Short Communication

Transection of Major Histocompatibility Complex Class I-Induced Neurites by Cytotoxic T Lymphocytes

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Damage to neurites with transection of axons and spheroid formation is commonly noted in the central nervous system during viral and autoimmune diseases such as multiple sclerosis, but it remains open whether such changes are caused primarily by immune mechanisms or whether they are secondary to inflammation. The present experiments explored whether neurites can be directly attacked by cytotoxic T lymphocytes (CTLs). Cultured murine neurons induced by interferon- γ and tetrodotoxin to express major histocompatibility complex class I were pulsed with a dominant peptide of the lymphochoriomeningitis virus envelope glycoprotein (GP33) and then confronted with GP33-specific CD8⁺ CTLs. Within 3 hours the neurites developed cytoskeleton breaks with adjacent solitary neuritic spheroids, as documented by confocal examination of the cytoskeletal marker β -tubulin III. At the same time cytoskeleton staining of the neuronal somata showed no damage. The CTLs selectively attacked neurites and induced segmental membrane disruption 5 to 30 minutes after the establishment of peptide-specific CTL-neurite contact, as directly visualized by live confocal imaging. Thus, major histocompatibility complex class I/peptide-restricted CD8⁺ T lymphocytes can induce lesions to neurites, which might be responsible for axonal damage during neuroinflammatory diseases. (*Am J Pathol* 2001, 159:809–815)

During viral, autoimmune, and neurodegenerative brain processes, major histocompatibility complex (MHC) class I expression is induced on resident cells including neurons, often followed by infiltration of inflammatory

cells into the affected brain tissue.^{1–3} It has been demonstrated *in vitro* that MHC class I inducibility of neurons is more strictly regulated than other cell types. Expression of MHC class I was inducible in neurons by interferon- γ (IFN- γ) when concomitantly the neuronal activity was blocked with tetrodotoxin (TTX).^{4,5} MHC class I expression should render neurons susceptible to cytotoxic T lymphocyte attack. However, we found that neuronal cell bodies, although expressing MHC class I, are protected against early peptide-specific or alloreactive cytotoxic T lymphocyte (CTL)-mediated lysis⁶ and might be killed later solely via Fas/CD95 mediated apoptosis.

Neurons are highly polarized cells⁷ and in most studies little attention has been paid to the conditions of the axons or dendrites following T lymphocyte cytotoxicity.^{8–10} Interestingly, while neuronal cell bodies seem to be relatively protected against overt cytotoxicity in most neuroinflammatory processes,¹¹ their neurites are often damaged during viral infections^{12–15} or central nervous system (CNS) autoimmune diseases.^{16,17} For example, axonal injury is a common neuropathological feature in the Theiler's murine encephalomyelitis virus model.¹² In this model the axonal damage was more closely related to the severity of clinical disease than the degree of demyelination.¹² Further, axonal damage and neurological deficits were absent in β 2-microglobulin-deficient mice lacking functional MHC class I molecules despite extensive demyelination.¹² Damage to neurites with transection of axons and formation of neurite spheroids were also observed in demyelinated, inflammatory multiple sclerosis (MS) lesions.^{16,17} In MS, neurite damage was associated with the number of CD8⁺ T lymphocytes infiltrating the lesion.¹⁸ Again, damage of neurites appears to be responsible for the persistent neurological deficits seen in these patients.¹⁹ Thus, in both viral and autoim-

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mune CNS disease, neurites are selectively and locally damaged during the inflammatory process by a mechanism which still has to be elucidated.

In this study we analyzed the interaction between cytotoxic T lymphocytes and neurites *in vitro*. By performing live imaging we directly visualized the CTL-neurite interaction and showed that antigen-specific CTLs are directly capable of damaging neurites in a MHC class I/peptide-restricted fashion.

Materials and Methods

Murine Hippocampal Cell Cultures

Hippocampal cell cultures were prepared from embryonic day 16 mice (C57BL/6; Max-Planck-Institute of Neurobiology and Biochemistry, Martinsried, Germany) as previously described.⁴ Dissociated neurons were cultured in Basal Medium Eagle (BME, Gibco, BRL) with the B27 supplements (Gibco, BRL), glucose (1% (v/v), Sigma), and fetal calf serum (FCS; 1% w/v; Pan System, Würzburg, Germany). Recombinant mouse IFN- γ (100 U/ml; Laboserv, Giessen, Germany) and TTX (1 μ mol/L, Sigma) were added to the cultures for 72 hours as indicated.

MHC Class I Immunohistochemistry

Hippocampal neuronal cultures were fixed in 4% paraformaldehyde and incubated with rat monoclonal antibody directed against mouse MHC class I (1:100, ER-HR52; Dianova, Hamburg, Germany) followed by secondary fluorochrome Cy3-conjugated goat antibody directed against rat immunoglobulin (IgG) (10 μ g/ml; Dianova). Cells were then washed and incubated with mouse monoclonal antibody specific for MAP2 (10 μ g/ml; Sigma) and secondary dichlorotriazinyl-aminofluorescein (DTAF)-conjugated goat antibody directed against mouse IgG (10 μ g/ml; Dianova). For triple labeling, cultures were then incubated with a mouse monoclonal antibody directed against β -tubulin III (2 μ g/ml; Sigma) and Cy5-conjugated goat antibody directed against mouse IgG (10 μ g/ml; Dianova). Optical sections along the z-axis were scanned with a confocal laser-scanning microscope (63 $\times$ oil objective, Leica, Inc., Deerfield, IL). Baseline labeling for MHC class I was revealed with primary purified rat immunoglobulin (10 μ g/ml; Dianova) and secondary fluorochrome-conjugated goat antibodies to rat IgG.

Generation of GP33-Specific Cytotoxic T Lymphocytes and Peptide Loading of Target Cells

Spleen cell suspensions were obtained from lymphochoriomeningitis virus (LCMV) infected C57BL/6 mice (200 plaque forming units LCMV-WE at least 3 months previously) and cultured in Iscove's modified Dulbecco's me-

dium (Gibco, BRL) supplemented with 10% FCS (Pan System), penicillin-streptomycin, 2-mercaptoethanol, 0.1 ng/ml mL-7 (Biosource Int., Camarillo, CA) and 10% IL-2 containing supernatant. Cultures were restimulated at 10-day intervals with irradiated RMA-S cells pulsed with peptide GP33 (10 ng/ml, peptide GP33-41, KAVYN-FATC, Neosystem S.A., Strasbourg, France). CTL lines were used for experimentation after three rounds of restimulation, a time at which the CTLs exhibited a CD8⁺, $\alpha\beta$ T cell receptor phenotype and were highly specific for the LCMV GP33 peptide, but not LCMV peptide GP276 or NP396, as determined by cytotoxicity assays.²⁰ CTL cultures were recovered from a gradient at day 7 after stimulation and then used for killing assays. Target cells were pulsed with 1×10^{-7} mol/L of the H-2D^b-restricted LCMV peptide GP33 or the H-2D^b-restricted influenza virus nucleocapsid peptide NP 366-374 (ASNENMETM, Neosystem S.A.).

Cytoskeleton Immunohistochemistry

Hippocampal neuronal cultures were fixed in 4% paraformaldehyde at 3 hours after addition of CTLs and then were incubated with the mouse monoclonal antibody specific for β -tubulin III (10 μ g/ml; Sigma) and secondary fluorochrome DTAF-conjugated goat antibody directed against mouse IgG (10 μ g/ml; Dianova). Optical sections along the z-axis were scanned with a confocal laser-scanning microscope (63 $\times$ oil objective, Leica, Inc.). Frequency of localized lesion sites with adjacent solitary neurite cytoskeleton spheroids were determined from at least three independent experiments. Degenerated neurons with "beaded" neurites sporadically detectable in neuronal cell cultures were excluded from the analysis.

Confocal Imaging of Plasma Membranes

During confocal microscopy, cells were maintained at 37°C in imaging buffer consisting of 142 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl₂, 0.8 mmol/L MgSO₄, 1 mmol/L NaH₂PO₄, 5 mmol/L glucose, 25 mmol/L Hepes, 0.1% bovine serum albumin, 1% (v/v) FCS, and 1 μ mol/L TTX, adjusted to pH 7.4. CTLs were added to the neuronal cultures at an effector/target ratio adjusted to 5:1. The fluorescence plasma membrane dye FM1-43 (15 μ mol/L, Molecular Probes) was applied to the cells and images were obtained with the confocal laser scanning microscope (40 $\times$ oil objective, Leica, Inc.). Laser intensity was reduced to a minimum to avoid phototoxicity. Optical sections of selected areas were obtained along the z-axis every 5 minutes. Percentage of transected neurites at the contact site of CTLs within 1 hour of GP33- or influenza virus peptide-pulsed and pretreated (IFN- γ plus TTX) neurons was determined from at least three independent experiments.

Figure 1. Induction of MHC class I molecules on neurites. Neuronal cell cultures were paralyzed with TTX ($1 \mu\text{mol/L}$) and pretreated with IFN- γ (100 U/ml) for 72 hours to induce MHC class I. Neurons were triple labeled with antibodies directed against MHC class I (red), the neuronal cytoskeleton protein β -tubulin III (green) and the dendritic marker protein MAP2 (blue). MHC class I expression is detectable in axons and dendrites. Baseline labeling for MHC class I was revealed with control antibody (Control Ab). Scale bar, $20 \mu\text{m}$.

Results

Induction of MHC Class I on Neurites

In culture, neurons derived from hippocampi of C57BL/6 mice did not show constitutive expression of MHC class I on their plasma membrane. However, expression of MHC class I molecules on the cell membrane is inducible on most neurons via treatment with IFN- γ and is increased by concomitant blockade of neuronal activity with TTX.⁶ The MHC class I surface expression induced

by 72-hour treatment with IFN- γ and TTX was not restricted to the neuronal somata, but also detected on neurites (Figure 1). Triple labeling with antibodies against MHC class I, β -tubulin III and microtubule-associated protein-2 (MAP2) detected MHC class I molecules both on dendrites stained for the dendritic cytoskeleton protein MAP2 and on axons positive for the neuronal cytoskeleton protein β -tubulin III, but not the dendritic marker protein MAP2 (Figure 1). After having confirmed inducibility of MHC class I on axons

and dendrites we investigated whether T lymphocytes have the potential to induce neurite damage like that observed in viral and autoimmune inflammatory CNS disease.

Neurite Cytoskeleton Disruption by Cytotoxic T Lymphocytes

To analyze the interaction between cytotoxic T lymphocytes and neurites we propagated CTLs specific for the dominant peptide of LCMV envelope glycoprotein (GP33). These CTLs have a CD8, $\alpha\beta$ TCR phenotype and are highly specific for LCMV peptide GP33,⁶ presented in context of H-2D^b. When neurons were pulsed with peptide GP33 the CTLs rapidly attached to neuronal cell bodies and neurites. To study structural changes in the neurites following CTL attack, we fixed the cultures 3 hours after CTL attack and performed immunocytochemistry for β -tubulin III. At this time neuronal cell bodies did not show any cytoskeleton pathology. In striking contrast, the neurite cytoskeleton was selectively disrupted at distinct sites (Figure 2A). The neurites showed segmental cytoskeleton damage over a length of 3 to 6 μ m. Adjacent to the local lesions, cytoskeleton spheroids of 2 to 3 μ m in diameter developed, demonstrating accumulation of cytoskeleton proteins (Figure 2, B and C). In most cases the β -tubulin III labeling was completely lost at the lesioned site, indicating disruption of the cytoskeleton and transection of the neurite. The number of single cytoskeletal spheroids adjacent to segmental neurite disruption was determined following CTL attack. On average, localized neurite lesion sites with adjacent solitary neurite cytoskeleton spheroids were detected at a frequency of 0.85/neuron in cell cultures expressing MHC class I and pulsed with peptide GP33 at 3 hours after CTL attack (Figure 2D). These localized cytoskeleton changes were not detectable in neurons lacking MHC class I expression, or neurons pulsed with the influenza virus control peptide (Figure 2D).

Direct Cell Membrane Damage of Neurites by Cytotoxic T Lymphocytes

To confirm that CTLs can directly cause local transection of neurites, live interactions between CTLs and neurites were sequentially recorded using confocal laser scanning microscopy over a 60-minute time period. Neurons were pretreated with IFN- γ (100 U/ml) and TTX (1 μ M/L) to induce MHC class I expression and plasma membranes were visualized with the fluorescent dye, FM1-43. Approximately 2 to 5 minutes after contact of the CTLs with neurites presenting peptide GP33, structural alterations were observed in neurite membranes (Figure 3A). First, the neurites showed irregular labeling with the fluorescence dye at the point of contact, indicating beginning discontinuity of the membrane. Within 10 to 30 minutes of contact with the CTL, transection of neurites was detected by complete disruption of the plasma membrane (Figure 3A). Transection of neurites was directly

Figure 2. Neurite cytoskeletal damage at 3 hours after attack by LCMV peptide-specific CTLs. Neurites were immunolabeled with antibodies directed against β -tubulin III and LCMV peptide GP33-specific CTLs were added to neurons pretreated for 72 hours with IFN- γ and TTX and pulsed with LCMV peptide GP33-pulsed. **A:** The neuronal cell culture shows single cytoskeleton damage and transected neurite with adjacent spheroids at 3 hours after addition of LCMV peptide-specific CTLs. **B** and **C:** The cytoskeleton protein β -tubulin III is disrupted over a distance of approximately 3–5 μ m and is associated with a cytoskeleton spheroid formation adjacent to the localized lesion. **D:** The frequency of single localized cytoskeletal damage of neurites with adjacent spheroid per neuron was determined 3 hours after CTL attack of cultures pulsed with LCMV peptide GP33 (peptide GP33) or influenza nucleocapsid virus peptide (control peptide). Neuronal cells were either untreated or pretreated with IFN- γ and TTX for 72 hours to induce MHC class I (MHC I-induced). Frequency of localized lesion site 3 hours after CTL attack in neurons pre-treated with IFN- γ and TTX and pulsed with peptide GP33 was significantly ($P < 0.01$) different to neurons pulsed with the control influenza virus peptide. Scale bars: **A**, 10 μ m; **B** and **C**, 5 μ m. **D:** Data in **D** are presented as mean $\pm$ SEM of at least three independent experiments.

observed at the region of contact with the CTL. The CTLs then either detached or remained adhered to one of the neuritic ends. The separated neuritic ends showed increased membrane dye uptake and appeared to reseal. Localized membrane damage of neurites at the point of CTL contact was dependent on the presentation of the

Figure 3. Live imaging of MHC class I-restricted neurite transection by cytotoxic T lymphocytes. CTL-neurite interactions were visualized with the membrane dye, FM1-43, and continuously scanned by confocal laser scanning microscopy over 1 hour. Neuronal cells were pretreated with IFN- γ and TTX for 72 hours to induce MHC class I expression. **A:** Within 15 minutes, enhanced uptake of dye and structural damage of the neurite was observed at the point of contact with the CTL. Total transection with apparent resealing of the separate ends of the neurite occurred 25 minutes after establishment of CTL-neurite contact. Scale bar, 10 μ m. **B:** Percentage of transected neurites after interaction between cytotoxic T lymphocytes and neurons was determined. LCMV GP33-specific CTLs were added to the neurons, which were previously pulsed with LCMV peptide GP33 (peptide GP33) or influenza nucleocapsid virus peptide (control peptide). The frequency of transected neurites after CTL attack of neurons pulsed with peptide GP33 was significantly ($P < 0.05$) different from neurons pulsed with the control influenza virus peptide. Data in **B** are presented as mean $\pm$ SEM of at least three independent experiments.

LCMV peptide GP33. In detail, 16 of 34 ($47.1\% \pm 16.6\%$ SEM) individual CTL-neurite interactions observed in five independent experiments using confocal microscopy over a 60-minute period resulted in localized transection

of neurites as visualized by disruption of the membrane fluorescence dye (Figure 3B). Membrane damage with transection of neurites was detectable, on average, 25.3 minutes (± 14.9 SD) after establishment of CTL-neurite contacts. In contrast, the majority of CTLs did not form stable conjugates with neurites of cultures pulsed with the control peptide. Most CTLs detached from established CTL-neurite contacts within 5 to 10 minutes. Only 5 of 51 ($9.8\% \pm 2.6\%$ SEM) of CTL-neurite interactions caused minor membrane changes in the presence of the control influenza virus peptide (Figure 3B).

Discussion

These data demonstrate a segmental cytoskeleton and membrane damage of neurites following attack by MHC class I/peptide-restricted CD8⁺ CTLs. Importantly, this T lymphocyte attack is selective for individual neurites, not detectable at the cell body, and well before the onset of apoptotic cell death. This is in line with our previous observation that neuronal cell bodies are protected against perforin-mediated effects of CTLs, while apoptotic cell death mediated via Fas/CD95 molecules can occur hours later in susceptible neurons.⁶ Damage of neuronal processes by immune mechanisms has been studied previously using different approaches. In one such study, addition of purified CTL granule-derived granzyme A to a mouse neuronal cell line caused rapid retraction of neurites,²¹ an effect that could be due either to direct neurite damage or activation of thrombin receptors on neurons. Another report described killing of cell bodies and damage to neurites by alloreactive lymphocytes of dissociated sympathetic neurons.¹⁰ This study showed that activated allogeneic lymphocytes have the potential to induce degeneration of neurons and neurites of the peripheral nervous system within 10 to 24 hours. However it remained open, whether lymphocytes, either CD4 or CD8, acted on neurites via cell-cell contact, soluble mediators, or indirectly via glial cells.

Neurite damage could be caused either directly by a local injury or secondary to neuronal cell death. In the present study we monitored live interactions between CTLs and neurites by continuous imaging, thus allowing localization of membrane lesions opposite to the CTL-neuronal contacts. No sign of neurite damage was observed in non-stimulated, non-MHC expressing neurons following attack by CTLs. Neurite damage depended on the induction of appropriate MHC class I molecules on the neuronal membrane, and on the presence of specific antigenic peptides. This observation and the rapid development of membrane lesions within 0.5 hour are consistent with a perforin mediated lysis pathway exerted by CD8⁺ CTLs.

Interestingly, about 50% of the stable CTL-neurite conjugates showed complete transection of the membrane. Further, no repair of the transected neurite was observed during our live imaging experiments. Thus, it must be hypothesized that a round of "hits" from a single CTL interaction is sufficient to induce irreversible damage to a neurite. In principle, damage to neurites might be trig-

gered by soluble mediators rapidly released in response to recognition of MHC/peptide by CTLs. However, such characteristic localized damage of neuritis with spheroid formation has not been observed after tumor necrosis factor- α or IFN- γ treatment (unpublished observation). Further, damage to neurites was not observed in identical neuronal cultures challenged with autoreactive CD4⁺ T cells (unpublished observations).

This early cytotoxicity of T lymphocytes was restricted to neurites and was undetectable at the neuronal cell bodies, confirming our previous observation that neuronal cell bodies are protected against CTL mediated lysis.⁶ Further, we did not observe neurite damage in close proximity to the neuronal cell bodies. Distinct susceptibility to rapid CTL-mediated lysis between neuronal soma and neurites might be explained by regional differences of neuronal membranes and underlying cytoplasm. After neuronal polarization, axonal membranes and cytoskeleton become distinct from the rest of the cell.⁷ In particular, membrane receptors and their molecular links to the cytoskeleton²² may differ in a way that influence neurite/soma susceptibility to CTL-mediated cytoskeleton disruption, as revealed in our experiments. One obvious candidate would be MHC class I. However, expression of MHC class I was not found to be expressed differentially in the cell soma and neurites.

The finding that neurites are selectively damaged, while the cell soma is protected against CTL lysis, was somehow unexpected but might have emerged during evolution to limit the spread of viral and microbial organisms. It has been shown that microbes are transported along axons and dendrites as well as *trans*-synaptically, which can lead to fast and fulminant spread of infections.²³ Transection of infected neurites would efficiently limit transport and spread of microbes, while intact neuronal soma could survive to minimize loss of neurons, which have a very low regenerative capacity.

Our data that CTLs can transect neurites may have implications for understanding neuroinflammatory diseases. While neuronal cell bodies seem to be relatively protected against overt cytotoxicity in most neuroinflammatory processes, their neurites are often damaged during viral infections or CNS autoimmune diseases. For example, this pattern of axonal injury is observed in the Theiler's murine encephalomyelitis virus model.¹² In this model, the axonal damage was also dependent on MHC class I expression and more closely related to the severity of clinical disease than the degree of demyelination.

In MS, a demyelinating inflammatory disease with putative autoimmune pathogenesis, axonal disruption is a prominent feature of the plaque.^{16,17} Formation of axonal spheroids and bulbs, as they reflect axonal dissection in the MS lesion, are caused by accumulation of cytoskeletal proteins, either resulting from retracted neurites or interrupted cytoskeletal transport. Very similar segmental cytoskeleton changes of neurites (spheroid and bulb formations) were detected in our culture system after 3 hours, while membrane disruption was already obvious after 0.5 hour after specific CTL attack.

This raises the possibility that autoimmune CTL-mediated mechanisms may contribute to axonal damage

commonly seen in MS plaques.¹⁷ According to current dogma, MS is initiated and controlled by an autoimmune reaction against brain white matter.²⁴ This immune attack induces focal demyelinated areas, which might enable CTLs to get in contact with neurites. It was assumed that these brain autoimmune T cells assault myelin presenting cells, but spare neurons, which express neither MHC products nor myelin autoantigen. More recent work revealed, however, that neurons are indeed inducible to produce MHC class I molecules, especially in a functionally compromised state.^{4,5} Furthermore, several myelin proteins have been demonstrated in neurons. They include proteolipid protein²⁵ as well as golli-myelin basic protein.²⁶ Thus, in demyelinated, inflammatory brain lesions, the structural prerequisites for CTL²⁷ attack against MHC class I-induced axons, are given. It is tempting to speculate that the CD8⁺-enriched immune infiltrates co-localizing with axonal damage, as recently described in certain MS lesions, indeed involve CTL-mediated immune attacks.²⁸

In conclusion, involvement of cytotoxic CD8⁺ T lymphocytes in damage of neurites during neuroinflammatory diseases should be considered as a possible pathogenic mechanism having important consequences for therapeutic immune intervention.

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