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Granule-Derived Granzyme B Mediates the Vulnerability of Human Neurons to T Cell-Induced Neurotoxicity

Yohannes Haile,* Katia Carmine Simmen,† Dion Pasichnyk,* Nicolas Touret,† Thomas Simmen,‡ Jian-Qiang Lu,§ R. Chris Bleackley,† and Fabrizio Giuliani*

Multiple sclerosis (MS) is considered an autoimmune disease of the CNS and is characterized by inflammatory cells infiltrating the CNS and inducing demyelination, axonal loss, and neuronal death. Recent evidence strongly suggests that axonal and neuronal degeneration underlie the progression of permanent disability in MS. In this study, we report that human neurons are selectively susceptible to the serine-protease granzyme B (GrB) isolated from cytotoxic T cell granules. In vitro, purified human GrB induced neuronal death to the same extent as the whole activated T cell population. On the contrary, activated T cells isolated from GrB knockout mice failed to induce neuronal injury. We found that following internalization through various parts of neurons, GrB accumulated in the neuronal soma. Within the cell body, GrB diffused out of endosomes possibly through a perforin-independent mechanism and induced subsequent activation of caspases and cleavage of α-tubulin. Inhibition of caspase-3, a well-known substrate for GrB, significantly reduced GrB-mediated neurotoxicity. We demonstrated that treatment of neurons with mannose-6-phosphate prevented GrB entry and inhibited GrB-mediated neuronal death, suggesting mannose-6-phosphate receptor-dependent endocytosis. Together, our data unveil a novel mechanism by which GrB induces selective neuronal injury and suggest potential new targets for the treatment of inflammatory-mediated neurodegeneration in diseases such as MS. The Journal of Immunology, 2011, 187: 4861–4872.

In healthy individuals, lymphocyte traffic into the CNS is very low and tightly controlled (9, 10). In contrast, under inflammatory conditions, circulating T cells are activated and readily cross the blood-brain barrier, gaining access to the CNS in a significant number (10). Indeed, T lymphocytes are among the main constituents of the inflammatory infiltrates within MS lesions (11, 12). Earlier studies have shown a correlation among infiltrating lymphocytes, axonal pathology, and neuronal death in particular within the progressive MS (13). More recently, it has been shown that natalizumab, a mAb that prevents lymphocyte migration across the blood-brain barrier, significantly reduced light neurofilament, a biomarker of axonal damage (14), in the CSF of relapsing remitting MS patients (15).

Activated T lymphocytes secrete granzyme B (GrB), a serine protease released from the granules of cytotoxic T cells, and induce target cell death by disrupting a variety of intra/extracellular protein substrates (16–18). Caspases are among GrB substrates (19, 20), and their activation leads to cell death by apoptosis. GrB-expressing cytotoxic T cells were observed in close proximity of oligodendrocytes or demyelinating axons in acute MS lesions (21). Indeed, T cells activated by a soluble anti-CD3 Ab induced severe neurotoxic effect in both allogeneic and syngeneic systems in vitro (22); however, the mechanisms of this T cell-mediated neurotoxicity have not been explored. It has recently been shown that recombinant GrB induces neuronal injury in vitro (23). However, recombinant GrB does not have the posttranslational changes occurring in the granule-derived GrB that are required to enter the target cell (24). Indeed, in the study by Wang et al. (23), the rate of neuronal injury was low (10–20%) and not comparable to what we observe in the T cell-mediated neuronal injury.

In the current study, we show that MS active lesions, characterized by the presence of infiltrating inflammatory cells, expressed high levels of GrB. In vitro, granule-purified human GrB and activated T cells induced severe neurotoxic effects on human neurons. Furthermore, in the murine system, T cells isolated from GrB knockout BL6 mice were not able to induce killing of neurons.
derived from the syngeneic naive mice. Interestingly, GrB entered neurons through the mannose-6-phosphate (M6P) receptor (M6PR) and induced cellular death by disrupting the cytoskeleton. Altogether, our data demonstrate that granule-derived GrB plays a significant role in T cell-mediated neuronal injury affecting neurodegenerative diseases such as MS.

**Materials and Methods**

**Culture of human T cells, RNA isolation, and real-time RT-PCR**

Human PBMCs were isolated from the blood of adult healthy volunteer donors using the Ficoll-Hypaque centrifugation separation and suspended in serum-free AIM-V T cell culture medium (Life Technologies, Burlington, ON, Canada). T cells were plated, at a density of 200,000 cells/well in 200 μl, on multiwell culture plates immobilized with either 5 μg/ml human anti-CD3 or anti-CD3/CD28 Ab (for induction of T cell activation) or on untreated wells (for unactivated control cells) for 3 d. After 3 d in culture, cells were collected and centrifuged to isolate cell pellets. In a separate experiment, frozen and paraffin-embedded tissue samples were collected from three noninflammatory control cases (1 amyotrophic lateral sclerosis, 1 Alzheimer’s dementia, and 1 cardiac arrest) and three MS patients, according to the guidelines approved by the local institutional ethics committee. These samples were obtained from the brain bank of the MS clinic at the University of Alberta. In the MS tissues, areas of active and chronic active MS lesions as well as normal-appearing white matter (NAWM) were identified based on the presence or absence of infiltrating inflammatory cells and demyelination in adjacent white matter sections, as described previously (12). For RT-PCR, the frozen brain tissues and cells (separately) were lysed and homogenized in TRIzol (1 ml/106 cells; Invitrogen, Carlsbad, CA). The concentration and quality of RNA were measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific). The amount of 2 μg RNA for each reaction was used to synthesize cDNA. RNA was treated with DNase I (Promega, Madison, WI), and cDNA was synthesized using oligo(dT) and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer’s recommended instructions. Semiquantitative RT-PCR was carried out using Bio-Rad iQ SYBR green supermix on either iQ5 pro-

**Human fetal neuron culture**

Human brain tissue was obtained from 15- to 20-wk fetuses according to the guidelines approved by the local institutional ethics committee. Human cortical fetal neurons (HFNs) were isolated, as previously described (22). Briefly, brain specimens were washed in PBS, followed by removing the meninges and blood clots. The fragmented brain pieces were transferred into 50-ml tube and digested in 4 ml 2.5% trypsin and 6–8 ml DNase I for 15 min in 37˚C water bath. The activity of trypsin was inhibited by the 2.5% trypsin and 6–8 ml DNase I for 15 min in 37˚C water bath. The activity of trypsin was inhibited by the 2.5% trypsin and 6–8 ml DNase I for 15 min in 37˚C water bath. The activity of trypsin was inhibited by 1% sodium pyruvate, 25 μM HEPES, 1% antibiotic/mycotic, 1% glucose, and 2% B-27 supplement. Cells were counted and plated in a concentration of 5 million cells/ml.

**Isolation and culture of mouse T cells**

Spleens were splenocytes from wild-type (strain: BL6) or GrB knockout male mice (strain: Bl6.129Gzmbs1Mlej). The spleen was mechanically dissociated, and T cells were isolated using the Ficoll-Hypaque centrifugation separation. They were then suspended in RPMI 1640 culture medium supplemented with 1% glucose, 1% HEPES, 1% antibiotic/10 μM 2-ME, and 1% B-27. T cells were plated into multiwell culture plates either immobilized with 5 μg/ml mouse anti-CD3 Ab (for induction of T cell activation) or on untreated wells for 3 d.

**Immunocytochemistry**

HFNs (100,000 cells/well) were cultured on poly-ornithine–coated 16-well culture plates for 3 d. In parallel, T cells were cultured either on untreated or anti-CD3/CD28–coated multiwells. After 3 d, the same amount (1:1) of activated T cells was applied into the neuron culture. Similarly, HFNs were treated with GrB (100 ng/100 μl) purified from the human NK cell line, YT-Indy, prepared as previously described (26), and the coculture was kept for 24 h. The control neuronal culture groups were treated with only AIM-V medium (without T cells) or unactivated T cells. Moreover, in the supernatant killing assay, supernatant either from unactivated or 3-d-activated T cells was added, with or without GrB, into neuronal culture.

**Mouse fetal neuron culture**

Mouse fetal neuron culture

Neurons were obtained from 15-d-old embryonic mice (strain: BL6). Pregnant mice were anesthetized and decapitated. The skin in the stomach area was incised, and the embryos were taken out into the petri dish. Each embryo was separated from the embryonic sack, and the head was cut and cultured on a separate petri dish. Under the microscope, the meninges were carefully peeled off. The cortical parts of the two hemispheres were collected into a 15-ml falcon tube containing buffer HEPES, HBSS, and antibiotics. The tissues were washed twice with the buffer and incubated in 1 ml trypsin for 10 min at 37˚C in the water bath. The activity of trypsin was stopped by PBS, and the tissue was homogenized using a pasteur pipette. The cells were centrifuged for 5 min at 1400 rpm and suspended in a neurobasal medium supplemented with 1% sodium pyruvate, 25 μM HEPES, 1% antibiotic/mycotic, 1% glucose, and 2% B-27 supplement. Cells were counted and plated in a concentration of 5 million cells/ml.
To evaluate the cytotoxic effect of T cells or GrB on neurons, six fields per well were randomly and manually counted using original magnification ×40 objective fluorescence microscopy. The mean value of the control neuronal culture that was not exposed to T cells was treated as 100%. The average number of MAP-2–, DAPI/MAP-2–, or β-III tubulin-positive neurons/group was inferred as a percentage of the control neuronal culture group. At least six wells were quantified from each condition. Every experiment was repeated at least three times.

Perforin inhibition study
It is well known that the lytic activity of perforin is dependent on the presence of calcium ions to allow the formation of pores on the target cells (27). Therefore, to avoid the possibility of perforin contamination within the purified GrB, both purified GrB and supernatants from 3-d–activated T cells were incubated with a Ca2+-chelating agent (0.4 M EGTA/0.8 M MgCl2) for 1 h before adding into neuronal culture, as previously described (28). HFNs were cultured for 3 d, followed by treatment either with purified GrB or supernatant from activated T cells in the presence/absence of the Ca2+-chelating agent. The control groups were treated with AIM-V media only. Similar experiments were conducted by preincubating activated T cells with the Ca2+-chelating agent. After 24-h coculture, neuronal viability was evaluated by immunocytochemistry using anti–MAP-2 Ab.

Comparison between GrB constitutively secreted from activated T cells and human purified GrB used in neuronal killing assay
T cells were activated for 3 d, and the conditioned media (CM; supernatants) from both activated and nonactivated T cells were analyzed on a 10% PAGE denaturing gel. The gel was loaded with the following four groups (control and experimental groups): 15 µl AIM-V media; 15 µl unactivated T cell CM; 15 µl T-activated CM; or 21.43 ng human purified GrB (hGrB) from YT-Indi cell lines. The proteins were transferred onto nitrocellulose overnight and probed against hGrB 2C5 Ab at a 1:500 dilution (Santa Cruz).

Measuring enzymatic activity of GrB constitutively secreted from activated T cells
hGrB and GrB secreted from T cells activated with either anti-CD3 or a combination of anti-CD3/CD28 were assayed for their enzymatic activity. GrB enzymatic activity was measured in RPMI 1640 reaction mix containing 50 mM HEPES (pH 7.5); 10% (w/v) sucrose, 5 mM DTT, 0.05% (w/v) CHAPS, and 300 µM acetyl-Ile-Glu-Pro-Asp-paraanitroanilide (Ac–I-EPD-pNA; Kamiya Biomedical). The plate was incubated for 6 h at 38˚C. Hydrolysis of Ac–I-EPD-pNA was measured at 405 nm at time 0 and every hour thereafter using a Multiskan Aspect spectrophotometer (Thermo Lab System).

Cleavage of GrB N-linked oligosaccharides by endoglycosidase H
The impact of endoglycosidase H (endoH) on GrB, either secreted from activated T cells (CD3 or CD3/CD28) (labeled CM), stored in their cells (labeled g), or purified from YT-Indi, was assessed. Native cell lysate, CM, or purified GrB protein was first denatured in 5% SDS and 0.4 M DTT at 100˚C for 10 min, and then was incubated with recombinant endoH, according to the manufacturer’s instruction (BioLabs), and digested at 37˚C for 2 h and 30 min. GrB glycoforms were resolved on a 14% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with anti-human GrB 2C5 Ab diluted in 1:500 (Santa Cruz Biotechnology).

GrB internalization and M6P blocking
Purified GrB was labeled using Alexa Fluor 488 microscale protein-labeling kit (Invitrogen, Molecular Probes), according to the manufacturer’s instructions. To assess whether GrB is acting on the surface or inside the cell, human neuronal cell cultures were treated with green fluorescence-tagged GrB (GrB488; 0.5–1 µg/ml) for either 3, 6, or 24 h at 37˚C. Similarly, some groups were treated with 25 mM M6P for 1–2 h before addition of GrB. Following the incubation period, the media were removed and cells were washed three times with PBS, followed by 3× washing with MEM media plus 0.5% BSA (pH 2) at RT. Cells were washed again three times with PBS and then fixed for 30 min with 4% PFA at RT. Finally, slides were slides mounted and viewed under the confocal microscope at original magnifications ×40–63.

In addition, the role of M6P in inhibiting GrB–induced neurotoxicity was evaluated. After 3 d in culture, HFNs were treated with 1–25 mM M6P for ~2 h, followed by incubation with GrB (1 µg/ml) or activated T cells. Control groups were treated with either GrB, activated T cells, or M6P alone, or remained untreated. After 24-h incubation, the viability of neurons was evaluated immunocytochemically using anti–MAP-2 Ab.

GrB488: colocalization with early endosome, intracellular diffusion, and neuronal apoptosis
To evaluate whether GrB is taken up by early endosomes, HFNs were cultured for 3 d, followed by incubation (blocking) with donkey serum (1:10,000 in PBS) to block the unspecific binding. Neurons were treated with 0.5–1 µg/ml GrB488 for 15 min, 20 min, 30 min, 1 h, or 2 h. After washing three times with PBS, neurons were fixed in 4% PFA and immune stained for early endosomal marker using mouse IgG1 anti-human endosomal Ag 1 (EEA1; 250 ng/ml or 1:1000 in PBS; BD Biosciences), following the protocol described above for immunocytochemistry. The culture was further incubated for 20 min with DAPI (1:1000 in PBS) to stain the nucleus. The colocalization of GrB and EEA1 or DAPI was assessed under confocal microscope.

To analyze the time scale of GrB–induced cellular apoptosis, GrB488–treated neuronal cultures (coverslips) were washed with acidic media (MEM media + 0.5% BSA [pH 2] at RT), followed by final washing with PBS. The neurons were further incubated for 20–30 min with the following apoptotic markers: annexin V 647 (1:200 in PBS) and propidium iodide (0.4 µg/ml). Nuclear fragmentation was evaluated by staining the neuronal nuclei with DAPI. After washing the culture with PBS, GrB488 internalization and induction of apoptosis were evaluated by confocal microscopy.

Western blotting
HFNs were cultured for 3 d. Simultaneously, human PBMCs were activated using anti-CD3 for 3 d. For caspase inhibition study, some groups of HFN cultures were treated with 200 µM Z-VAD-FMK (pan-caspase inhibitor) for 2 h. Neurons were then further treated with purified GrB (1 µg/ml), activated T cells, or unactivated T cells. Controls remained untreated, and Hela cells were used as a positive control. After 24 h, T cells were carefully washed, and proteins from HFNs were isolated and loaded into 10% electrophoresis gel. The protein was transferred into nitrocellulose membrane (1 h at 100 V). The nonspecific binding in the membrane was blocked with 1× TBS/casein blocker (Bio-Rad). After several washes with PBS, the cleavage of the cytoskeletal protein was evaluated using Ab against α-tubulin (1:3000).

Statistical analysis
Results were statistically analyzed as mean ± SD using GraphPad Prism 5. The groups were compared using one-factor ANOVA, followed by Tukey posthoc test for normally distributed data. A two-tailed unpaired t test was applied to compare two groups with normally distributed data. The p values <0.05 were considered significant. Asterisks correspond to *p < 0.05, **p < 0.01, and ***p < 0.001.

Results
Active lesions of MS and activated T cells express GrB
Human brain tissue was obtained from three MS patients as well as three noninflammatory disease control patients, and the lesion types were identified, as previously reported (12). Luxol Fast Blue staining showed that MS active lesions are significantly demyelinated and are characterized by massive infiltration of inflammatory cells (Fig. 1aA), whereas in the chronic active lesion, there is significant demyelination and decreased density of inflammatory cells compared with the active lesion (Fig. 1aB). No demyelination or inflammatory infiltrates were observed in the NAWM as well as in the healthy control tissue (Fig. 1aC). Immunostaining for infiltrating T lymphocytes showed a significant number of CD8+ T cells in the active lesions compared with the chronic active lesions. No CD8+ cells were identified in the NAWM (Fig. 1aD–aF, respectively) as well as no CD3+ T cells (data not shown). The presence of double GrB/CD3+ T cells in acute MS lesions has been previously reported (21). In this study, we assessed the expression of GrB in the different lesion types. RNA was isolated from active and chronic active lesions as well as NAWM. Control tissue was obtained from normal subjects. RT–PCR revealed that MS active lesions expressed >100-fold change...
in GrB when compared with normal controls. On the contrary, the
eexpression of GrB in chronic active lesions was significantly lower
than the active lesions, but significantly higher than the NAWM
\( p < 0.001; \) Fig. 1b). The difference in GrB expression between
the different lesion types correlated with the variation in density of
CD8\(^+\) T cells, as depicted in Fig. 1aD–aF. Similarly, anti-CD3–
avivated T cells expressed \( \sim 40 \)-fold change of GrB compared
with the control unactivated T cells (Fig. 2a; \( p < 0.001 \)). We have
previously shown that GrB is mainly expressed by CD8\(^+\) T cells,
although CD4\(^+\) cells also express it, but to a lower level (29).

\textit{Activated T cells and GrB kill human neurons}

To investigate whether expression of GrB has a neurotoxic effect,
a neuronal killing assay was performed using MAP-2 as a marker of
neuronal viability, as previously described. Disappearance of MAP-2
immunoreactivity has been shown to be associated in vivo and

\textbf{FIGURE 1.} Identification of MS lesions and measurement of GrB expression. \( a \), Luxol Fast
Blue (LFB) staining in active, chronic active, and NAWM lesions of MS (A–C respectively; original
magnification \( \times 20 \)). The lower panels demonstrate immunohistochemical staining for CD8\(^+\)
T cells in active, chronic active, and NAWM lesions (D–F; original magnification \( \times 40 \)). \( b \), RT-PCR showing the expression of GrB in the normal control or active, chronic active, or NAWM
lesions of MS patients (\( ^* p < 0.05, ^{**} p < 0.001 \)). This difference in GrB expression may
attribute to the variation in density of infiltrated T lymphocytes in the different lesion types. The
data in the graph are pooled from three independent experiments.

\textbf{FIGURE 2.} GrB expression and activated T cells mediated neuronal killing in vitro. \( a \), Expression of
mRNA for GrB in unactivated or anti-CD3-activated T cells. \( b \), Immunocytochemical staining using a neu-
ronal viability marker, MAP-2. Human neurons either remained untreated control (A, B) or were cocultured
with unactivated T cells (C), cocultured with activated T cells (D), or treated with GrB (E). Scale bars, 50 \( \mu \)m; 200 \( \mu \)m. \( c \), Quantification of MAP-2-positive neurons in the control group or the groups treated with unac-
tivated T cells, activated T cells, or hGrB. The percentage indicates the ratio between viable MAP-2–
positive neurons and untreated control. \( d \), Quantification of DAPI/MAP-2 double-positive (merged) cells in
the control group or the groups cocultured with unac-
tivated T cells, activated T cells, or hGrB. The per-
centages indicate the ratio between viable DAPI/MAP-
2–positive cells and controls. Asterisks indicate signif-
icant difference between compared groups (\( ^* p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001 \)). Data are represen-
tative of at least three individual experiments.
in vitro with neuronal injury and death (22). Activated T cells were cocultured with HFNs for 24 h. Immunocytochemical analysis using anti–MAP-2 Ab revealed that untreated control neurons (Fig. 2bA, 2bB) and the group cocultured with unactivated T cells (Fig. 2bC) remained viable, whereas a severe neuronal loss was noticed in the group cocultured with activated T cells (Fig. 2bD). Furthermore, the addition of purified GrB (1 μg/ml) to the neuronal culture induced dramatic neuronal killing (Fig. 2bE). Quantification of MAP-2–stained neurons clearly showed that both activated T cells and purified GrB induce the same extent of neuronal death (>60%), whereas the control groups did not produce any toxic effect (Fig. 2c; p < 0.01). Furthermore, quantification of DAPI/MAP-2 double-positive (merged) neurons showed similar results (Fig. 2d; p < 0.001). Comparing the neurotoxic effects of CD4+ and CD8+ T cells revealed that the majority of neuronal death is inflicted by the latter, and correlates to the level of GrB expressed by CD8+ T cells (29). These findings suggest the relevance of GrB on neurotoxicity. Because neuronal cultures had a purity of ~95%, and there was no difference between MAP-2– and DAPI-based quantification, we used MAP-2 immunoreactivity alone as a marker of neuronal viability in most of our experiments.

Activated T cells from GrB knockout mice fail to kill mouse neurons

To further confirm whether GrB induces neuronal killing, T cells were isolated from the spleens of GrB knockout mice, activated with anti-CD3, and cocultured with mouse embryonic day 15/16 neurons. Control neuronal cultures were treated with activated or unactivated T cells isolated from wild type or treated with only medium without T cells. As shown in Fig. 3a, a viability assay using anti–β-III tubulin Ab revealed that the neurons in the control group (Fig. 3aA–aC) remain healthy with unaltered morphology. In contrast, significant neuronal loss was observed in the group treated with activated T cells from control wild type (Fig. 3aD). Interestingly, activated T cells from GrB knockout mice did not induce neurotoxicity (Fig. 3aE). Quantification of β-III tubulin-stained neurons showed that activated T cells from wild-type mice induce a significant neurotoxic effect (>50%) compared with activated T cells from GrB knockout mice, untreated control, or control neurons cocultured with unactivated T cells (Fig. 3b).

Supernatants from activated T cells do not induce killing of human neurons

To assess whether GrB-mediated neuronal killing was induced by constitutively secreted GrB or by the stored/degranulated GrB, supernatants from activated T cells were applied to HFN cultures and incubated for 24 h. Staining with anti–MAP-2 Ab showed that the supernatants do not induce any neurotoxic effect. On the contrary, the addition of purified GrB to the supernatant of activated T cells induced severe neurotoxicity, matching the effect of GrB alone (Fig. 4a; p < 0.05).

**FIGURE 3.** GrB knockout activated mouse T cells do not induce neuronal killing. a, Immunocytochemistry, using anti–β-III tubulin Ab, showing embryonic day 15 mouse neurons untreated control (A, B) or cocultured with unactivated mouse T cells (C), cocultured with activated mouse T cells from wild-type mice (D) or activated mouse T cells from GrB knockout mice (E). Scale bars, 50 μm (A), 200 μm (B–E). b, Quantification of β-III tubulin-positive viable mouse neurons from the groups shown in a. The percentage indicates the ratio between viable β-III tubulin-positive mouse neurons and untreated control (*p < 0.05, **p < 0.01, ***p < 0.001). Mouse T cells were harvested from spleens of control wild-type or GrB knockout mice. Data are representative of three individual experiments.
To address the difference in neurotoxic effect between the constitutively secreted and granule-derived GrB, Western blotting analysis on T cell lysates and supernatants was performed. The results indicated that the secreted GrB differs in molecular mass from the granule-purified human GrB (Fig. 4b, lanes 3, 4). There was no trace of GrB in the supernatant or lysate of unactivated T cells (Fig. 4b, lanes 1, 2, respectively). GrB derived from the anti-CD3– or anti-CD3/CD28–activated T cell lysates had comparable molecular mass to the granule-purified hGrB (molecular mass 32 kDa), whereas the secreted/released GrB had a higher molecular mass (molecular mass 35 kDa). To investigate whether the different molecular mass were related to a difference in glycosylation, the GrB proteins were enzymatically digested by endoH that cleaves asparagine-linked mannose. Following digestion, the granule-stored GrB was cleaved into two distinct fragments of ~30 and 27 kDa, whereas the secreted GrB in the supernatant resulted in ~35-, 32-, and 27-kDa fragments (Fig. 4c). These findings suggest two distinct isoforms of GrB: one stored within the granules and another constitutively secreted via a non-granule route. These GrBs differ in the amount of complexed mannose, and this is in agreement with previous reports (24).

Moreover, the enzymatic activity of the supernatants obtained either from anti-CD3– or anti-CD3/CD28–activated T cells was compared with purified granule-derived hGrB. Experiments using GrB-ELISA showed high levels (beyond the kit’s detection limit) of GrB secreted by activated T cells into the supernatant (data not shown). These findings suggest two distinct isoforms of GrB: one stored within the granules and another constitutively secreted via a non-granule route. These GrBs differ in the amount of complexed mannose, and this is in agreement with previous reports (24).

FIGURE 4. Granule-derived GrB is required to mediate neuronal injury. a, Human fetal neurons were treated either with AIM-V cell media (control) or supernatants from unactivated and anti-CD3–activated T cells in the absence or presence of purified GrB or GrB alone in the T cell media (*p < 0.05). b, Western blotting showing the relative molecular mass of GrB secreted into the supernatant obtained from activated T cells (lane 3; 35 kDa) and hGrB (lane 4; 32 kDa); lanes 1 and 2 are controls containing only cell culture media or supernatant attained from unactivated T cells, respectively. c, Comparison of GrB in the CM or cell lysates (¢) of unactivated T cells, anti-CD3–activated T cells, anti-CD3/CD28–activated T cells, or hGrB. – or +, Indicates the absence or presence of endoH. Addition of endoH-induced different fragments of GrB with molecular mass of 32 kDa (denoted by blue circle), 30 kDa (blue asterisk), and 27 kDa (red asterisk). d, Measurement of GrB enzymatic activity against time. The enzymatic activity of human GrB (89 ng) was compared with constitutively secreted GrB from unactivated T cell, anti-CD3–activated T cells (100% and diluted to 50 or 25%), or anti-CD3/CD28–activated T cells (100% and diluted to 50 or 25%). e, Assessment of perforin inhibition, by chelating calcium using 0.4 M EGTA/0.8 M MgCl₂, of constitutively secreted or purified GrB-mediated neuronal killing (columns 3–5). The percentage indicates MAP-2–stained viable neurons in relation to the controls (*p < 0.05). Data are representative of two to three individual experiments.
shown). Thus, the supernatants were diluted in media into 50 or 25%, and hydrolysis of the GrB substrate Ac-IEPD-pNA was measured at 405 nm. Only the higher concentrations of hGrB (>178 ng) showed more activity than the supernatants. Constitutively secreted GrB from activated T cells cleaved IEPD-pNA substrate in vitro, meaning significantly higher GrB activity compared with supernatant derived from unactivated T cells (Fig. 4d). These findings demonstrate that the difference in neurotoxic effect between secreted and granule-derived GrB is not related to a difference in their enzymatic activity, but possibly associated with posttranslational modifications (30). In addition, an assay was performed to test whether perforin, a pore-forming protein, played a role in the GrB-mediated neuronal killing. Previous reports have shown that the lytic activity of perforin is dependent on the presence of calcium ions (27, 28, 31–33). Granule-purified GrB does not contain perforin; however, to exclude any possible contamination with this protein, both purified GrB and/or supernatant from activated T cells were incubated with calcium chelator (0.4 M EGTA and 0.8 M MgCl₂) for 1 h before adding them to the neuronal culture. Quantification of MAP-2–positive neurons showed that perforin inhibition does not prevent neuronal killing either in the neuronal culture treated with purified GrB or in the culture treated with supernatant plus GrB (Fig. 4e; p < 0.05).

Similarly, perforin inhibition (Ca²⁺ chelation) did not prevent GrB-mediated events (e.g., caspase 3 activation, α-tubulin cleavage) or activated T cell-mediated neuronal death (data not shown). These findings suggest that GrB-mediated neuronal killing on human neuronal cells is independent of perforin.

**Purified GrB internalization and neurotoxicity are blocked by M6P**

Our study showed that GrB-mediated neuronal injury is independent of perforin. Thus, we investigated the possible entry site of GrB. Based on our findings that the secreted and granule-derived GrB differ in the amount of mannose, and considering previous reports on the role of M6PR as a receptor for GrB (30) and its selective expression on neurons but not on astroglia (22, 30, 34), we investigated the role of this receptor on GrB-induced neurotoxicity. As illustrated in Fig. 5a, unlike the untreated controls in which neurons show a regular morphology (Fig. 5aA, 5aA’), there is significant amount of GrB internalized into the neuronal cells in the group treated with the fluorescent labeled GrB488 (see arrows in Fig. 5aB’ indicating internalized GrB). This internalization was completely blocked when the neurons were preincubated with 25 mM M6P (Fig. 5aC’). Unlike the GrB-treated group (Fig. 5aB, 5aB’), the neurons preincubated with M6P (Fig. 5aC, 5aC’) had normal morphology similar to untreated neuronal control cultures (Fig. 5aA, 5aA’). Further immunocytochemical analysis using anti–MAP-2 staining revealed no changes in neuronal density and morphology in the M6P plus GrB– or M6P alone-treated groups compared with control untreated neurons (Fig. 5bA, 5bC, 5bD). In contrast, a severe neuronal loss was observed in the GrB alone-treated group (Fig. 5bB). Quantitative analysis showed that the viability of neurons in the GrB-treated group is only 16.84 ± 11.25%, and M6P pretreatment significantly increases neuronal viability to 66 ± 26.76% (Fig. 5c; p < 0.001). However, in the T cell–neuron coculture, pretreatment of neurons with M6P did not prevent neuronal loss, although there was a trend toward neuroprotection at 25 mM M6P (Fig. 5d). This suggests that activated T cells use alternative mechanisms to deliver GrB or to induce target cell death.

**Purified GrB is taken up by early endosomes and diffused in the cytoplasm of neuronal cells independent of perforin**

To assess whether the internalized GrB is initially stored in the endosomes, neurons were treated with GrB488 for 15 min to 2 h. Immunostaining revealed that GrB488 is essentially colocalized with the endosomal marker EEA1 within 15 min of GrB treatment (see Fig. 6aA–aE), and then gradually diffuses out into the cytoplasm in the absence of any lytic agent (Fig. 6aF–aJ).
We further evaluated GrB-induced cellular apoptosis. Immunostaining showed colocalization of GrB488, annexin V 647, and propidium iodide in single neurons (see arrows in Fig. 6a, 6a’). Moreover, some DAPI-stained neurons showed nuclear fragmentation surrounded by GrB (data not shown), suggesting an apoptotic cell death. This GrB-mediated neuronal apoptosis was rapid and occurred within 40 min; however, not all of the neurons were dying at the same time, and this can be related to the fact that the culture is a mixture of heterogeneous neuronal cells with potentially different sensitivity to GrB cytotoxicity.

**GrB-induced neuronal death is caspase dependent**

To investigate the substrate/target of GrB during neuronal apoptosis, HFNs were treated with either activated T cells or GrB. Control groups were cocultured with unactivated T cells, remained uncleaved (Fig. 7a). This indicates that activated T cells release granule-derived GrB, which mediates neuronal killing by destabilizing the cytoskeletal protein of the neuronal cells (Fig. 7a [lanes 3, 4], 7a’ [lanes 4, 5]). The bands in the control groups, such as the untreated HFNs, HeLa cells, or neurons cocultured with unactivated T cells, remained uncleaved (Fig. 7a, 7a’). To address whether GrB acts directly on the substrate, α-tubulin, or induces apoptosis indirectly through caspase activation, HFNs were incubated with the pan-caspase inhibitor Z-VAD-FMK prior to the coculture or treatment with purified GrB. As previously described, both activated T cells and GrB-induced α-tubulin cleavage (Fig. 7b, lanes 3, 5). This cleavage was absent when the neurons were pretreated with Z-VAD-FMK (Fig. 7b, lanes 2, 6), suggesting that the α-tubulin cleavage is caspase dependent. To further elucidate the role of caspase-mediated neuronal killing, another set of experiments was conducted using caspase-3 inhibition. When neuronal cultures were treated with GrB alone, almost 80% of neurons were killed (Fig. 7cB, 7cE, column 2; p < 0.05). In contrast, neuronal cultures pretreated with Z-VAD-FMK or the caspase-3 inhibitor significantly reversed this severe neuronal loss (Fig. 7cA, 7cC, 7cD, 7cE, columns 3, 4). These data show that GrB-induced neuronal apoptosis is mainly mediated by caspase-3 activation.

**Discussion**

Our study addresses the detailed mechanisms of GrB-mediated neuronal injury that might contribute to neurodegenerative processes of MS (Fig. 8). It is well documented that T cells express and release GrB upon activation following the encounter with a foreign Ag. Indeed, active lesions of MS patients are characterized by the presence, among others, of infiltrating inflammatory T cells. In this study, the expression of high levels of GrB in the active lesions of MS is possibly a consequence of the presence of inflammatory T cells, mainly CD8+ T cells, within the tissue. GrB-expressing T cells were found in close apposition to demyelinated axons in the parenchyma of acute MS lesions (21). Similarly, in a rat model of spinal cord injury and cerebral ischemia, it has been shown that neuronal death occurs in close proximity to GrB-positive cells (36, 37). In our study, we show that GrB-expressing activated T cells induced neuronal killing (60%) comparable to granule-purified human GrB. Furthermore, activated T cells isolated from wild-type mice were significantly neurotoxic, whereas activated T cells from GrB knockout mice failed to induce any neuronal injury. Our findings are in agreement with a previous report by others who have shown that GrB-deficient CTLs were unable to induce DNA fragmentation in target cells (17). Although it is known that there are significant differences between human

![FIGURE 6](http://www.jimmunol.org/)
and murine granzymes, our results support the evidence that GrB is responsible for the induction of neuronal injury in the mouse system (38).

More recently, Wang et al. (23) have shown that anti-CD3/CD28–activated T cells release GrB into the supernatant, which induces mild cytotoxicity (∼20%) on human neurons. In contrast, we found that supernatants from activated T cells could not induce neuronal injury, whereas cell-to-cell contact-dependent neuronal killing induces severe (60–80%) neuronal death. Using a transwell system in which neurons and activated T cells are cocultured in two separate compartments divided by a porous membrane, allowing only the exchange of soluble factors, we have previously reported that no neurotoxicity occurs. In addition, we have shown that neurons are highly susceptible to activated T cell–mediated cytotoxicity independently of MHC class I or II expression and in the absence of added Ag (22). It is well known that the expression of MHC-I on healthy and fully differentiated neurons within the CNS is minimal or negligible (39, 40). Furthermore, most infiltrating T cells within MS lesions are Ag nonspecific, and it has been shown that T cells are able to induce collateral bystander axonal damage and/or neuronal death within the CNS. These findings underline the importance of cell–cell contact in delivering the “kiss of death” to the target neurons and induce effective neurotoxicity. This interaction may occur independent of Ag specificity (41–43).

Moreover, we observed that GrB constitutively secreted in the activated T cell supernatant has higher molecular mass (35 kDa versus 32 kDa) and different glycosylation compared with the granule-derived GrB released following degranulation at the immune synapse. It was also recently reported that CTLs release mainly inactive GrB (GrB zymogen) through the constitutive pathway (44), which might explain the low neuronal killing induced by the supernatant of activated T cells. Interestingly, in our study, the activity of the constitutively secreted GrB was comparable to that of granule-purified GrB. However, endoH, which cleaves asparagine-linked mannose oligosaccharides, digested the constitutively secreted GrB into three different fragments (∼32, 30, and 27 kDa), whereas granule-derived GrB produced two fragments of 30 and 27 kDa, consistent with previous reports (24).

These data suggest that the two GrBs differ in their post-translational modification and mannose content. To effectively exert its cytotoxic effect, GrB needs to enter the target neuronal cell. It has been shown that mannose is essential for GrB entry into some target cells and that the M6PR is the death receptor for GrB. M6PR is highly expressed on the surface of neurons, but not on glial cells (30, 45). Indeed, neurons are sensitive to CTL-mediated killing (46). On the contrary, no T cell– or GrB-mediated cytotoxicity has been described in glial cells such as astrocytes and oligodendrocytes (22, 23). These observations suggest M6PR as the potential candidate receptor for GrB entry into neuronal cells. In this study, we show that blocking the M6PR with its high-affinity ligand M6P effectively inhibited granule-purified human GrB internalization and prevented neuronal killing (Fig. 5a–c), confirming that the main route of purified GrB entry into neurons is through M6PR. Therefore, unlike the constitutively secreted GrB, which contains highly complex carbohydrates, possession of M6P enabled the granule-purified GrB to enter to the target cell via M6PR and induce apoptosis. In contrast, Wang et al. (23) have shown that M6P is not able to prevent GrB-mediated neuronal killing. However, they found that the killing is prevented by pertussis toxin, suggesting the mediation of a G12–coupled receptor such as M6PR. This apparent discrepancy can be related to the low dose of M6P used (1 mM compared with 25 mM in our study) because, as previously reported, M6P inhibits GrB-mediated cellular killing in a dose-dependent manner (30). Similarly, in our study, both purified GrB– and T cell-based neurotoxicity assays showed that low concentration of M6P (1–10 mM) did not protect neurons from death, and any concentration of M6P higher than 25 mM displayed different levels of toxicity on the cells. We have previously reported that half-maximal inhibition of target cell fragmentation was only evident with M6P at unphysiological concentrations (>25 mM) (30). In addition, in Wang et al. (23), the use of recombinant GrB without posttranslational modification and subsequent addition of M6P can also explain these differences. Moreover, although M6PR may interact with several non-M6P–containing ligands, they have much lower affinity for the receptor (47). Furthermore, the constitutively secreted 35-kDa GrB, usually found in the supernatant of activated T cells, lacks...
the M6P-targeting motif, and this explains its failure to acquire mannose and enter the target cell (24) and to induce significant neuronal death. Overall, these findings would explain the divergence in the rate of GrB-mediated cell death between Wang et al. (23) (10–15%) and our study (60–80%).

As described above, granule-purified GrB internalization into neuronal cells is dependent on M6PR. In contrast, M6P pre-treatment of neurons did not prevent T cell-mediated neuronal death. This lack of protection could be related to the contemporary release of perforin and activation of a membrane-repair response, as recently described by Thiery et al. (48). This recent study supports previous reports showing membrane receptors as being not crucially important for the delivery of GrB into the target cell during the T cell-mediated cellular death; and the binding of GrB to the target cell can be facilitated by the expression of surface molecules such as heparan sulfate (49, 50). Similarly, other reports showed M6PR-independent target cell death using M6PR knockout mice, as well as employing various cell lines that overexpress/lack M6PR (51). Nevertheless, this does not imply that M6PR is not absolutely essential for the endocytosis of GrB; rather, it suggests that M6PR is not the only port of entry for GrB directly released by the T cells into the target neurons. Indeed, we have previously reported that CTL-mediated DNA fragmentation is significantly higher on M6PR-overexpressing compared with M6PR-deficient cells, and the cellular apoptosis increases with the augmentation of the ratio between effector and target cells. In addition, it is possible that high concentrations of GrB are released at T cell–neuron interface such that some uptake can occur in a M6PR-independent fashion or GrB could possibly antagonize M6PR on the receptor (30). Moreover, lymphocytes may possess multiple lytic mechanisms to induce apoptosis on the target cell (22, 52).

Perforin has been reported as a key mediator in the CTL-induced axonal/neuronal damage in various mice models of autoimmune diseases (53–57). In contrast, our study showed that GrB internalization into neurons as well as purified GrB and T cell-mediated neuronal killing are independent of perforin (Figs. 4e, 6a). Following its internalization through M6PR, GrB is immediately taken up by early endosomes. Previous works, in other cell types, demonstrated that internalized GrB does not induce death until endosomes are disrupted by perforin or adenovirus with subsequent release of GrB within the cytoplasm (48, 58, 59). Interestingly, in this study, GrB was released out of the early endosomes in the absence of any lytic agents, diffused in the cytoplasm, and rapidly induced neuronal apoptosis. Using annexin V as an early apoptotic marker, we showed colocalization of annexin V 647, GrB, and propidium iodide. DAPI staining revealed nuclear fragmentation surrounded by GrB-positive endosomes. In addition, GrB-mediated responses such as caspase 3 activation and α-tubulin cleavage were not affected by perforin inhibition. These findings suggest a perforin-independent cell death following internalization of GrB, although we cannot exclude the possibility of a concomitant effect on the extracellular matrix. It has been shown that a perforin-independent GrB-mediated smooth muscle cell death occurs through cleavage of extracellular matrix and anoikis (60). These findings suggest that GrB-mediated target cell apoptosis could be cell specific and eventually pursue different pathways depending on the cell type. The inability of preventing T cell-mediated neuronal death by inhibiting perforin does not decrease the relevance of this pore-forming protein; instead, it highlights the existence of other routes and mechanisms such as M6PR, contributing to the entry of GrB and subsequent induction of neuronal cell apoptosis. Nevertheless, the release of GrB from the endosomes without addition of perforin or any other lytic agent is absolutely novel and requires further investigations.

The mechanisms that lead to diffuse neuronal and axonal injury in MS have not been thoroughly explored (61). In the cerebral cortex of chronic MS patients, axonal and neuronal degeneration occurs in the absence of parenchymal inflammatory infiltrating lymphocytes (7, 62), and it strongly correlates with clinical disability (63, 64). However, the mechanisms of neuronal loss in MS are not yet defined, although a possible role is played by a “dying back” mechanism consisting of a retrograde degeneration of neuronal soma following axonal injury (8, 46, 65). Gray matter atrophy characterizes the chronic stages of the MS neuronal injury. In contrast, acute MS neuronal injury is represented by axonal transection and formation of axonal spheroids suggestive of a cytoskeletal disruption (6). In the animal model of MS, experimental autoimmune encephalomyelitis, T cells mediated the disruption of the microtubule network within neurons (66). Indeed, the release of GrB within the target cell initiates apoptosis by cleaving a variety of protein substrates that have either direct or indirect consequences in DNA fragmentation and cell death (16, 17, 67). A microtubule component, α-tubulin, was recently shown to be a new substrate for GrB in HeLa cells (35). In this study, to our knowledge, we showed for the first time that α-tubulin is cleaved by GrB in human neurons. This cleavage could explain the disruption of the fast axonal transport and subsequent neurite transection and spheroid formation at the proximal end (6, 8).

It is widely accepted that GrB induces apoptosis by activating caspases in target cells (19). In our study, activated T cells or GrB-induced death was blocked by the pan-caspase inhibitor Z-VAD-FMK, suggesting that this GrB-mediated cleavage of α-tubulin is dependent upon caspase activation. Caspase-3 inhibitors reversed the severe neuronal killing induced by GrB, confirming the previous reports that this caspase is a key substrate for GrB-mediated cell death and is highly expressed within neuronal cell bodies and mediates the death of the soma (16, 23, 67, 68).

In conclusion, we demonstrate that neurons are highly susceptible to T cell-mediated cytotoxicity because of their selective expression of M6PR, which allows GrB internalization. Inside the neuron, GrB releases out of the endosomes independent of perforin

**FIGURE 8.** Schematic diagram summarizing the mechanism of GrB-mediated neuronal apoptosis. Anti-CD3–activated or CTL release granule-derived GrB into the target cell (neuron). GrB enters into neurons through M6PR and accumulates within the endosomes. GrB diffuses out from the endosome and activates caspase-3. The activation of caspase-3 results in either a direct cellular apoptosis or cleavage of a cytoskeletal protein (α-tubulin) and subsequent neuronal apoptosis.
and cleaves both caspases and α-tubulin with subsequent neuronal death (Fig. 8). Inhibiting the activity of GrB together with targeting the binding site of GrB to M6PR or blocking activation of caspases can offer potential novel approaches for future therapies of neuroinflammatory diseases of the CNS such as MS.

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Disclosures

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