	48,622 ± 1,916[b]

Mixed hippocampal neuron–glia cultures were incubated for 12 h with inserts containing 2×10^5 RBL-2H3 cells treated with anti-DNP IgE/DNP-HSA ("antigen"). L-NAME (1 mM), anti-TNF-α antiserum (1 μg/ml), or both were added to the hippocampal cells at this time. The hippocampal cell cultures were fixed 60 h after removing the inserts. Neurofilament-immunopositive (NF^+) neurons were quantified as described in Materials and Methods. Values are means ± SD (four experiments).

[a] $p < 0.05$, compared with the control group; [b] $p < 0.001$, compared with the antigen + IL-1β group and $p < 0.05$ compared with the control group.

Cytokines directly cause delayed neurotoxicity by activating the NO· pathway in astrocytes

Incubation of mixed neuron–glia hippocampal cultures with TNF-α (100 U/ml) for 60 h reduced neuron numbers to 83.7 ± 2.0% of control ($p < 0.01$, three experiments). Although IL-1β up to 100 ng/ml failed to induce neuronal injury in these mixed cultures, IL-1β and TNF-α acted synergistically, such that 10 ng/ml IL-1β plus 100 U/ml TNF-α caused neuron survival to fall to 27.8 ± 3.3% after 60 h ($p < 0.001$, three experiments). Interferon-γ was able to substitute for IL-1β (data not shown). The cytotoxic action of TNF-α/IL-1β appeared to require activation of astrocyte NOS, as either 1 mM L-NAME or 200 μM N^{ω}-nitro-L-arginine (another potent NOS inhibitor) prevented neuron death (Table 4). Addition of 1 mM L-

TABLE 4. *Cytokine-treated astrocytes can produce delayed neuronal injury via NOS*

Addition	% of neuron survival
TNF-α/IL-1β	27.8 ± 3.3
+*N*-Arg	84.6 ± 8.3[a]
+*N*-Arg/L-Arg	32.4 ± 9.0
+*N*-Arg/D-Arg	91.4 ± 2.4[a]
+L-NAME	87.6 ± 8.4[a]
TNF-α/IL-1β (astrocyte-depleted)	79.0 ± 12.2[a]

Mixed hippocampal neuron–glia cultures (8 DIV) were treated with TNF-α (100 U/ml) plus IL-1β (10 ng/ml), alone or together with 200 μM N^{ω}-nitro-L-arginine (*N*-Arg), 1 m*M* L-arginine (L-Arg), 1 m*M* D-arginine (D-Arg), or 1 m*M* L-NAME. Neuronal viability was assessed by direct counts of fixed cultures under phase-contrast microscopy, and was confirmed using trypan blue dye exclusion. Values are means ± SD (three experiments).

[a] $p < 0.001$, compared with TNF-α/IL-1β group.

arginine, but not D-arginine, to the exposure medium completely reversed the effect of N^{ω}-nitro-L-arginine. Further, TNF-α/IL-1β decreased neuron survival by only 22% in astrocyte-poor cultures (Table 4). Antimitotics did not adversely affect the neuronal population in the mixed cultures. The astrocytoma C6 cell line, which contains a cytokine-inducible NOS (Simmons and Murphy, 1993), was used in place of primary astrocytes to generate neuron–glia cocultures. Incubation of mixed hippocampal neuron–C6 cell cultures with TNF-α/IL-1β also induced a delayed neurotoxicity (data not shown), thus making unlikely a possible contribution by microglia in the mixed neuron–astroglia hippocampal cultures.

Down-modulation of mast cell activation by palmitoylethanolamide reduces neuronal injury

Palmitoylethanolamide is formed in peripheral tissues (Natarajan et al., 1982) and by CNS neurons (Di Marzo et al., 1994), and is reported to down-modulate mast cell activation in vitro (Facci et al., 1995) and in vivo (Aloe et al., 1993; Mazzari et al., 1994) and to decrease tissue inflammation (Perlìk et al., 1971; Mazzari et al., 1994). This *N*-acylamide has thus been suggested to exert, via mast cells, a local autacoid antiinflammatory function (Facci et al., 1995). We tested the ability of palmitoylethanolamide to protect neurons in the mixed hippocampal cultures from injury triggered by mast cell activation. Palmitoylethanolamide (30 μM) was added to the cultures at the time of mast cell activation. The concentration of MBP was reduced to 20 μM to produce an extent of neuron loss comparable to that seen upon antigen stimulation of mast cells. Palmitoylethanolamide limited neuronal injury provoked by either antigen- or MBP-treated mast cells, raising cell survival from 46 to 81% ($p < 0.001$) and from 48 to 74% ($p < 0.01$), respectively (Fig. 3). The *N*-acylamide, however, did not protect neurons from injury caused by direct cytokine activation of astrocytes in the absence of mast cells (data not shown).

DISCUSSION

The experiments described here demonstrate that mast cells can participate in delayed injury to CNS neurons. MBP-activated mast cells were able to trigger hippocampal neuron death selectively in mixed neuron–astroglia cultures. An immunogenic stimulus applied to mast cells also proved to be efficacious in initiating the neuronal injury process. Further, palmitoylethanolamide, an endogenous *N*-acylamide suggested to exert, via mast cells, a local autacoid antiinflammatory function (Facci et al., 1995), significantly reduced neuron losses caused by either MBP- or antigen-activated mast cells. A possible participation of mast cells in CNS inflammation has often been assigned to demyelination processes. Inflammation, however, may also play a role in some neurodegenerative diseases (Eikelenboom et al., 1994).

Mast cell activation injures neurons by astrocyte/NO·-dependent and -independent pathways

There are several potential sources of NO· production in the brain, including neurons and endothelial cells that express NO· constitutively and produce NO· for brief periods in response to receptor activation (Nathan, 1992). Astrocytes and microglia can be induced by endotoxin and cytokines to express a NOS isoform similar to macrophage NOS, which synthesizes NO· for extended periods (Boje and Arora, 1992; Nathan, 1992; Lee et al., 1993; Murphy et al., 1993). NO· is a relatively stable free radical that can easily cross cell membranes and has the potential to travel significant distances in tissue before reacting with a target. In neuronal cultures, including hippocampus, acute exposure to high concentrations of NO· by overactivation

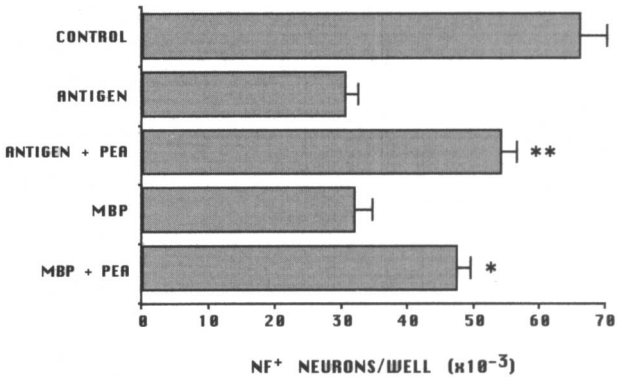

FIG. 3. Palmitoylethanolamide reduces hippocampal neuron death caused by antigen- or MBP-treated mast cells. Mixed neuron–glia cultures were incubated for 12 h with inserts containing 5×10^4 mast cells treated with either anti-DNP IgE/DNP-HSA ("antigen") or 20 μM MBP, alone or together with 30 μM palmitoylethanolamide (PEA). Hippocampal cell cultures were fixed 60 h after insert removal. Neurofilament-immunopositive (NF^+) neurons were quantified as described in Materials and Methods. Values are means ± SD (four to five experiments). *$p < 0.01$ or **$p < 0.001$ compared with the same condition but without palmitoylethanolamide.

of glutamate receptors or by addition of high concentrations of NO• donors results in neuronal cell death (Dawson et al., 1991, 1993).

Neuron injury caused by exposure of the hippocampal cultures to activated mast cells appeared to be a consequence of astrocyte NOS induction. Neurotoxicity was effectively inhibited by the arginine analogue L-NAME and by oxyhemoglobin, demonstrating that NO• is, at least in part, responsible for this cell death. Further, neuron loss provoked by activated mast cells was greatly diminished, although not entirely eliminated, in astrocyte-poor cultures. This last result strengthens the evidence for the participation of an inducible glial NOS in this experimental paradigm of delayed neurodegeneration. The mast cell mediators that take part in inducing astrocyte NOS remain to be identified. Neutralizing antibodies to TNF-α partially, albeit significantly, reduced neuron injury, suggesting that mast cell TNF-α (Galli, 1993), possibly in concert with other cytokines (Gordon et al., 1990), acts to induce the enzyme. This idea is supported by results obtained using antigen-activated RBL-2H3 cells, where neuronal toxicity in the mixed hippocampal cultures occurred only in the presence of exogenous IL-1β (or interferon-γ), and was strongly reduced by anti-TNF-α antibodies. RBL-2H3 cells are known to produce de novo TNF-α upon IgE receptor crosslinking (Ohno et al., 1990). In addition, the data with RBL-2H3 cells favor the interpretation that the mast cell effects were not due to peritoneal-derived non-mast cells.

A small (15–25%) but significant loss of neurons was consistently observed in the presence of activated mast cells, and this loss was refractory to inhibitors of NOS and to glial cell depletion. This may have resulted from a direct neuronal action of mast cell mediators not involved in the induction of glial NOS (e.g., proteases, arachidonate metabolites). Participation of cytokines like TNF-α and IL-1β seems unlikely, as they were not neurotoxic per se. The direct neurotoxic effects of activated mast cells were also of a delayed nature. To the best of our knowledge, such a phenomenon has not been described previously.

The number of mast cells in whole rat brain reportedly varies from about 1,500 to 19,000, and they are found mainly in the thalamus, hypothalamus, circumventricular organs, and meninges (Hough et al., 1984). More recent studies using electron microscopic techniques suggest that mast cell numbers in brain, observed at the light microscopic level, may be an underestimate of the total mast cell population present (Manning et al., 1994). We have also observed that nervous tissues processed by standard histological methods can easily lead to the apparent "loss" of otherwise identifiable mast cells, unless special precautions are taken (authors' unpublished data). In this study, the number of mast cells added to the hippocampal cultures exceeds that expected to be present in a comparable volume of brain tissue. In examining the effects of mast cell secretion on brain myelin changes, Theoharides et al. (1993) cultured brain slices with numbers of peritoneal mast cells far in excess of the quantity of endogenous mast cells expected to be present in the brain slices used. Mast cell mediators released in in vitro cultures are subject to variable degrees of dilution, especially in the present experiments, before reaching the neural cell monolayer. It is not unreasonable to expect that in vivo such mast cell effects may take place on a more local level, leading to induction of astrocyte NOS and neuronal exposure to deleterious quantities of NO• wherever mast cells may be found.

Exposure of hippocampal neuron–glia cultures to high concentrations of TNF-α for 48–60 h caused a small, but significant, reduction in neuron number. The concentration–response curve for TNF-α was shifted downward in the presence of IL-1β, consistent with the neurotoxicity elicited in mixed cultures by antigen-activated RBL-2H3 cells and exogenous IL-1β. Cytokine induction of astrocyte NOS was sufficient to cause neuron death, in accord with other findings (Dawson et al., 1994; Skaper et al., 1995). Activated microglia can also mediate neuronal death via a NO•-dependent mechanism (Boje and Arora, 1992; Chao et al., 1992). Although the culture procedures used here were chosen so as to minimize contamination by microglia, their participation, to a limited extent, cannot be completely ruled out. Because a homogeneous glial cell line (C6) was able to substitute for primary astrocytes in these responses, the latter possibility seems unlikely.

NO• as a neurotoxin

NO• has been associated with neurotoxicity, but the mechanism(s) by which NO• kills cells has not been elucidated. The reaction of NO• with superoxide anion results in the formation of the potent oxidant species peroxynitrite which can, either by itself or through hydroxyl radical-like decomposition products, initiate lipid peroxidation and loss of cellular integrity (Beckman et al., 1990; Lipton et al., 1993). NO• and its degradation products may also mediate cytotoxicity through formation of iron–NO complexes of respiratory enzymes, oxidation of protein sulfhydryls, and DNA deamination (Radi et al., 1991; Wink et al., 1991). NO• activation of poly(ADP-ribose) synthetase (Zhang et al., 1994) can lead to cell death through depletion of β-nicotinamide adenine dinucleotide (the source of ADP-ribose) and ATP (Cosi et al., 1994; Zhang et al., 1994). Alternatively or in addition, NO• could react with hydrogen peroxide to form highly reactive singlet oxygen (Noronha-Dutra et al., 1993).

Acute elevations of neuronally derived NO•, e.g., upon *N*-methyl-D-aspartic acid receptor stimulation, are sufficient to kill neurons (Dawson and Snyder, 1994). The slow onset of neurotoxicity elicited either indirectly by activated mast cells or directly by cytokines in mixed neuron–glia cultures may reflect the time needed to reach cytotoxic concentrations of NO• in the extracellular milieu, consequent to induction of astrocyte NOS. In addition, the lengthy (12 but not 1

h) exposure of mixed neural cultures to activated mast cells, needed to provoke neuronal injury, could be explained by a time-dependent accumulation of astrocyte NOS-inducing molecules released from the mast cells themselves and/or from astrocytes stimulated by mast cell-derived mediators (e.g., TNF-α-induced expression of astrocyte TNF-α). A direct mast cell origin for NO$^\bullet$ can probably be excluded, as neuronal cell death was only first detectable 24–36 h after removal of the activated mast cells, a time at which any mast cell-derived NO$^\bullet$ would have long since dissipated. Significant, but reduced, neuroprotection could still be achieved when NOS inhibitors were first added to the mixed neural cultures after withdrawing the mast cells (authors' unpublished observations).

Palmitoylethanolamide decreases neuronal injury by down-regulating mast cell activation

Palmitoylethanolamide reduced the delayed toxicity to hippocampal neurons caused by antigen-stimulated mast cells, consistent with its reported ability to down-modulate the immunogenic activation of mast cells in vitro (Facci et al., 1995). The *N*-acylamide was also effective in rescuing neurons from injury provoked by MBP-treated mast cells. Palmitoylethanolamide has not been shown previously to be effective against an MBP stimulus to mast cells, although it can improve the neurological outcome of mice with experimental allergic encephalomyelitis (Leon et al., 1994*b*). The effects of palmitoylethanolamide were not exerted directly at the neuronal level. Nerve cell death caused by cytokine induction of astrocyte NOS in the absence of mast cells was not sensitive to palmitoylethanolamide, nor was the compound able to protect against the neurotoxic effects of nonenzymatically generated NO$^\bullet$ (authors' unpublished data). Mast cells express the peripheral cannabinoid receptor CB2 which, upon binding palmitoylethanolamide, controls their activation (Facci et al., 1995). It is of interest that palmitoylethanolamide down-modulates mast cell activation in vivo (Aloe et al., 1993; Mazzari et al., 1994) and decreases tissue inflammation (Perlìk et al., 1971; Mazzari et al., 1994). Palmitoylethanolamide and other *N*-acylamides are synthesized in peripheral tissues (Schmid et al., 1990) and by CNS neurons (Di Marzo et al., 1994), and accumulate in some pathological conditions known to be associated with inflammatory reactions (Natarajan et al., 1982). This suggests that palmitoylethanolamide may exert, via mast cells, a local autacoid antiinflammatory function—hence the acronym autacoid local inflammation antagonism ("ALIA") (Aloe et al., 1993; Facci et al., 1995).

A role for NO$^\bullet$ in CNS pathologies

NO$^\bullet$ has been suggested to play a role in the neuropathologic sequelae of various CNS disorders. Neuronal (constitutive) NOS may be sufficient to cause neurotoxicity due to glutamate receptor overactivation (Dawson and Snyder, 1994). Induction of NOS also occurs in the CNS with viral infection, traumatic injury, ischemia, and experimental autoimmune encephalomyelitis (Koprowski et al., 1993; Endoh et al., 1994; Van Dam et al., 1995; Vizzard et al., 1995). In the acquired immunodeficiency syndrome, HIV-1-induced nerve cell loss and dementia are associated with the presence of infiltrating leukocytes and the accumulation of inflammatory cytokines, including TNF-α, IL-1, and interleukin-6 (Merrill and Chen, 1991). The cellular site(s) of origin for these cytokines remains to be defined precisely, and a role for mast cells cannot be excluded. Mast cells are involved in virus-induced inflammation in the CNS (Mokhtarian and Griffin, 1984), and their activation can be triggered by certain viruses (Sugiyama, 1977). The deleterious neuronal consequences of glia-derived NO$^\bullet$ are potentially dual, namely, direct nerve cell injury (Dawson et al., 1994; Skaper et al., 1995), and the rendering of neurons more sensitive to other toxic insults, e.g., those associated with excitatory amino acid receptor activation (Hewett et al., 1994). Involvement of inflammation in the pathology of certain neurodegenerative diseases has also been proposed (Eikelenboom et al., 1994). Pharmacological strategies aimed at modulating uncontrolled mast cell activation, which make use of endogenous molecules and mechanisms, may be of therapeutic benefit.

Several studies have documented the presence of NO$^\bullet$ or its products in the affected tissues or cells of mice and rats with experimental autoimmune encephalomyelitis (Koprowski et al., 1993; Lin et al., 1993), with inhibitors of inducible NOS ameliorating the disease (Cross et al., 1994). There is also evidence for increased NO$^\bullet$ production (Johnson et al., 1995) and astrocytic NOS expression (Bö et al., 1994) in multiple sclerosis. Mast cells appear to have an active role in eliciting experimental autoimmune encephalomyelitis (Dietsch and Hinrichs, 1989), where they can be found in a degranulating state in areas of demyelination (Theoharides et al., 1991). Mast cells have also been localized to multiple sclerosis plaques (Olsson, 1974; Toms et al., 1990). Release of MBP from demyelinated axons and/or myelin fragments from the action of mast cell proteases (Johnson et al., 1988; Dietsch and Hinrichs, 1991) could provide a further trigger for mast cells, leading to a feed-forward reaction. Immune-mediated processes have been reported to cause degeneration of neurons in the substantia nigra (Le et al., 1995). Neuronal loss, although not a hallmark of remitting–relapsing multiple sclerosis, may ultimately prove to be an underestimated feature in the chronic progressive form of the disease, perhaps as a result of protracted glial NOS expression. Experiments are currently in progress to address this issue.

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