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T cell-activated microglia as stand-by glutamate scavengers: the role of interferon-γ

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Abstract

Neuronal degeneration in the central nervous system (CNS) is largely caused by destructive self-compounds such as glutamate. Studies in vivo have suggested that memory T cells directed to self-antigens resident at the site of injury, upon homing to the lesion, facilitate protection against neurodegeneration. In this in vitro study we show that activated T helper (Th1) cells can significantly increase the ability of microglia not only to perform their usual immune-related function of phagocytosis and antigen presentation, but also to scavenge neurotoxic extracellular glutamate under physiological conditions or in a hostile oxidative environment. Gene array analysis showed that T cell-activated microglia exhibit upregulation of gene clusters associated with interferon (IFN)-γ-dependent signaling, and of genes encoding for chemokines and immunoproteasomes or associated with defense against oxidative stress. IFN-γ indeed simulated, in part, the effect of the activated T cells on microglia. It also increased microglial expression of glutamate transporter-1 but not of inducible nitric oxide synthase, enabling the microglia to tackle glutamate toxicity without the risk of redox imbalance. The physiological relevance of these findings is supported in part by the negative effect of systemic injection of anti-IFN-γ antibodies on neuronal survival after optic nerve injury. The results suggest that Th1 cells, partly through the mediation of IFN-γ, can activate microglia not only as resident innate immune cells but also as stand-by neural cells in situations of stress. These findings have far-reaching basic and clinical implications for neuronal death secondary to mechanical injury and for neurodegenerative diseases such as Alzheimer's and Parkinson's, amyotrophic lateral sclerosis, glaucoma, and other disorders characterized by glutamate toxicity.

Introduction

Glutamate is a ubiquitous mediator of toxicity in acute trauma of the central nervous system (CNS) such as brain or axonal injury (Alessandri and Bullock, 1998), ischemia (Lipton, 1999) and epilepsy (Fountain, 2000), and as well as in chronic neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and glaucoma (Choi, 1988). Research efforts world-wide are aimed at stopping the glutamate-associated progressive degeneration by the use of compounds that block glutamate toxicity (Chen et al., 1992; Choi, 1994) or increase the ability of local clearance mechanisms to restrict extracellular glutamate to nontoxic amounts (Gorovits et al., 1997; Shaked et al., 2002).

Recent studies of rodent models in our laboratory, using the mechanically injured optic nerve or spinal cord or employing retinal ganglion cells (RGCs) directly exposed to glutamate toxicity, have shown that an additional self-protective mechanism against glutamate and other self-compounds present in toxic excess of their physiological concentrations is provided by the immune system (Moalem et al., 1999b; Hauben et al., 2000b; Hauben et al., 2000a; Kipnis et al., 2000; Hauben et al., 2001b; Hauben et al., 2001a; Schori et al., 2001b; Mizrahi et al., 2002). An immune-mediated self-protective mechanism is spontaneously evoked against a physiological compound such as glutamate but not against nonphysiological neurotoxic compounds such as NMDA (Schori et al., 2001a). This protective mechanism is wiped out in the absence of T cells, pointing to the existence of a physiological T cell-dependent mechanism that protects the individual from glutamate toxicity (Schori et al., 2001b; Schori et al., 2002). This mechanism can be boosted by immunization with specific antigens located at the lesion site (Mizrahi et al., 2002). Since the ability to exhibit this beneficial effect is antigen specific (Moalem et al., 1999b; Mizrahi et al., 2002), we postulated that to be protective the T cells need to be locally activated by specific antigens presented to them at the lesion site (or in other words, that T cells homing to the

lesion site should encounter and become activated by their specific antigen there), and that thereafter their activity will be mediated, independently of T cells, by cytokines and other T cell-derived products.

Early activation of microglia as antigen-presenting cells correlates with T cell-mediated protection and repair of the injured CNS (Shaked et al., unpublished observations). Moreover, the glial glutamate transporter GLT-1 is strongly expressed in activated microglia after axotomy (Lopez-Redondo et al., 2000). The above and other observations, coupled with the finding that protection is preferentially mediated by T helper 1 (Th1) cells (known to facilitate macrophage activity) (Kipnis et al., 2002), led us to suggest that once activated, T cells coordinate the local innate protective response by activating resident microglia.

We show here that activated T cells can significantly increase the ability of microglia not only to perform their usual immune function of phagocytosis and antigen presentation, but also to scavenge neurotoxic extracellular glutamate under physiological conditions or in a hostile oxidative environment.

Gene array analysis showed that T cell-activated microglia exhibit enhanced expression of gene clusters associated with interferon (IFN)-γ-dependent signaling (e.g. STAT-1, IRF-1, SOCS-1). The effect of the activated T cells on microglia could indeed be partially simulated by IFN-γ. Moreover, treatment with IFN-γ increased microglial expression of GLT-1 but had little effect on the expression of inducible nitric oxide synthase (iNOS), so that the IFN-γ-activated microglia could fight off glutamate toxicity without risk of redox imbalance. Also upregulated were genes encoding for chemokines and immunoproteasomes, and genes associated with defense against oxidative stress. The physiological relevance of these findings is demonstrated by the negative effect of systemic injection of anti-IFN-γ antibodies on neuronal survival after optic nerve injury.

Materials and Methods

T cell line. The T cell line (T_{MBP}) was generated from draining lymph node cells obtained from Lewis rats immunized with myelin basic protein (MBP), as previously described (Ben-Nun and Cohen, 1982a, b, c). The antigen was dissolved in phosphate-buffered saline (PBS; 1 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected (in 0.1 ml of the emulsion) into the rats' hind footpads, the rats were killed and draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 μg/ml) in a proliferation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), nonessential amino acids, and autologous rat serum 1% (volume/volume). After incubation for 72 h at 37°C, 90% relative humidity and 7% CO_2, the cells were transferred to a propagation medium consisting of DMEM, L-glutamine, 2-mercaptoethanol, sodium pyruvate, nonessential amino acids, and antibiotics, in the same concentrations as above, as well as 10% fetal calf serum (FCS; volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A-stimulated spleen cells. The cells were incubated in the propagation medium for 4 to 10 days prior to restimulation with their antigen (10 μg/ml) in the presence of irradiated thymus cells (10^7 cells/ml; 2000 rad) in proliferation medium. The T cell lines were expanded by repeated stimulation and propagation. The T_{MBP} cells used in this study were previously characterized as Th0 cells with a Th1 bias (Moalem et al., 2000).

Co-culturing of microglia and T_{MBP} cells. Microglia were purified from the cerebral cortex of newborn (day 0) Lewis rats as previously described (Corradin et al., 1993). The cells were

grown as a mixed glial culture for 7 days. Microglia were separated from other glial cells by shaking for 6 h at 200 rpm at 37°C. The cells were then seeded on coverslips coated with poly-D-lysine (20 µg/ml) (Sigma–Aldrich, Rehovot, Israel) in 24-well plates (10^5 cells/ml/well), and incubated at 37°C, 5% CO_2 for 3 days before T_{MBP}-activated cells were added. Microglia and T_{MBP}-activated cells (2×10^4 cells/ml/well) were co-cultured for 24 h in RPMI-1640 medium (Sigma–Aldrich) containing 10% FCS, 50 µM 2-mercaptoethanol (Sigma–Aldrich), 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The microglia were then washed three times with PBS and prepared for immunocytochemistry. In a separate experiment, microglia and T_{MBP} cells were co-cultured in a transwell using BD Falcon™ cell culture inserts, pore size 0.4 µM (BD Biosciences City, State).

Microglia phagocytosis of Zymosan A. The engulfment of zymosan particals was used as an indicator of phagocytosis. Basically, zymosan A florescent stained particles (Sigma, Israel) were added to microglial cultured in 24-well plates (10^5 cells/ml/well). After incubation for 90 minutes (37°C, 5% CO_2), confocal imaging with superimposed light transmission mode was used to assess that the particles were engulfed inside the microglia cells. The number of engulfed particles per microglia was automatically counted using Image-Pro Plus 4.5 software from eight fields (200 μm^2) of slide in duplicates from three independent experiments.

Glutamate uptake. Uptake of glutamate was measured essentially according to the method of Swanson et al. (Swanson et al., 1997). Briefly, the medium in the wells was replaced with 0.5 ml of Hanks's balanced salt solution (HBSS) and maintained for 30 min at 37°C in 10% CO_2. Following this preincubation, each well was incubated at 37°C with 0.5 ml of reaction mixture (2 µM/0.05 µl L-[U]-^{14}C glutamate/HBSS or 40 µM/0.05 µl L-[U]-^{14}C glutamate/HBSS).

After incubation for 30 min, each well was rinsed twice with cold HBSS and its contents were then solubilized with 200 µl of lysis buffer (0.5 N NaOH/0.05% sodium dodecyl sulfate). ^{14}C-glutamate was determined in 100-µl aliquots using a scintillation counter. Protein content was determined using the Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL). In experiments aimed at examining the dependence of glutamate uptake on sodium, sodium was replaced with choline chloride (137 mM). Values are presented as means ± SD of one of three separate experiments, each repeated 8 times, with similar results.

Immunocytochemistry. Cells on coverslips were fixed (20 min at room temperature) in 4% paraformaldehyde diluted in PBS, then incubated (15 min at room temperature) in permeabilization/blocking solution (10% FCS, 2% bovine serum albumin, 1% glycine, 0.1% Triton X-100 in PBS). For double staining we used primary anti-B7-2 and primary anti-ED1 antibodies, both originating in mouse. To avoid any false staining with the secondary antibodies (both of which are directed against mouse IgG), the staining was carried out as follows: cells were incubated with anti-ED1 antibody alone for 1 h at room temperature as described above and then with the secondary goat anti-mouse antibody (FITC) at twice the normal concentration. This was done in order to saturate all free sites on the primary antibody, thus preventing it from binding later to the secondary Cy3 goat anti-mouse antibodies. The cells were then washed with PBS containing Tween-20, incubated for 1 h with primary mouse anti-rat antibody to B7-2, washed again, and incubated with the secondary Cy3-conjugated goat anti-mouse antibody. To confirm the lack of binding of the Cy3-conjugated goat anti-mouse antibody to the first primary antibody (ED1), a negative control reaction was carried out by following the first and second steps described above but without the primary anti-B7-2 antibody. For triple staining (to examine the expression of class II major histocompatibility complex proteins (MHC-II) on microglial cells and for nuclear

labeling), we used I-B4 (which is directly conjugated to FITC), Cy5 (the secondary antibody for MHC-II), and propidium iodide (PI; 0.25% volume/volume; Molecular Probes, Eugene, OR). The results were analyzed using Image-Pro Plus 4.5 software. For immunocytochemical analysis of glutamate transporter (GLT)-1, we used guinea pig anti-rat GLT-1 polyclonal antibody (Chemicon, USA), the staining was carried out as follows: cells were incubated with anti-GLT-1 antibody over night at 4°C and then with the Cy3 conjugated anti- guinea pig affinity purified secondary antibody (Chemicon, USA).

Microarrays. Changes in microglial gene expression after co-culturing with T cells were examined by the use of spotted oligonucleotide microarrays. A collection of 4,854 oligonucleotides specific for 4,803 rat cDNA clusters was purchased from Compugen (Jamesburg, NJ). In addition, a set of 113 oligos was designed and synthesized by MWG-Biotech AG (Ebersberg, Germany) based on a set of GenBank accession numbers that we provided. All bioinformatics for the oligonucleotides are provided on our searchable web site (http://www.ngelab.org). Oligos were spotted onto poly-L-lysine-coated slides by the use of standard procedures. Hybridization probes were prepared using the Genisphere 3DNA dendrimer system (Genisphere, Montvale NJ). Samples were hybridized and washed on a Ventana Discovery System (Ventana Medical Systems, Tuscon, AZ) using protocols developed by us, then imaged on an Axon 4000B scanner. All microarray data were loaded into the MIAME-compliant database BASE (Saal et al., 2002) and subjected to LOWESS normalization (Yang et al., 2002b). Normalized arrays were then imported into GeneSpring 5.0 (Silicon Genetics, Redwood City, CA). For the replicated data of each gene, a one-sample Student's t-test (two-tailed) was used to examine whether the mean normalized expression level for the gene was statistically different from 1.0. This procedure yielded a 473-gene data set at $P < 0.05$. To control for errors due to multiple comparisons, we estimated the false discovery rate (FDR; the expected ratio of erroneous rejections to the number of rejected

hypotheses) according to the approach introduced by Benjamini and Hochberg (Benjamini and Hochberg, 1995). Individual P values were compared with their corresponding FDR thresholds, and 462 genes were retained. Finally, we sorted genes by their normalized expression levels and obtained a list of 84 genes by applying a conservative 2-fold threshold.

Reverse transcription PCR. We assayed specific mRNA expression using quantitative reverse transcription PCR (RT–PCR) with selected gene-specific primer pairs. The primers used were inducible nitric oxide synthase (iNOS), sense TGAGACAGGAAAGTCGGAAG, antisense TCCCATGTTGCGTTGGAA, and L19, sense CTGAAGGTCAAAGGGAATGTG, antisense GGACAGAGTCTTGATCTC. RNA was reverse-transcribed with SuperScript II as recommended by the manufacturer. RT–PCR reactions were carried out using 1 μg of cDNA, 35 nmol of each primer, and ReadyMix PCR Master Mix (ABgene, Epsom, UK) in 30-μl reactions. The PCR products were assessed using 1.5% gel agarose.

Quantitative reverse transcription PCR. We assayed specific mRNA levels using quantitative reverse transcription PCR (Q-RT–PCR) with selected gene-specific primer pairs. The primers used were: STAT-1, sense AACGGTCCCAAAATGGAGGT, antisense TGTAGGGCTCAACAGCATGG; GLAST, sense TCGGAATGCCTTTGTGCTACT, antisense GCCATTCCCACAATGACAGC; EAAC-1, sense AATATTCCGCAAGTTGGGCC, antisense GCAAGCCCACTCAGGACAGT; GLT-1, sense AGCATTGGTGCAGCCAGTATT, antisense GAGGAGCATGGTGACCAACC; and GAPDH as a housekeeping gene, sense AACTCCCTCAAGATTGTCAGCAA, antisense GGCTAAGCAGTTGGTGGTGC. RNA was reverse-transcribed with SuperScript II and random primers as suggested by the manufacturer. PCR reactions were carried out using 10 ng cDNA, 50 nM of each primer, and SYBR® Green PCR Master Mix (Applied Biosystems,

Foster City, CA) in 10-μl reactions. The Q-RT–PCR products were assessed by measuring the SYBR Green fluorescence (DyNAmo™ SYBR® Green qPCR Kit, Applied Biosystems) (Ririe et al., 1997; Wittwer et al., 1997) collected during real-time PCR on an Applied Biosystems 7900HT Micro Fluidic card. A control cDNA dilution series was created for each gene to establish a standard curve. Each reaction was subjected to melting-point analysis to confirm single amplified products.

Optic nerve injury model and assessment of RGC survival

Animals. C57Bl/6J mice were obtained from The Weizmann Institute of Science, Israel.

Antibodies. The primary antibodies used were rat anti-mouse CD11b (mac-1) monoclonal antibody (Pharmingen, Becton-Dickinson, Franklin Lakes, NJ), rat anti-mouse IFN-γ monoclonal antibody (R4-6A2) (Endogen, Woburn, MA), and normal rat IgG (Sigma, St Louis, MO) and IgG1 (R&D Systems, Minneapolis, MN). As second antibody we used FITC-conjugated goat anti-rat IgG (Jackson ImmunoResearch, West Grove, PA).

Crush injury. A severe crush injury was inflicted on the intraorbital portion of the optic nerves of wild-type male mice aged 8–12 weeks. Two days before the injury all RGCs were stereotactically labeled with the neurotracer dye FluoroGold (5% solution in saline; Fluorochrome, Denver, CO). On day 14 after the injury the retinas were excised and whole-mounted and the RGCs were counted. Statistical significance was determined using a two-tailed unpaired *t*-test (Fisher et al., 2001).

Treatment with antibodies. Two days before crush injury all RGCs were stereotactically labeled with the neurotracer dye FluoroGold. Immediately after injury, neutralizing rat anti-

mouse IFN-γ mAb (R46A2) or control rat IgG was injected intravenously (i.v.) into the mouse's tail vein (15 or 60 μg). After 14 days the retinas were excised and whole-mounted and the RGCs were counted.

Immunohistochemistry: Nerves were excised for immunohistochemical staining 14 h and 1, 4, and 7 days after crush injury. Longitudinal cryosections of the nerves, 10 μm thick, were picked up onto gelatin-coated glass slides. Sections were fixed in absolute ethanol for 10 min at room temperature, washed twice in double-distilled water, and incubated for 3 min in PBS containing 0.05% polyoxyethlene–sorbitan monolaurate (Tween-20). They were then incubated for 1 h at room temperature with primary antibodies diluted in PBS containing 3% FCS and 2% bovine serum albumin. The sections were washed 3 times with PBS and Tween-20 (0.05%) and incubated with second antibody for 1 h at room temperature. After further washing with PBS containing Tween-20, the sections were viewed under a confocal microscope.

Results

Interaction with activated T_{MBP} cells in vitro converts microglia into an active phenotype

Experiments in vivo have shown that in rats subjected to contusive injury of the spinal cord, passive transfer of T cells activated by stimulation with their specific antigen MBP (T_{MBP} cells) (Moalem et al., 1999b) causes upregulation of the costimulatory molecule B7-2 (Butovsky et al., 2001) and MHC-II, both reminiscent for antigen presentation. These findings prompted us to examine the direct effects of activated autoimmune T cells on microglia, potentially a primary target for activation by T cells. This was done by co-culturing an enriched population of microglial cells (of which 98% were ED-1 positive according to Corradin et al. (Corradin et al., 1993)) with activated T_{MBP} cells. After 24 h we analyzed the

microglia for expression of B7-2 or MHC-II using ED1 antibodies (recognizing CD68, a glycosylated lysosomal antigen expressed on activated phagocytes (Damoiseaux et al., 1994)), or with IB-4 (which labels microglia of any phenotype (Dailey and Waite, 1999)), together with anti-B7-2 or anti-MHC-II antibodies, respectively (Fig. 1). Control microglia (cultured without T cells) expressed only small amounts of B7-2 (Fig. 1A) and negligible amounts of MHC-II (Fig. 1C). In the presence of activated T_{MBP} cells, both B7-2 (Fig. 1B) and MHC-II (Fig. 1D) were upregulated.

Activated T_{MBP} cells enhance the capacity of microglia for glutamate uptake

The above demonstration that T_{MBP} cells can modify the phenotype and activity of microglia encouraged us to examine the direct effects of these T cells on the ability of microglia to remove extracellular glutamate. An enriched population of rat microglia was co-cultured with activated or resting T_{MBP} cells for 24 h. Glutamate uptake by the microglia was measured after the addition of [^{14}C]-glutamate (2 μM or 40 μM) for 30 min at 37°C in uptake medium containing 150 mM NaCl. The two glutamate concentrations represent physiological and stress-related extracellular glutamate levels, respectively (van Landeghem et al., 2001). Microglial cultures, incubated with 137 mM NaCl or 137 mM choline chloride in the absence of T cells, served as a control. Intracellular glutamate concentrations were determined after extensive washing to remove T cells. Figure 2A, B shows microglial uptake of glutamate at the physiological concentration (2 μM), expressed as nmol glutamate per well or nmol glutamate per mg protein, respectively. Both parameters show the same pattern of increased glutamate clearance by T_{MBP} cell-activated microglia. By presenting the data in two ways we were able to verify that the assays of glutamate uptake and of protein content were unaffected by T cell traces that might have remained after washing of the cultured microglia. In all

subsequent experiments the data are therefore presented only as nmol glutamate per mg protein. Resting memory T cells had only a modest effect on uptake.

Glutamate uptake is most commonly mediated via high-affinity sodium-dependent transporters (Billups et al., 1996; Zerangue and Kavanaugh, 1996; Levy et al., 1998). To verify that this was the mechanism operating in the present study, we replaced the sodium in the uptake medium with choline. As expected, this completely blocked the uptake activity, both at the physiological glutamate concentration (0.084 ± 0.04 with choline compared to 4.8 ± 1.3 with NaCl; $P < 0.001$, Fig 2B) and at the stress-related glutamate concentration (2.8 ± 0.6 compared to 24.3 ± 7.2, respectively; $P < 0.001$, Fig. 2 C).

In general, for immunological tasks, specificity of Th cells is known to be required for their activation; once the T cells have been activated, however, their activity on microglia is antigen independent and is mediated via soluble substances such as cytokines. To determine whether the observed ability of activated T_{MBP} cells to scavenge extracellular glutamate was indeed mediated by soluble factor(s), we co-cultured microglia with T_{MBP} cells using a Falcon transwell filter (pore size 0.4 μM) that allows the passage of soluble substances from the activated T cells but does not allow direct contact between the two cell populations. Under these conditions we observed that the activated T cells, although deprived of any cell–cell interaction, nevertheless induced microglial upregulation of glutamate uptake at both the physiological and the stress-related glutamate concentrations (Fig. 2A, B and C). These results suggest that the T cell effect on microglia is mediated by soluble factors (such as cytokines, the amounts of which were increased after activation). Glutamate uptake as a function of the number of co-cultured T cells is shown in Figure 2D. In terms of specific activity values, glutamate uptake by microglia reportedly reaches as much as 50% of that observed in astrocytes (Nakajima et al., 2001a), generally acknowledged as the professional clearance cells for glutamate in vivo.

Similar changes in microglial gene expression induced by activated T cells and IFN-γ

To examine the nature of the activation acquired by the microglia through their exposure to activated T cells, we first conducted a gene array analysis of T cell-activated microglia. The analyzed microglia had been co-cultured with activated T cells without the transwell apparatus, to allow their activation both via cell–cell interaction and via soluble compounds. At the end of the incubation we washed the microglia thoroughly to remove the T cells; it is possible, however, that some T cell traces might have remained in the microglial RNA, as discussed below. The microarrays used for the analysis comprised a collection of approximately 5,000 oligonucleotide probes specific for most known rat genes; however, since they had not been specifically developed for this research, the array lacked some important immune-related probes, such as MHC-II-encoding genes and neuronal genes encoding for glutamate transporters. Total RNA was extracted from the T cell-activated microglia and compared to RNA from control cultures without T cells.

Examination of the *t*-test results corrected by the false discovery rate (FDR) procedure (see Methods) on the normalized data sets revealed 60 significantly upregulated and 24 significantly downregulated genes in the RNA from the co-cultured microglia. The normalized gene expression levels are given with P values for replicated genes. Changes of more than threefold were seen in 18 of the 60 upregulated genes, while 22 of the 24 downregulated genes showed changes of two- to fourfold. Selected microarray results were confirmed by SYBR ® Green RT–PCR on seven upregulated genes and one downregulated gene (results not shown).

Among the clusters of activated genes, but not all of them, were genes related to IFN-γ-signaling pathways. The T_{MBP} cells used in this study were primarily Th0 with a bias

towards Th1 (Moalem et al., 1999a). Th1 cells are characterized by production of a battery of pro-inflammatory cytokines, such as interleukin-2 (IL-2), interleukin-3 (IL-3), tumor necrosis-α (TNF-α), lymphotoxin (TNF-β), and IFN-γ. We therefore considered the possibility that the activation by T_{MBP} cells was partially mediated by IFN-γ. In light of this possibility, coupled with our concern that some of the genes found to be upregulated in the T_{MBP}-cell-activated microglial RNA preparation might have been derived from traces of T cells in our preparation and not from the microglia, we repeated the gene array analysis; this time we compared genes from IFN-γ-activated microglia to those from nonactivated microglia and searched for genes that overlap those found in the T_{MBP}-cell-activated microglia. This comparison yielded 24 upregulated and six downregulated genes that overlapped between IFN-γ-activated and T_{MBP}-cell-activated microglia. For the 30 overlapping genes the Pearson correlation coefficient between T_{MBP} co-culture ratios and IFN-γ treatment for listed genes was 0.504, with a P value of 0.002 (Table 1). Additional genetic alterations in the T_{MBP}-cell-activated microglia, other than those that overlapped genetic alterations induced by T_{MBP} cells, are currently being investigated. The comparison between T_{MBP}-cell- and IFN-γ-activated microglia confirmed that the change in mRNA expression seen after T_{MBP} activation is derived from the microglia and not from possible T cell traces. Gene clusters detected in the microglia following their activation by T_{MBP} cells or by IFN-γ were constituents of the JAK/STAT pathway (Darnell et al., 1994), such as IRF-1 (11.8-fold change, $P = 1.7 \times 10^{-4}$), STAT-1 (6.5-fold change, $P = 5.6 \times 10^{-5}$), SOCS-1 (4.6-fold change, $P = 0.007$), JAK-1 (1.4-fold change relative to control, $P < 0.01$) and JAK-3 (1.4-fold change, $P = 0.05$) (data not shown due to the arbitrary 2-fold cut-off), as well as other genes known to be induced by IFN-γ, such as p67 GBP (Vestal et al., 1996) (10.8-fold change, $P = 0.001$) (Table 1). Additional genes clusters found to be upregulated in the T_{MBP}-cell activated microglia were those encoding for chemokines and chemokine receptors. These clusters include genes such

as MCP-1 (6-fold change, P = 0.008), MIP-1α (5.5-fold change, P = 0.02), CXCR3 (2.0-fold change, P = 0.04), and a dramatic induction of the rat homolog to IP-10, Mob-1, a 10-kDa IFN-γ-inducible protein (23.4-fold change, $P = 2.3 \times 10^{-3}$).

Genes that participate in amino acid transport, such as the neutral amino acid transporter ASCT2 (Broer et al., 1999) (2.6-fold change, P = 0.006) and the neuronal glutamine transporter GLT-1 (1.6-fold change, P = 0.003) were both upregulated. Of great interest was the observed upregulation of genes related to anti-oxidation, such as SOD2 (Murakami et al., 1998) (2.7-fold change, P = 0.01), thioredoxin (Lovell et al., 2000) (2.0-fold change, P = 0.02), glutaredoxine (Daily et al., 2001) (2.45 fold change, P = 0.001), and the antioxidant enzyme B166 (Knoops et al., 1999) (2.51-fold change, P < 0.002). MTP-1 (Burdo et al., 2001), an iron exporter that may have an antioxidant function (Yang et al., 2002a) was also substantially upregulated (8.5-fold change, $P = 1.8 \times 10^{-4}$) (Table 1).

We also observed significant upregulation in the expression of genes related to antigen presentation and antigen processing. Immunoproteasomes (Rivett et al., 2001), a group of proteins responsible for antigen processing, such as proteasome RC1 (3.0-fold change, P = 0.006), macropain protease 28 subunit (4.8-fold change, P = 0.002), major vault protein (2.7-fold change, P = 0.003), and proteasome RC6-1 (2.19-fold change, P = 0.004), were all upregulated, as was MHC Ib RT1 (2.6-fold change, P < 0.0001). Also worth noting was a modest upregulation in the expression of genes associated with CNS metabolism, such as brain glucose transporter (2.5-fold change, P = 0.015), phosphofructokinase 1 (PFK1) (1.8-fold change, P = 0.14), and hexokinase II (HKII) (2.3-fold change, P = 0.003).

Among the few genes that were reduced by more than 2-fold were HMG-1 (high mobility group 1), the late mediator of endotoxin lethality (0.43-fold change, P = 0.022) (Wang et al., 1999) and the metabolic enzyme glutamine synthetase (0.46-fold change, P = 0.024) (Table 1).

The T-cell effect on glutamate uptake can be reproduced by IFN-γ but not by IL-4 or lipopolysaccharide

In light of the gene array results, we examined whether the observed effect of activated T_{MBP} cells on the clearance of glutamate by microglia can be reproduced, at least in part, by IFN-γ. Figure 3A presents a dose–response curve showing the effect of IFN-γ on glutamate uptake. Under the same conditions glutamate uptake was not affected by interleukin 4 (IL-4), a cytokine characteristic of Th2 (glutamate uptake was 24.8 ± 2.1, 14.5 ± 3.5, and 15.0 ± 2.2 for IFN-γ, IL-4, and control, respectively; i.e., glutamate uptake in the IFN-γ-treated culture was significantly higher (P < 0.0001) than in the control cultures (Fig. 3B). These results prompted us to investigate whether the observed function of microglia to act as scavengers of glutamate is a general feature of the pro-inflammatory activation of microglia or if it is specific to IFN-γ activation. We therefore examined whether the increase in glutamate uptake induced by activated T_{MBP} cells can be reproduced by lipopolysaccharide (LPS), a potent pro-inflammatory agent shown to greatly enhance the phagocytic activity of microglia and the production of free radical production (Smith et al., 1998), on the clearance of extracellular glutamate by microglia. We found that LPS (100 ng/ml) had no effect on microglial uptake of glutamate (Fig. 3C).

We then compared LPS-activated and IFN-γ-activated microglia with respect to induction of NO production. Production of NO by activated microglia, mediated through iNOS, has been viewed as a major threat to oligodendrocytes (Merrill et al., 1993). As previously reported (Smith et al., 1998), LPS and IFN-γ both induced upregulation of phagocytic activity (Fig. 4); however, whereas inducible nitric oxide synthase (iNOS) was strongly expressed by LPS-activated microglia, its expression by IFN-γ-activated microglia was weak. These findings demonstrate that the microglial phenotype activated by T_{MBP} cells

via IFN-γ is unique, and is distinct from that induced by LPS (Fig. 4A, B), i.e., unlike the LPS-induced phenotype its induction is not accompanied by oxidative stress. These findings indicate that the activity of microglia as glutamate scavengers depends on a specific stimulus, which is supplied by activated T_{MBP} cells, in part via IFN-γ production, and is not simply a general feature of inflammatory conditions.

Next we examined the induction by IFN-γ of transcripts encoding for glutamate transporters. Using SYBR Green RT–PCR, we assayed the expression of two glial glutamate transporters, glutamate transporter–1 (GLT-1) and glutamate aspartate transporter (GLAST), and of the neuronal transporter excitatory amino acid carrier-1 (EAAC-1) (Kanai and Hediger, 1992). Only GLT-1, which is known to be expressed by microglia (Nakajima et al., 2001b), was strongly induced by IFN-γ (3.3-fold change, P = 0.01; Fig. 5A). The specificity of this regulation was demonstrated by comparison with GAPDH, which we assayed independently in the same cDNA. GAPDH mRNA levels did not change significantly in the presence of IFN-γ (P = 0.13). Analysis of GLT-1 expression at the protein level by immunocytochemistry showed that the protein was expressed by both IFN-γ-activated and nonactivated microglia (Fig. 5B). However, the analysis did not allow accurate assessment of the difference in amount of protein expressed by the IFN-γ-activated and the nonactivated cultured microglia.

Activate T_{MBP} cells prevent inhibition of microglial glutamate uptake mediated by oxidative stress

The observed upregulation of genes associated with protection against oxidative stress, and the fact that a modest increase in iNOS was induced by IFN-γ but not by LPS, prompted us to examine whether T cells can enable microglia to maintain their capacity for glutamate uptake under conditions of oxidative stress, known to cause a significant decrease in glutamate

clearance (Piani et al., 1993; Volterra et al., 1994; Trotti et al., 1996; Agostinho et al., 1997; Sorg et al., 1997; Muller et al., 1998). Oxidative stress can be induced by ferrous ions (Schori et al., 2001b). Incubation of microglia in the presence of increasing concentrations of ammonium-ferrous sulfate (0.01–1 mM) showed that glutamate uptake was inhibited in a dose-dependent manner by the ferrous salt (Fig. 6A). Co-culturing of the microglia with activated T_{MBP} cells counteracted the inhibitory effect of the ferrous ions and partially restored the glutamate-clearing ability of the microglia (Fig. 6A). The ability of INF-γ (20 ng/ml) to counteract the effect of oxidative stress was significantly lower than that of the T_{MBP} cells (Fig. 6B). IFN-γ effectively counteracted the inhibitory effect of the lower concentration of ferrous salt on glutamate clearance by the microglia (Fig. 6B), but was less effective with the higher concentration (Fig. 6B).

To determine whether the relatively weak effect of IFN-γ with the higher concentration of ferrous salt is attributable to glutamate toxicity or the potency of IFN-γ or both, we assessed the effect of activated T_{MBP} cells in the presence of ferrous salt at 0.5 mM. The ability of the activated T_{MBP} cells, but not of IFN-γ, to counteract oxidative stress was still significant at this concentration of ferrous salt (Fig. 6C). These results further indicate that T cells, as a source of cytokines and growth factors, can exert their controlling effect on microglial cells via IFN-γ and additional factors yet to be determined.

Retinal ganglion cell survival is decreased by anti-IFN-γ antibodies administered to wild-type mice immediately after optic nerve injury

The results described above suggest that after a CNS insult, IFN-γ contributes to the body's ability to cope with the stress. To evaluate the physiological relevance of IFN-γ to an individual's spontaneous ability to cope with injurious conditions in the CNS, we used the mouse optic nerve crush model (Fisher et al., 2001). In this model, microglial activation

(manifested by Mac-1 immunoreactivity) could be seen within 14 h of the injury (Fig. 7A). To examine the possibility that IFN-γ activity is linked to the ability to resist injurious consequences, we assessed the ability of anti-IFN-γ antibodies to block neuronal survival after a CNS insult. Immediately after optic nerve injury, we injected neutralizing rat anti-mouse IFN-γ antibodies (R46A2) or control rat IgG into the tail veins of C57Bl/6J mice. The numbers of surviving RGCs per square millimeter (mean ± SD) in mice injected with 15 μg or 60 μg of control rat IgG were 1121 ± 138 and 770 ± 78, respectively. These values are significantly higher than the corresponding values obtained in mice injected with 15 μg or 60 μg of the anti-IFN-γ antibodies R46A2 (957 ± 67 or 635 ± 97; $P < 0.05$ or $P < 0.03$, respectively; Fig. 7B).

Discussion

Studies in our laboratory, using several animal models of CNS injury, have shown that autoimmune T cells directed against antigens residing in the site of lesion have a beneficial effect on the damaged tissue (Moalem et al., 1999b; Mizrahi et al., 2002). It was proposed that T cell specificity be exploited as a means of promoting homing of T cells and boosting their local activation. In this in-vitro study we show that activated T helper (Th1) cells can significantly increase the ability of microglia to scavenge neurotoxic extracellular glutamate under physiological conditions or in a hostile oxidative environment. Gene array analysis showed that T cell-activated microglia exhibit enhanced expression of gene clusters associated with IFN-γ-dependent signaling (e.g. STAT-1, IRF-1, SOCS-1). The effect of the activated T cells on microglia could indeed be simulated, in part, by IFN-γ. Moreover, treatment with IFN-γ increased microglial expression of the glutamate transporter GLT-1 but had little effect on the expression of iNOS, so that the IFN-γ-activated microglia could fight off glutamate toxicity without risk of redox imbalance. Also upregulated were genes encoding

for chemokines and immunoproteasomes, and genes associated with defense against oxidative stress. The physiological relevance of these findings is supported in part by the negative effect of systemic injection of anti-IFN-γ antibodies on neuronal survival after optic nerve injury.

A. Glutamate as a common mediator in neurodegenerative disorders

The amino acid glutamate is a pivotal neurotransmitter needed for day-to-day functioning of the brain, spinal cord, and visual system (Fonnum, 1984; Ottersen and Storm-Mathisen, 1984; Collingridge and Lester, 1989; Headley and Grillner, 1990). To protect itself against extreme conditions of glutamate stress, the CNS possesses a remarkable ability to remove glutamate excess via transporter proteins present in the plasma membranes of glial cells and neurons (Danbolt, 2001). Under normal conditions these transporters represent the only significant mechanism for removal of glutamate from the extracellular space, and thus for restoring extracellular glutamate to nontoxic amounts. However, when glutamate exceeds physiological concentrations (as in the case of neurodegenerative disorders) and hence also exceeds the capacity of local glutamate-buffering mechanisms, it becomes a major cause of cytotoxicity (Danbolt, 2001).

B. T cells can protect against glutamate toxicity

Recent results from our laboratory suggest that T cells activated by encountering their own specific antigens, which are presented to them at the CNS lesion site by antigen-presenting cells, can serve as a backup for the local neural cells by counteracting glutamate toxicity (Schori et al., 2001b; Schori et al., 2002). The T cells found to mediate neuroprotection were Th0 with a bias toward Th1 (Moalem et al., 2000). Neuronal protection is preferentially mediated by Th1 cells (Kipnis et al., 2002). These observations led our group to suggest that Th 1 cells, once activated, coordinate the local innate response by activating resident

microglia. Activated microglia exhibit a variety of effector functions, such as phagocytosis and antigen presentation (Aloisi, 2001). They can also express neuroprotective proteins such as neurotrophins (Nakajima et al., 2001b) and glutamate transporters (Nakajima et al., 2001a, Rimaniol, 2000 #793). The glial glutamate transporter GLT-1 is strongly expressed in activated microglia after nerve axotomy (Lopez-Redondo et al., 2000). On the other hand, microglia reportedly mediate neuronal death by releasing glutamate (Piani et al., 1991; Piani et al., 1992), NO (Bal-Price and Brown, 2001), and TNF-α (Downen et al., 1999).

C. IFN-γ as a T cell-mediated cytokine invoked in neuroprotection

The present study throws some light on the opposing effects of activated microglia leading to neurodegeneration or to neuroprotection. Gene array analysis was carried out in a search for the mechanism underlying the T cell-mediated activation of microglia for neuroprotection. The finding that genes expressed by IFN-γ-activated microglia overlap those expressed by microglia activated as a result of co-culturing with T_{MBP} cells suggested to us that the mechanism through which T cells influence microglial activity might involve IFN-γ, considered to be a potent regulator of several features of the immune and the principal means by which T cells activate macrophages response (Boehm et al., 1997). In addition, this cytokine is thought to play a prominent role in host defense (Boehm et al., 1997). The observed overlapping of gene clusters associated with microglial activation by T_{MBP} cells and by IFN-γ is in line with the reported pluripotent effect of IFN-γ ascribed to several gene products that are regulated by the JAK–STAT pathway (Ramana et al., 2002). It was therefore not surprising to find here that IFN-γ was capable of partially simulating the effects of the activated T_{MBP} cells on microglial activity. IFN-γ increased glutamate uptake, probably by inducing expression of the glial glutamate transporter GLT-1. It is interesting to note that the microglial capacity for glutamate clearance in this study was not affected by an

inflammatory reaction induced by LPS, supporting the notion that the observed effect of T_{MBP} cells on microglia requires a specific cytokine (such as IFN-γ) and does not simply reflect a general proinflammatory condition. In addition, the enzyme iNOS was found to be strongly expressed by LPS-treated microglia but not by mieroglia treated with IFN-γ. It thus appears that the T cell-dependent activation of microglia that is mediated in part by IFN-γ can produce a microglial phenotype distinct from the one induced by bacterial activation; the former represent cytotoxic microglia, whereas the latter represents cells whose function is protection and repair.

In terms of specific activity values, the glutamate clearance capacity of the microglia was reported to be up to as much as 50% of that reported in astrocytes (Nakajima et al., 2001a), the cells generally acknowledged as chiefly responsible for glutamate removal in vivo. After activation by T_{MBP} cells the capacity of microglia for glutamate clearance was significantly increased, approaching that of astrocytes.

The possible participation of IFN-γ in the post-traumatic events leading to neuronal repair gains support in this study from the experiment in which antibodies against IFN-γ in mice subjected to optic nerve crush injury reduced neuronal survival. The mice used in this experiment were of the C57Bl/6J strain, in which the ability to spontaneously harness a T cell-mediated protective immune response is relatively limited. In this case, therefore, the limited neutralizing effect of IFN-γ was partly a reflection of the small number of nerves that survived secondary neuronal death after the primary injury (Kipnis et al., 2001).

D. Microglia as stand-by cells in the CNS

It thus seems likely that in an injured site deprived of astrocytes (Blaugrund et al., 1993), migrating microglia can take over the usual functions of the missing glial cells (Lopez-Redondo et al., 2000). In the normal healthy CNS, clearance of neurotoxic agents is done primarily by astrocytes. Under degenerative conditions, however, astrocytes at the lesion site may be in short supply (Blaugrund et al., 1993). Astrocytes delineate the lesion site and form a glial scar (Faber-Elman et al., 1996). Microglia, on the other hand, appear to be capable of migration (Lopez-Redondo et al., 2000), and in addition they express both chemokines and chemokine receptors (Aloisi, 2001). It thus seems from the present work that microglia serve a dual function, acting both as antigen-presenting cells (i.e., immune cells) and as glutamate-scavenging cells (i.e., neural cells). In view of their ability to function in both capacities, microglia might be regarded as stand-by cells at the service of the two systems when needed. The two functions are complementary, as glutamate removal from the extracellular microenvironment is required not only for the neurons, but also for T lymphocyte activation (Angelini et al., 2002). Our study is in line with recent data suggesting a link between pro-inflammatory microglia and tissue repair (Gasque et al., 2002) (Shaked et al., unpublished observations). It should be noted, however, that T cell-mediated products, including IFN-γ, might also exert a direct effect on other cells then microglia such as oligodendrocytes, astrocyes, and neurons (Dell'Albani et al., 1998; Schwaiger et al., 2000; Stuve et al., 2002).

E. Summary

The results of this study suggest that autoimmune Th1 cells, once activated (for example upon homing to the lesion site), act—at least partially through the secretion of IFN-γ—by conferring on the microglia a phenotype with enhanced capacity for glutamate clearance. This finding thus substantiates the contention that the innate immune response, in coping with the

threat of local damage by destructive self-components, is assisted by a T cell-mediated autoimmune response, just as it is assisted by a T cell-mediated immune response when confronted with invading microorganisms (Nevo et al., 2001; Schwartz and Kipnis, 2002). In patients with neurodegenerative diseases, microglia have often been implicated in the ongoing process of degeneration (Perry et al., 2003). Our results suggest that activated microglia, or at least those activated by IFN-γ, may comprise a beneficial phenotype, whereas those activated by LPS might be destructive. The beneficial effect of IFN-γ for CNS tissue has already been recognized. Thus, while IFN-γ is believed to play an essential role in the pathogenesis of immune-mediated demyelinating disorders (Panitch et al., 1987; Hartung et al., 1992; Olsson, 1995), deficiency in IFN-γ enhances the severity of the disease in susceptible animals (Tanuma et al., 1999; Espejo et al., 2002). Glutamate uptake was recently shown to be increased in rats with an experimental autoimmune disease, a finding that was described as unexpected (Ohgoh et al., 2002). However, since our results show that the same mechanism is responsible for both protection and destruction (Kipnis et al., 2002) that finding is not unexpected, as it substantiates the notion that microglia are capable of both release and uptake of glutamate, and that their net behavior is a reflection of the particular context. It further corroborates the contention that the same autoimmune T cells participate in protection and in autoimmune disease, with the final result determined by the timing of their arrival at the site of injury, the duration of their presence in the CNS, and their quantity (Shaked et al., unpublished observations). Protection by potentially encephalitogenic T cells was recently demonstrated in the context of brain injury (Hofstetter et al., 2003). It thus seems that although anything more than a modest constitutive level of immune activity is not needed by the healthy brain and might even have a destructive effect in individuals susceptible to autoimmune disease (Hickey and Kimura, 1987; Wekerle, 1993; Hohlfeld, 1999), a

heightened immune activity is both necessary and beneficial—as long as it is well regulated—in the diseased or damaged brain.

Figure legends

Figure 1. Co-culturing of microglia and T_{MBP}-activated cells results in induced expression of B7-2 and MHC-II on microglial cells. Confocal micrographs show microglia that were untreated (A,C) or treated with T_{MBP}-activated cells (B,D), and stained for ED-1 (green) and B7-2 (red) (A,B) or for I-B4 (membrane staining of microglia both activated and nonactivated) (green), MHC-II (red), and propidium iodide (PI) (blue) (C,D).

Figure 2. MBP-activated T cells enhance the capacity of microglia for uptake of extracellular glutamate at physiological and stress-related concentrations. Rat microglia were co-cultured for 24 h with activated (T_{MBP}^{*}) or resting (T_{MBP}) T_{MBP} cells, allowing (without filter) or preventing (with filter) cell–cell interactions, and were then incubated for 30 min at 37°C in uptake medium containing 150 mM NaCl or 150 mM choline chloride at two [^{14}C]-glutamate concentrations. A,B. Physiological glutamate concentration (2 μM/0.05 μCi L-[u]^{14}C-glutamate), expressed as nmol glutamate per well (A) or nmol glutamate per mg protein (B). C. Stress-related glutamate concentration (40 μM/0.05 μCi L-[u]^{14}C-glutamate). D. Stress-related glutamate concentration as a function of the number of T_{MBP}^{*} cells. The intracellular glutamate concentration was determined as described in Materials and Methods and is presented as nmol glutamate per mg protein. Values are mean ± SD of eight replicate determinations from one of three separate experiments with similar results.

Figure 3. Effect of activated T_{MBP} cells on the uptake of glutamate by microglia is reproduced by INF-γ but not by IL-4 or LPS. Rat microglia were co-cultured with activated T_{MBP} cells (T_{MBP}^{*}) or incubated with (A) the indicated concentration of IFN-γ; (B) 0.2 ng/ml of IL-4 or 20 ng/ml of IFN-γ; or (C) 100 ng/ml of LPS. After being incubated for 24 h they were further

incubated for 30 min at 37°C in uptake medium containing the stress-related glutamate concentration. Intracellular glutamate concentration was determined as described in Materials and Methods and is presented as nmol glutamate per mg protein. Values are means ± SD of eight replicate determinations from one of three separate experiments with similar results.

Figure 4. Phagocytic activity and expression of inducible nitric oxide synthase (iNOS) by different stimuli. (A) The photographs show the extent of phagocytosis by untreated and treated microglia, scanned by confocal microscopy with two channels: green represents zymosan A-engulfed particles and blue represents ED-1-positive microglia. Confocal imaging with superimposed light transmission verifies that all particles were engulfed by the microglia (lower right, light transmission). (B) Quantitative analysis of phagocytosis. Microglia treated with LPS, IFN-γ, or T_{MBP} engulfed significantly more particles than naïve cells (two-tailed Student's t-test, $P < 0.001$). The number of engulfed particles per microglial cell was automatically counted using Image-Pro Plus 4.5 software from eight fields (200 μm^2) of the slide, prepared in duplicate from three independent experiments and divided by the total number of microglial cells. (C) Rat microglia were incubated for 12 h with IFN-γ (20 ng/ml) or with 100 ng/ml of LPS. Expression of specific mRNAs was assayed by reverse transcription PCR (RT–PCR), using selected primer pairs specific for iNOS, and L-19 as housekeeping gene. Expression of iNOS transcripts by microglia was dramatically increased after stimulation by LPS but not by IFN-γ.

Figure 5. IFN-γ upregulates microglial expression of mRNA of the glutamate transporter GLT-1. Rat microglia were incubated for 12 h with IFN-γ (20 ng/ml). (A) Specific mRNA levels were assayed by quantitative reverse transcription PCR (Q-RT–PCR), using selected primer pairs specific for GLAST, EAAC-1, GLT-1, STAT-1, and GAPDH. (B)

Immunocytochemical analysis of GLT-1 expression in untreated (control) microglia and in microglia activated by IFN-γ. IFN-γ-treated cultures were also stained for secondary antibodies without any primary antibodies (no antibodies). Arrows point to GLT-1 immunoreactive microglial processes. The asterisks denote differences between control and IFN-γ treated cells.

Figure 6. Activated T_{MBP} cells or INF-γ counteract oxidative stress-mediated inhibition of glutamate uptake. Rat microglia were incubated for 24 h with the indicated concentration of ammonium-ferrous (II) sulfate hexahydrate (which increases the formation of toxic oxygen species) and co-cultured with or without T_{MBP+} (A,C), or incubated with 20 ng/ml INF-γ (B). Microglia were then incubated for 30 min at 37°C in uptake medium containing glutamate at a physiological concentration. Intracellular glutamate concentrations were determined as described in Experimental Procedures and presented as a percentage of the uptake in untreated controls (taken as 100%) (A,B), or in nmol glutamate per mg protein (C). In all cases the results are given by means ± SD of eight replicate determinations from one representative experiment out of three separate experiments.

Figure 7. Inflammatory response after optic nerve injury and contribution of IFN-γ to neuronal survival. C57Bl/6J mice were subjected to a crush injury of the optic nerve, as described in Experimental Procedures. (A) Accumulation and activation of microglia in crush-injured optic nerves at 14 h and 1, 4, and 7 days after injury. The excised optic nerves were cryosectioned and immunostained for microglia using Mac-1 antibody. The results shown are of one of three separate experiments with similar results. Arrows point to the site of injury. (B) Survival of RGCs after optic nerve crush injury in C57Bl/6J mice injected with anti-IFN-γ antibodies. Labeling of RGCs and crush injury were as described in Materials and

Methods. Immediately after the injury the mice were injected i.v. with neutralizing rat anti-mouse IFN-γ antibodies or control rat IgG, and 14 days later the retinas were excised and flat-mounted. Labeled RGCs from four randomly selected fields of identical size in each retina (all located at approximately the same distance from the optic disk) were counted under the fluorescence microscope and their average number per mm^2 was calculated. Significantly fewer RGCs were observed in mice injected with anti-mouse IFN-γ antibodies than in control mice ($P < 0.05$). Each group contained five or six mice.

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Figure 1.

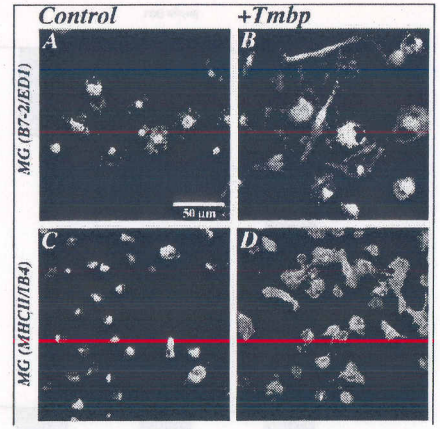

Figure 2.

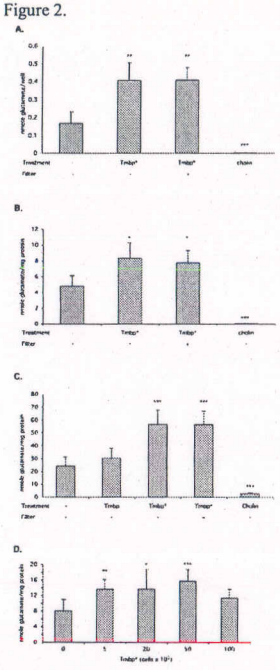

Figure 3.