Vulnerability of Human Neurons to T Cell-Mediated Cytotoxicity

Fabrizio Giuliani, Cynthia G. Goodyer, Jack P. Antel and V. Wee Yong

*Journal of Immunology* 2003; 171:368-379; doi: 10.4049/jimmunol.171.1.368

http://www.jimmunol.org/content/171/1/368

---

**Why The JI?**

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**

This article cites 69 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/171/1/368.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Vulnerability of Human Neurons to T Cell-Mediated Cytotoxicity

Fabrizio Giuliani, Cynthia G. Goodyer, Jack P. Antel, and V. Wee Yong

Axonal and neuronal loss occurs in inflammatory diseases of the CNS such as multiple sclerosis. The cause of the loss remains unclear. We report that polyclonally activated T cells align along axons and soma of cultured human neurons leading to substantial neuronal death. This occurs in an allogeneic and syngeneic manner in the absence of added Ag, requires T cells to be activated, and is mediated through cell contact-dependent mechanisms involving CD4+ and CD8+ T cell subsets are equally neuronal cytotoxic. In contrast to neurons, other CNS cell types (oligodendrocytes and astrocytes) are not killed by T cells. These results demonstrate for the first time the high and selective vulnerability of human neurons to T cells, and suggest that when enough activated T cells accumulate in the CNS, neuronal cytotoxicity can result through Ag-independent non-MHC class I mechanisms. The Journal of Immunology, 2003, 171: 368–379.

Materials and Methods

Isolation of T cells

Mononuclear cells were isolated from the blood of healthy volunteers by ficoll-hypaque centrifugation as previously described (24–26) and suspended in serum-free AIM-V medium (Life Technologies, Burlington, Ont., Canada). To activate T cells, 1 μg/ml of an anti-CD3 Ab (OKT3) was added once for a period of 3 days. The floating cells were then removed from any adherent monocytes, and a fixed density was then used for testing cytotoxicity. Unless otherwise stated, the density of anti-CD3-activated T cells was 100,000 in 100 μl of AIM-V, and this was added to 50,000 neurons previously prepared (see below). Some mononuclear cell preparations did not receive OKT3, and the floating cells that were collected 3 days after were referred to as unactivated T cells. Flow cytometry analyses of the floating cells collected after 3 days of initiation of OKT3 treatment
indicated that CD3+ T cells constituted over 90% of the total cell population; these were ~60% CD4+ and 40% CD8+ in cell ratio. B lymphocytes (CD19+) and NK cells (CD56+) consisted of the rest of the floating cell population and no monocytes (CD14+) were detected; when analyzed further, NK cells constituted ~3% of the population. There was no significant difference in the proportion of the unactivated and activated lymphocyte populations. Henceforth, given that the majority of cells in the floating population are T cells, these will be referred to as T lymphocytes. T lymphocytes were also obtained from the spleen of fetuses and tested on syngeneic neurons. Each spleen was diced into small cubes and then placed on a filter containing 130-μm pore size. Tissue fragments were mechanically dissociated into single cells that were collected into PBS. Cell suspensions were then subjected to Ficoll-Hypaque centrifugation, and the T cells were then cultured and activated as previously described for cells from adult donors. Using flow cytometry analyses, the purity of CD3+ T cells after 3 days of culture was in excess of 95%.

In experiments that involved T cell subsets, mononuclear cells were first isolated from the blood of healthy adult volunteers by Ficoll-Hypaque centrifugation and then suspended in AIM-V medium as previously described. The T cells were then subdivided into CD3+ and CD8+ populations using magnetic separation columns (Miltenyi; Miltenyi Biotec, Auburn, CA). The subsets were activated by incubation for 72 h in control plates or anti-CD3-coated plates (BD Pharmingen, Bedford, MA) at a concentration of 200,000 cells per well.

**Human fetal neuron and astrocyte cultures**

Brain and spleen tissues from human fetuses of 10–18 wk fetal age were obtained following therapeutic abortion according to guidelines approved by local institutional ethics committees. For the preparation of brain cell cultures, 5–15 g of brain tissue diced into fragments of ≤1 mm with a pair of scalpels was incubated in 40-ml volume for 15 min at 37°C with 0.25% trypsin and 200 μg/ml DNase I in PBS. The suspension was then washed through a filter containing 130-μm pore size, and the filtrate was centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in PBS and centrifuged. Following a final washing step in feeding medium (see below), the pellet was suspended in feeding medium and cells were plated into T-75 flasks coated with 10 μg/ml polyornithine. Plating density was 50 million cells in 25 ml of medium. Feeding medium was MEM-supplemented with 10% FBS, 20 μg/ml gentamicin, 0.1% dextrose, 1× nonessential amino acids, 10 μM glutamine, and 1 mM sodium pyruvate. All medium constituents were from Life Technologies.

To obtain neuron-enriched cultures, cells in the T-75 flasks were subjected, immediately upon seeding, to 3 cycles of 25°C trypsin and 200 μg/ml DNase I in PBS. The suspension was then washed through a filter containing 130-μm pore size, and the filtrate was centrifuged at 1200 rpm for 10 min. The cell pellet was resuspended in PBS and centrifuged. Following a final washing step in feeding medium (see below), the pellet was suspended in feeding medium and cells were plated into T-75 flasks coated with 10 μg/ml polyornithine. Plating density was 50 million cells in 25 ml of medium. Feeding medium was MEM-supplemented with 10% FBS, 20 μg/ml gentamicin, 0.1% dextrose, 1× nonessential amino acids, 10 μM glutamine, and 1 mM sodium pyruvate. All medium constituents were from Life Technologies.

**Isolation of human oligodendrocytes**

Specimens were obtained during surgery for epilepsy. Viable cells were isolated using Percoll gradient centrifugation, and oligodendrocytes of over 95% purity were obtained as detailed elsewhere (28).

**Immunocytochemistry and evaluation of cytotoxicity**

Following fixation with 4% paraformaldehyde in PBS, astrocytes were identified with a rabbit polyclonal Ab to glial fibrillary acidic protein (GFAP) (1:100 dilution; DAKO, Carpinteria, CA), which is an intermediate filament protein specific to astrocytes in the CNS. Oligodendrocytes were identified with a mouse mAb to galactocerebroside (29). Neurons were labeled by a mouse mAb to microtubule-associated protein (MAP)-2 (1:5000; Sigma-Aldrich) or by a rabbit polyclonal Ab to τ protein (1:400; Sigma-Aldrich). MHC-I expression was identified by a mouse anti-human HLA-ABC Ab (2.5 μg/ml, W6/32 clone; Serotec, Oxford, U.K.), MHC class II (MHC-II) expression was assessed using a mouse anti-human HLA-DR, -DP, and -DQ Ab (2.5 μg/ml, Tu39 clone; Research Diagnostics, Flanders, NJ).

Appropriate secondary Abs conjugated to Cy3 or Alexa 488 (Molecular Probes, Leiden, The Netherlands) were then employed and immunofluorescence was analyzed by using a Leica (Deerfield, IL) immunofluorescence microscope. Cultures were counterstained with Hoescht dye (33258) to label all nuclei.

To evaluate cytotoxic effects on neurons and to achieve consistency in quantitation between samples, each well was assessed along its equatorial axis counting six random fields using the ×40 objective. All the MAP-2-positive cells in each field were enumerated. For every condition in each set of experiments, 4 wells were counted to obtain the mean number of neurons remaining in culture. The mean value for control neuronal cultures (i.e., wells not exposed to T cells) was thus obtained, and results from experimental groups were then expressed as a percentage of that of control cultures. Finally, each bar in the figures represents the mean ± SD of the results from 4 wells, and at least 800 cells were counted in the control cultures. All results were reproduced in at least three sets of experiments.

**MITT viability assay**

In some experiments, neuronal toxicity was evaluated using the MITT spectrophotometric assay. Neural and astrocytic cell populations were exposed to test agents in 100 μl culture medium for 48 h. Twenty microliters of 0.5 mg/ml solution MITT was then added per well. Following incubation for 1 h at 37°C, and after two washes with PBS, 200 μl DMSO was added for 10 min. The amount of solubilized MITT product was read on a spectrophotometer at 550 nm.

**Cytokines**

Cytokines and chemokines were tested alone or in different pairs. These were recombinant human (rh)IL-1β (BioSource International, Cambridge, CA), rhTNF-α (BD Biosciences, Bedford, MA), rhIFN-γ (Boehringer Mannheim, Mannheim, Germany), rhIL-12 (R&D Systems, Minneapolis, MN), rhGM-CSF (Upstate Biotechnology, Lake Placid, NY), recombinant human monocyte chemoattractant protein 1 (CCL-2; R&D Systems), recombinant human monocyte chemoattractant protein 3 (CCL-7; R&D Systems), recombinant human inflammatory protein 10 (CXCL-10; R&D Systems), and recombinant human macrophage inflammatory protein 1α (CCL-3; R&D Systems).

**Abs tested for their capacity to attenuate T cell cytotoxicity**

The following mAbs were used: mouse anti-human CD40 (Genzyme, Cambridge, MA), mouse anti-human 96-well-plated IgG1 (FasL; BD Pharmingen), mouse anti-human CD11a (LFA-1; BD Pharmingen) and mouse anti-human ICAM-1 (Chemicon International, Temecula, CA). These Abs were used at a concentration of 5 μg/ml. The function-blocking mouse anti-human MHC-I (W6/32 clone) or MHC-II (Tu39) Abs were used at a final concentration of 10 μg/ml each. T cells or neurons were incubated for 30 min with the respective Ab, following which excess Abs were washed off using PBS before the coculture.

To ensure that the anti-MHC-I Ab (W6/32) was indeed a function-blocking Ab, the MHC-I-restricted killing by CD8+ T cells of the L6Y cell line, a non-Hodgkin B cell lymphoma, was used. A 3HCr release assay was used to measure cytotoxicity as previously described (30).

**Statistical analysis**

Because all experiments involved multiple groups, statistical analyses were conducted using one-way ANOVA with Tukey’s post hoc test. Statistical analysis was performed using SPSS version 11.0 (SPSS, Chicago, IL).

**Results**

**Neurons are highly susceptible to T cell cytotoxicity**

We first determined whether T cells could kill neurons. Human fetal neurons were cocultured with unactivated or anti-CD3-activated T cells from adult human donors without any apparent neuroglial diseases. After 24 h, cultures were stained for MAP-2 because the disappearance of MAP-2 immunoreactivity is associated in vivo and in vitro with neuronal injury and death (31–37). We found that while unactivated T cells did not produce neuronal death, activated T cells consistently resulted in a significant loss of neurons (Fig. 1 and Fig. 2). The mode of cell death for
some neurons was apoptotic, as these cells were annexin V-positive before their detachment from the culture substrate (Fig. 2D). Fig. 3A documents the number of neurons remaining in culture expressed as a percentage of that in control conditions (i.e., neurons not exposed to T cells). Although unactivated T cells did not produce loss of neurons, activated T cells from three adult donors caused the death of 60–75% of neurons by 24 h.

Besides MAP-2 immunoreactivity, two other means to detect cytotoxicity were used. These methods involved the detection of free nucleosomes liberated into the cytoplasm of dying cells or of cellular MTT levels. It was found that the nucleosome content was consistently increased in neurons exposed to activated, but not unactivated, T cells compared with neurons alone (data not shown). Furthermore, MTT analyses showed a marked reduction in cultures exposed to activated T cells (data not shown).

We evaluated the rapidity of the onset of T cell cytotoxicity. We found that the number of MAP-2-positive cells started to decrease significantly by 3 h of coculture and progressively declined over the 72 h of study (Fig. 3B). Thus, the manifestation of T cell toxicity occurs quickly. In contrast to activated T cells, unactivated T cells did not reduce neuronal numbers at all the time points examined. To assess the relative ratio of T cells to neurons required to produce neuronal death, 50,000 neurons were co-cultured with different numbers of T cells. Although 10,000 T cells were observed to reduce neuronal numbers, a statistically significant result was obtained when the number of T cells was increased to 50,000 or 100,000 T cells with a ratio of T cells-neurons of 1:1 and 2:1, respectively (Fig. 3C).

In contrast to the T cell-mediated killing, monocytes seeded at a concentration of 100,000 cells (ratio of monocytes-neurons of 2:1) were not able to kill neurons. Activation of monocytes with LPS (100 ng/ml) did not further elicit any toxicity to neurons (data not shown).

To evaluate which T cell subset was responsible for the neuronal cytotoxicity, human fetal neurons were cocultured for 24 h with CD4+ or CD8+ subsets obtained through magnetic separation. Both subsets were able to kill neurons after anti-CD3 activation, producing a reduction of >80% of neurons compared with untreated cultures or neurons cocultured with unactivated CD4+ or CD8+ T cells (Fig. 4).

The previous results were from fetal neurons cocultured with T cells from adult donors. The MHC mismatch might have resulted in an MHC-I-mediated killing such as that occurring in a graft-vs-host reaction. Thus, we obtained T cells from the spleen of the same fetal brain donor. Neurons cultured with activated syngeneic splenocytes demonstrated an over 60% reduction in cell numbers compared with control neurons or neurons exposed to unactivated syngeneic T cells (Fig. 5A).
To provide more compelling evidence that T cell-mediated toxicity is not through an MHC-I mechanism, neurons were treated with an MHC-I function-blocking Ab at the concentration of 10 μg/ml for 30 min before their coculture with activated T cells. Neuronal loss was not prevented by this Ab (Fig. 5B) nor was it attenuated by the MHC-II function-blocking Ab (data not shown). That the MHC-I blocking Ab is efficacious in the first place is demonstrated by its ability to attenuate an MHC-I-mediated CD8\(^+\) cytotoxicity to a B cell line as evident using a 51Cr release assay. Here, a 55% specific killing of the LY8 B cell line by CD8\(^+\) T cells is attenuated to 15% by 10 μg/ml of the anti-MHC-I Ab. To confirm the efficacy of the MHC-II Ab, we assessed its ability to block the proliferation of glatiramer acetate-specific T cell lines in response to class II-dependent Ag presentation. In these experiments the Ab was able to reduce the proliferation by 80% compared with control cocultures.

Consistent with the lack of involvement of MHC-I in the killing of neurons by T cells is the finding that human neurons in culture do not express MHC-I unlike their astrocyte counterparts (Fig. 5C). In summary, neurons are highly susceptible to toxicity mediated by T cells, as long as the T cells are activated. There is no obvious difference in neuronal death mediated by allogeneic or syngeneic T cells. Activated CD4\(^+\) and CD8\(^+\) T cell subsets are equally cytotoxic for neurons. Once activated, T cells are not dependent on MHC-dependent mechanisms of cytotoxicity.

Cell-cell interaction is required for the neuronal killing by cytotoxic T cells

Upon activation, T cells produce various soluble factors including cytokines that potentially can damage target cells. There is conflicting data on the neuroprotective vs neurotoxic effect of proinflammatory cytokines both in vivo and in vitro (38). To assess the effect of cytokines in our model, we added different concentrations and combinations of proinflammatory cytokines and chemokines to neurons. No cell death was evident by MTT assay (Fig. 6, A and B). Human neurons could be susceptible to a combination of inflammatory cytokines (39) and thus toxicity might not have manifested when we applied cytokines individually or in pairs. To address this, the conditioned medium from activated T cells, which contains a variety of cytokines, was tested to determine whether this was toxic to neurons. There was absence of killing when neurons exposed to the conditioned medium were assessed after 24 h of culture (Fig. 6C). Finally, in Transwell experiments where T cells and neurons were exposed to each of the other soluble factors, but where both cell types were physically separated by a filter, no killing of neurons was evident (data not shown).
The data from the cytokine and conditioned medium studies suggest that T cell cytotoxicity was likely mediated by a cell-cell contact mechanism. In support, we noted that when T cells were cocultured with neurons, they aggregated around neuronal soma and neurites; importantly, where T cells were in contact with neuronal elements, the latter lost their MAP-2 immunoreactivity (Fig. 7).

**FIGURE 3.** Quantitative loss of neurons exposed to activated T cells. A, Comparison of the number of MAP-2 neurons that remain after 24 h of allogeneic neurons-T cells coculture. The results of activated (act.) or unactivated (unact.) T cells from three different donors on neuronal toxicity are displayed; similar results were seen in at least 10 other experiments involving different neuronal and leukocyte samples. Furthermore, while most neuronal cultures were used 2 wk after isolation, neurons that were “aged” in vitro for 1–2 mo were equally susceptible to toxicity by T cells. All results have been normalized to the number of neurons present in control cultures that were not exposed to T cells. B, Time course of neuronal loss when 100,000 allogeneic T cells were added to 50,000 neurons. C, Different numbers of activated T cells were added to 50,000 neurons and the number of surviving neurons was assessed 24 h after. A total of 100,000 unactivated T cells were also used. All results were normalized to neuronal cultures that were not exposed to T cells. One-way ANOVA, Tukey’s post hoc test: *, p < 0.005.

The data from the cytokine and conditioned medium studies suggest that T cell cytotoxicity was likely mediated by a cell-cell contact mechanism. In support, we noted that when T cells were cocultured with neurons, they aggregated around neuronal soma and neurites; importantly, where T cells were in contact with neuronal elements, the latter lost their MAP-2 immunoreactivity (Fig. 7).

**FIGURE 4.** Both activated CD4+ and CD8+ T cells can promote extensive neuronal loss. Comparison between the percentage of MAP-2-positive cells that remains after 24 h of neurons-T cells coculture show that while unactivated (unact.) T cells did not kill neurons, and both the CD4+ and CD8+ activated (act.) subsets were equipotent as the CD3+ whole T cell population. Prior BrdU proliferation assays performed to assess the activation status of cells showed a significant increase in cell proliferation of microbeads-separated cells only after anti-CD3 treatment; these data suggest that microbead purification did not activate T cells nonspecifically or contribute to neuronal toxicity unless the purified populations received anti-CD3 treatment. One-way ANOVA, Tukey’s post hoc test: *, p < 0.005.
To investigate whether neuronal death induced by T cells is cell-cell contact dependent, we investigated whether blocking cell surface molecules could attenuate the T cell toxicity. We used neutralizing Abs to selected molecules and focused on those implicated in T cell-mediated killing in other systems. Thus, T cells were treated for 30 min with 5 μg/ml of function-blocking Abs to FasL, LFA-1, or CD40, then washed and cocultured with neurons for 24 h. These treatments protected neurons as 70–80% remained viable, compared with <20% in cultures not exposed to Abs (Fig. 8). The selectivity of the anti-FasL, anti-LFA-1, or anti-CD40 Abs in protecting neurons was indicated by the lack of effect of anti-ICAM-1 pretreatment of T cells to confer neuroprotection.

In parallel experiments, neurons were treated for 30 min with 5 μg/ml anti-ICAM-1 or anti-CD40. These Abs were then removed through washing, and activated T cells were added to the neuronal cultures. Both Abs protected neurons from T cell-mediated cytotoxicity (Fig. 8).

Because the results of the Ab blocking experiments suggested the presence of ICAM-1 and CD40 on neurons, we used immunohistochemistry to determine whether these molecules could be detected on human neurons in culture; a mixed culture of neurons and astrocytes was used to maximize the possibility of detection of these molecules on either cell type. Fig. 9 shows that neurons were positive for CD40 although not all neurons expressed this molecule. Similarly, neurons also expressed ICAM-1, although the level tended to be lower than that of astrocytes (Fig. 9).

In summary, the toxicity of activated T cells on neurons requires cell-cell contact, and is mediated in part through FasL, LFA-1, and CD40.

**T cell cytotoxicity is selective for neurons**

We investigated whether activated T cells indiscriminately destroy all CNS constituents or whether toxicity was limited to neurons. Thus, T cells were incubated with human oligodendrocytes or astrocytes. No death was evident for oligodendrocytes as determined by morphologic analysis and cell counts (Fig. 10). When T cells were cocultured with astrocytes, there was no obvious reduction in astrocytic cell numbers (Fig. 10); however, the morphology of astrocytes was altered in that cells converted from a flat, fibroblast-like morphology to those with multiple stellate processes (Fig. 10D), indicative of a more reactive state.

Thus, the toxicity of activated T cells is selective for neurons, and oligodendrocyte and astrocyte numbers are not obviously altered upon confrontation with T cells during the period (24–48 h) of study.

**Discussion**

It has become evident in recent years that many inflammatory diseases of the CNS, including MS, Rasmussen’s encephalitis, and paraneoplastic encephalomyelitis, have a substantial degenerative component where neurons and axons are lost in large numbers (2, 6, 7, 40–42). This degenerative process is of crucial importance in determining clinical outcomes. In MS, the loss of neurons and axons beyond a critical threshold likely accounts for conversion of the relapsing remitting form of the disease to SPMS with permanent disabilities (43). Approximately 70% of patients with RRMS convert to SPMS at some stage in their disease (44). Furthermore, in a cohort of patients followed for 5 years the SPMS patients had a higher inflammatory activity during the time before the transformation from RRMS compared with patients still in the relapsing-remitting phase (45).

The direct cause of the neuronal and axonal injury has not been clear although this occurs in regions of accumulation of various inflammatory cell types (3, 5, 6, 46–48). Recently, it was reported that an average of 7% of CD8+ T cells within the CNS parenchyma of Rasmussen’s encephalitis were present in close apposition to neurons, and even appeared to invade them (42). Despite the correspondence of neuronal and axonal loss with inflammation, a direct cause and effect relationship of inflammatory cells destroying neurons has not been demonstrated.
In this study, by using defined sets of neural cultures, we describe that T cells are potent effectors of neuronal death. This occurs rapidly and necessitates that T cells are activated. Both CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets were neurotoxic. Unlike other examples where Ag-specific T cells were lethal to mouse neurons only upon the induced expression of MHC-I on neurons by prior treatment

**FIGURE 6.** Soluble factors do not mediate toxicity to neurons. A and B, Representing MTT results from two separate experiments, cytokines or chemokines in pairs (100 U/ml each) or alone did not result in neuronal toxicity. The positive control staurosporine (SP, 4 μM), a nonspecific kinase inhibitor, resulted in extensive death. One-way ANOVA, Tukey’s post hoc test: *, p < 0.05. C, The presence of activated T cells is required to kill neurons and this cannot be reproduced by conditioned medium (CM) collected from activated T cells. One-way ANOVA, Tukey’s post hoc test: *, p < 0.005.

**FIGURE 7.** Contact between activated T cells and neuronal structures results in the disappearance of MAP-2 immunoreactivity. A, A neurite (arrow) surrounded by activated T cells (arrow) is negative for MAP-2, whereas a neurite that is not (arrowhead) continues to be MAP-2-positive. A, MAP-2 immunofluorescence. B, Corresponding phase-contrast micrograph. The morphology of T cells suggests that they are likely also killed following the encounter with neurons.
with tetrodotoxin and IFN-γ (19–21), there was no such requirement for MHC-I or Ags in this study so long as the T cells have been polyclonally activated through anti-CD3 treatment. A 1:1 ratio of T cells-neurons is sufficient for toxicity to manifest. This ratio is achievable in vivo because many T cells are known to cluster around neurons in neuroinflammatory diseases of humans and experimental models (41, 42).

It is noted that the T cells used in this study were not pure populations and it is conceivable that the few contaminating non-T cell types may be responsible for the cytotoxicity observed. This is

FIGURE 8. The toxicity of T cells can be neutralized. Whereas activated T cells result in 80% loss of neurons in 24 h, this toxicity was attenuated by the treatment of T cells with Abs to LFA-1, FasL, and CD40, but not ICAM-1. Treatment of neurons with anti-ICAM-1 or CD40 also prevented the subsequent loss of neurons upon coculture with activated T cells. One-way ANOVA, Tukey’s post hoc test: *, p < 0.005.

FIGURE 9. Human neurons in culture express CD40 and ICAM-1. A mixed culture of neurons and astrocytes was used to maximize the possibility of detection of CD40 and ICAM-1 on either cell type. A–C, Shows that some neurons are positive for CD40: phase contrast (A), MAP-2 (B), CD40 (C). Similarly, neurons also expressed ICAM-1: phase contrast (D), MAP-2 (E), ICAM-1 (F), although the levels tended to be lower than that of astrocytes (flat cell). Some neurons positive for either CD40 or ICAM-1 are indicated by arrows in B and E, respectively.
unlikely because these contaminating cell types would also be present in the non-anti-CD3-treated preparations, which were not cytotoxic. Furthermore, CD3⁺-selected T cells were similarly cytotoxic to neurons (Fig. 4). Moreover, human T cell lines were also able to destroy human neurons in our studies (F. Giuliani, A. Bar-Or, and V. W. Yong, unpublished observations). Nonetheless, future studies will aim to derive pure primary T cells from human blood to obviate any possibility of other cell types having a cytotoxic response on human neurons.

We note that the neuronal cultures used in this study were over 90% pure. Most of the contaminating cell types are astrocytes and it is plausible that the killing of neurons might have been mediated through astrocytes. This is unlikely for at least two reasons. First, in a few cultures in which there were virtually no astrocytes,
neuronal toxicity was still elicited by activated T cells. Second, the T cell-neuron contact in Fig. 7A, which resulted in the loss of MAP-2 staining in neurites, occurred in the absence of any astrocytes.

The T cell cytotoxicity in this study requires cell-cell contact and cannot be reproduced by soluble products such as cytokines. In this regard, our experiments do not confirm the data of others that proinflammatory cytokines such as TNF-α can kill human fetal neurons in vitro (39). In support of a contact-mediated mechanism, our results show that T cells localized to the soma and processes of neurons causing the disappearance of MAP-2 staining (Fig. 7). The rapid loss of MAP-2 immunoreactivity in injured axons has been attributed to the activation of calpains, which promptly proteolyze MAP-2 leading to its nondetectability (49). Cell surface molecules that contributed to the T cell toxicity to neurons included FasL, LFA-1/ICAM-1, and CD40, but not MHC-I or MHC-II; of note, CD40 and ICAM-1 were detected on some but not all neurons (Fig. 9). We do not yet know the mechanisms by which the engagement of these molecules contribute to neuronal death. However, it has been reported that Fas-FasL interaction (50), or CD40 ligation (51), can alter intracellular pathways that regulate apoptosis of cells.

It should be noted that some neurons in vivo may express MHC molecules, particularly during a disease process. For example, neurons in Rasmussen’s encephalitis tissue (42) or following infection with Theiler’s virus (52) have been shown to express components of MHC-I, indicating that neurons in vivo can be susceptible to MHC-I-dependent killing. However, our results would emphasize that non-MHC-I-dependent mode of neuronal killing can also occur (53).

The mode of death of neurons following their encounter with T cells in this study is not clear and it is possible that both apoptotic and necrotic cell death occur to some degree. Some neurons were found to express annexin V (Fig. 2D), a marker of apoptosis, before their detachment from the culture substrate.

It is instructive to note that when autoreactive T cell lines are adoptively transferred into animals, a fate of these cells that migrate into the CNS is death; indeed, the expression of FasL by neurons is thought to account for the demise of these T cells (54, 55). Nonetheless, while the CNS has high potential for the elimination of T cell-dependent inflammation (56), this state is overwhelming in conditions such as MS where T cells infiltrate into the CNS in significant numbers. In our experiments, we observed that many of the T cells that aggregate around neuronal elements appear pyknotic and dead (Fig. 7), in correspondence with the concept that neurons can evoke T cell death as a mechanism of neuroprotection. However, before the elimination of T cells, significant axonal and neuronal injury had already occurred. It is also possible that T cells are first killed by the neuronal contact, and that it is the dying T cells that then release cytotoxic factors to neurons. However, we have tested this by adding dying T cells to neurons but no toxicity was found (F. Giuliani, C. G. Goodyer, J. P. Antel, and V. W. Yong, unpublished observations).

The effector molecules that account for the T cell cytotoxicity may be through two different pathways, one mediated by granzymes and perforins and the other mediated by the interaction of Fas with FasL. We did not evaluate whether one or both are operational in our model. Discordant data are present in the literature where the neuronal killing of neurons by T cells could be perforin-mediated (57) or Fas-mediated (58). In a model of MS, Theiler’s murine encephalomyelitis, it was shown that perforin-deficient mice showed no clinical disease (59). Others have reported that CNS neurons are refractory to cytotoxic granule-mediated lysis (60) and that, after the induction of MHC-I expression, neuronal killing is via an apoptotic mechanism mediated by a Fas-FasL interaction (58). In our model the treatment of T cells with blocking Abs anti-FasL, anti-LFA-1, and anti-CD40 can prevent the neuronal damage. This result is in accordance with previous reports (61–63) that all the pathways mediated by those molecules are involved in T cell-mediated cytotoxicity. The block of ICAM-1 on T cells did not have any effect on killing, consistent with the lack of expression of ICAM-1 on T cells. On the other hand, the targeting of ICAM-1 present on neurons prevented the killing by activated T cells. The ICAM-1 expressed on neuronal membranes could interact with the LFA-1 over-expressed on activated T cells. Consistent with these data is the report that neurons do express ICAM-1, Fas, FasL, and CD40 (54, 55, 64–66). Human fetal neurons specifically have also been found to express Fas (67), although we were not able to demonstrate this convincingly by immunohistochemistry in our cultures (data not shown). However, we were able to document CD40 and ICAM-1 on human neurons in culture (Fig. 9).

The data that showed an anti-CD40 blocking Ab applied to neurons diminished T cell cytotoxicity is consistent with CD40 on neurons mediating the interaction with T cells to produce toxicity. When we applied the CD40 blocking Ab to T cells, this also alleviated subsequent toxicity of T cells on neurons. Although this implies that the CD40 on T cells also has a role in causing neuronal death, an alternate explanation is that the CD40 Ab treatment of T cells decreased other aspects of T cell activation; in this regard, blocking CD40 on T cells has been reported to produce their anergy (68).

Finally, our results demonstrate that the T cell cytotoxicity is selective in that oligodendrocytes and astrocytes do not appear to be susceptible; however, we must emphasize that the adult nature of the oligodendrocytes might have contributed to this differential response compared with fetal neurons. Previously, we have found that human oligodendrocytes were not susceptible to killing by activated CD4+ or CD8+ T cells (69). Thus, oligodendocyte vulnerability is likely through other mechanisms, such as Ab-mediated cytotoxicity or soluble factors such as TNF-α (70). We noted that although astrocytes were not killed by activated T cells, a modification of their shape from flat to stellar was evident probably indicating an activation of these cells. The resistance of astrocytes to T cell killing could be facilitated by the induction of apoptosis of T cells in accordance with previous reports (71). We do not know why neurons are more vulnerable than oligodendrocytes or astrocytes to the T cell-mediated killing described in this report. This circumstance may involve a differential spectrum of adhesion molecules on neurons that facilitate T cell attachment, but the adhesion molecules on neurons that we studied are also expressed on oligodendrocytes or astrocytes (64–66). It remains possible that a neuron-specific adhesion molecule not examined in this study is indeed the predominant ligand for an interacting partner on activated T cells to evoke neuronal death.

T cells are known to patrol the CNS under normal conditions; why these do not kill neurons in the normal state could be related to their relatively low numbers in the normal state compared with diseased conditions. It is also possible that the level of activation of the T cells that patrol the CNS in the normal state is lower than that of T cells that infiltrate into the CNS in neuro-inflammatory diseases. This study highlights the need to examine more carefully the possibility that activated T cells under some conditions will be toxic to neurons in the CNS. This emerging area of study is supported by the recent demonstration in Rasmussen’s encephalitis that CD8+ T cells are in close apposition to dying neurons (42).

We would like to emphasize that fetal human neurons were used in this study, and their level of maturity might have contributed to their relative susceptibility to T cell-mediated killing. To test this, one would need to use differentiated neurons from adult brains, but
these do not survive the isolation and culture process. Another means is to “age” the neurons in culture. In this regard, while most neuronal cultures were used 2 wk after isolation, neurons that were aged in vitro for 1–2 mo were equally susceptible to toxicity by T cells (Fig. 3). It has been shown that enriched fetal human cortical neurons, in vitro, undergo morphological and biochemical differentiation with the formation of synapses within 1 wk after isolation. Furthermore, they develop excitable properties between day 7 and day 21 after isolation (72). Finally, we believe that our results are relevant to neuronal death in human diseases given the recent realization that neurons and axons are lost in large numbers in MS, Rasmussen’s encephalitis, and paraneoplastic encephalomyelitis (2, 3, 7, 40–42).

In summary, this study reveals for the first time that neurons are selectively vulnerable to T cell cytotoxicity and that this could be responsible for the neurodegeneration observed in inflammatory diseases. Our results would suggest that while the initiation of MS may be attributed to T cells that are Ag-specific, the subsequent arrival of activated T cells of many specificities then damage the CNS in a cell-contact-dependent non-Ag-restricted manner.

**Acknowledgments**

We gratefully acknowledge the superb technical assistance of Tammy Wilson and Ellie McCrea.

**References**


