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INFLUENCE OF INJURY SEVERITY ON THE RATE AND MAGNITUDE OF THE T LYMPHOCYTE AND NEURONAL RESPONSE TO FACIAL NERVE AXOTOMY

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Abstract

The temporal relationship between severity of peripheral axonal injury and T lymphocyte trafficking to the neuronal cell bodies of origin in the brain has been unclear. We sought to test the hypothesis that greater neuronal death induced by disparate forms of peripheral nerve injury would result in differential patterns of T cell infiltration and duration at the cell bodies of origin in the brain and that these measures would correlate with the magnitude of neuronal death over time and cumulative neuronal loss. To test this hypothesis, we compared the time course of CD3⁺ T cell infiltration and neuronal death (assessed by CD11b⁺ perineuronal microglial phagocytic clusters) following axonal crush versus axonal resection injuries, two extreme variations of facial nerve axotomy that result in mild versus severe neuronal loss, respectively, in the facial motor nucleus. We also quantified the number of facial motor neurons present at 49 days post-injury to determine whether differences in the levels of neuronal death between nerve crush and resection correlated with differences in cumulative neuronal loss. Between 1-7 days post-injury when levels of neuronal death were minimal, we found that the rate of accumulation and magnitude of the T cell response was similar following nerve crush and resection. Differences in the T cell response were apparent by 14 days post-injury when the level of neuronal death following resection was substantially greater than that seen in crush injury. For nerve resection, the peak of neuronal death at 14 days post-resection was followed by a maximal T cell response one week later at 21 days. Differences in the level of neuronal death between the two injuries across the time course tested reflected differences in cumulative neuronal loss at 49 days post-injury. Altogether, these data suggest that the trafficking of T cells to the injured FMN is dependent upon the intensity of peripheral nerve injury and associated neuronal death.

Keywords

facial nerve axotomy; crush; resection; T cells; microglial phagocytic clusters; neuronal death

Introduction

The effect of T cells in the central nervous system (CNS) appears to be context-dependent, as their presence has been shown to promote neuronal survival following certain types of injuries or, conversely, contribute to CNS pathology, such as in experimental autoimmune encephalomyelitis (EAE) and infection (Byram et al., 2004; Martino and Hartung, 1999; Nau and Bruck, 2002; Schwartz, 2001; Serpe et al., 1999). Peripheral transection of the facial nerve

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in adult mice induces retrograde neuronal cell loss in the facial motor nucleus (FMN) that is accompanied by a site-specific infiltration of T cells across an intact blood-brain-barrier to the injured motor neurons (Moran and Graeber, 2004; Raivich et al., 1998). Although trafficking of T cells to the injured FMN has been assumed to depend on injury severity, findings from independent studies are conflicting. On the one hand, the presence of T cells has been shown to be associated with substantial neuronal death in some studies, while others have reported significant T cell trafficking following injury despite minimal neuronal death (Galiano et al., 2001; Ha et al., 2007b; Ha et al., 2006; Raivich et al., 2003; Raivich et al., 2002). Differences in animal models and methodology used in these studies may contribute to the disparate findings. Understanding the significance of T cells in relation to neuronal death may thus be critical to gain further insight regarding the bi-directional effects of T cells in the injured CNS.

Although the time course of T cell accumulation in the FMN following facial nerve transection had been studied (Raivich et al., 1998), it was unknown how the T cell response would be altered by other forms of facial nerve axotomy that vary in injury severity. Thus, the present study was designed to address whether the degree of neuronal cell death induced by the severity of peripheral nerve axotomy influences the T cell response in the injured CNS. To address these issues, we used two extreme variations of the facial nerve axotomy model, nerve crush and resection, to compare injuries under well-controlled conditions that are of the same nature (i.e., mechanical lesion) but that differ in their severity. In contrast to nerve crush, where injured axons readily regenerate within a neural sheath that remains intact and the majority of neurons survive, facial nerve resection results in the removal of a portion of the nerve, leading to severe neuronal atrophy and loss (Guntinas-Lichius et al., 1994; Kuzis et al., 1999; Moran and Graeber, 2004). A comparison of the T cell response between these two injuries, which differ considerably in their capacity for nerve regeneration and in the extent of neuronal damage, enabled us to examine T cell trafficking in relation to disparate levels of neuronal degeneration. We tested the hypothesis that the differences in the level of neuronal injury induced by facial nerve crush versus resection would be associated with changes in the rate of accumulation and magnitude of the T cell response in the facial motor nucleus (FMN). Comparisons were made across time from 1 to 49 days post-injury in the FMN of C57BL/6 mice given facial nerve crush or resection and the number of CD3⁺ T cells and CD11b⁺ microglial phagocytic clusters, which represent dead or dying neurons, were quantified. In addition, we quantified the number of neurons at 49 days post-injury in both injury groups to determine whether differences in the levels of neuronal death at the various time points tested reflect cumulative neuronal loss over time.

Materials and Methods

Animals

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice used in this study were cared for in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were housed under specific pathogen-free conditions in individual microisolator cages.

Animal Surgery

Mice were 8-weeks-old at the time of surgery. Animals were anesthetized with 4% isoflurane. For nerve crush injury, the right facial nerve was exposed and the main branch of the facial nerve was crushed for 10 seconds using a fine-tipped hemostat, as described previously (Fendrick et al., 2005). For nerve resection injury, a portion of the main branch of the facial nerve was removed to prevent nerve reconnection. We assessed the whisker response of all mice immediately after surgery to ensure complete whisker paralysis. Mice were sacrificed at 1, 3, 5, 7, 14, 21, 28, or 49 days post-injury. Prior to sacrifice, each mouse was assessed for

functional recovery, by comparing the whisker response of the injured side to the uninjured, contralateral side. For euthanasia, mice were anesthetized by intraperitoneal injection of a 0.5 mg/ml ketamine cocktail (ketamine/xylazine/acepromazine given in a 3:3:1 ratio) and were perfused with 4% paraformaldehyde. Brains were collected, post-fixed in 4% PF for 2 hours at room temperature, and cryoprotected in 30% sucrose overnight at 4°C. Using the ambiguous nucleus and the facial nerve root as the starting and ending points, respectively, 15 µm coronal sections were cut throughout the caudal-rostral extent of the facial motor nucleus. Sections were collected on Superfrost/Plus slides (Fisher Scientific) and stored at -80°C.

Light microscopic immunohistochemistry

Tissue sections were incubated in normal goat serum (NGS) (Vector) for 1 hour at room temperature followed by overnight incubation with anti-mouse CD3 (17A2; PharMingen) or anti-mouse CD11b (5C6; 1:500, Serotec) at 4°C. Sections were washed in 1 × PBS after each incubation step. Visualization of the primary antibodies was performed by incubation of sections in goat anti-rat secondary antibody (1:2000, Vector Labs) for 1 hour at room temperature followed by incubation in avidin-peroxidase conjugates (1:500, Sigma) for 1 hour. No signal was obtained with each of the primary or secondary antibodies alone. The chromagen reaction was revealed by incubation in 3,3'-diaminobenzidine (DAB)-H₂O₂ solution (Sigma; 0.07% DAB/0.004% H₂O₂). Sections were counterstained with cresyl violet, dehydrated in ascending alcohol washes, cleared in xylenes, and coverslipped.

Quantification and statistical analysis

The number of CD3⁺ T cells or CD11b⁺ microglial phagocytic clusters was quantified in sections throughout the FMN by an experimenter under blind conditions. Eight sections per mouse (approximately 1/5 of the entire FMN) were used to assess each variable. As statistical analysis revealed that the distribution of the data was not normal, Spearman's rank correlation analysis was used to determine the relationship between T cells and neuronal death (as measured by the number of microglial phagocytic clusters). For neuronal cell counts, facial motor neurons were quantified in 8 representative sections of the FMN, as described previously (Deboy et al., 2006; Ha et al., 2007a). The medial nucleus, which receives innervation from the auricular branch of the facial nerve, remained uninjured and thus was excluded from the analysis. Cell counts were expressed as percentage of neurons lost [% neuronal loss = (neurons in uninjured FMN - neurons in injured FMN)/neurons in uninjured FMN]. Analysis of variance (ANOVA) was used to make statistical comparisons.

Results

Effect of injury severity on kinetics of T cell response and neuronal death

In Figure 1, the time course of the T cell and neuronal response to facial nerve crush and resection is shown. The levels of neuronal death (dashed line) in the FMN for both treatment groups was not apparent at day 1 post-injury but increased to less than one microglial phagocytic cluster/section between days 3-7 post-injury. T cell infiltration (solid line) to the FMN in both treatment groups was minimal (<1 T cell/section) by day 1 post-injury and showed comparable increases of 3-4 T cells/section by day 7 post-injury. T cell infiltration in the contralateral, uninjured FMN across all time points was negligible, with an average of less than 0.1 cells per 15 µm section. Differences in the rate of accumulation and magnitude of the T cell response became apparent by 14 days post-injury, when the levels of neuronal death reached their peak in both treatment groups. The number of CD11b⁺ microglial phagocytic clusters reached a maximal 0.5/section following facial nerve crush (open circles, dashed line) and coincided with a peak number of 6.5±0.27 CD3⁺ T cells/section (open circles, solid line). By contrast, facial nerve resection induced a peak number of 2.54±0.45 CD11b⁺ microglial phagocytic clusters/section (closed circles, dashed line) by 14 days post-injury that was

followed by a maximum number of 26.1 ± 2.29 CD3⁺ T cells/section (closed circles, solid line) one week later at 21 days. Interestingly, there was a second increase in the T cell response between 21 and 28 days post-crush, where the average number of T cells rose from 1.63 ± 0.85 cells/section at 21 days to 4.10 ± 1.57 cells/section at 28 days. By 49 days post-injury, the rate of neuronal death was decreased in both treatment groups and the number of T cells in the FMN declined to 0.19 ± 0.06 cells/section (crush) and 2.50 ± 0.78 cells/section (resection). Photomicrographs of the peak levels of neuronal death and T cells following facial nerve crush and resection are shown at their respective time points in Figure 2. In Figure 3A, Spearman's rank correlation coefficient (ρ) revealed that the number of CD11b⁺ microglial phagocytic clusters was highly correlated with the number of CD3⁺ T cells when mice from all time points and both treatment groups were combined ($\rho=0.68$, $p<0.01$). Similar significant p -values were obtained for nerve crush (Fig. 2B, $\rho=0.57$, $p<0.01$) and nerve resection (Fig. 2C, $\rho=0.74$, $p<0.01$) alone.

Neuronal cell loss and functional recovery following facial nerve crush and resection

As seen in Figure 4E, neuronal cell loss at 49 days post-injury was significantly greater in mice that received facial nerve resection compared to nerve crush [$F(1,4)=20.853$, $p<0.05$]. Photomicrographs of representative Nissl-stained sections of the injured and uninjured FMN following facial nerve crush and resection are shown in Figures 4A-4D. Functional recovery of the whisker response was observed in mice between 14-21 days post-crush but remained impaired in resected mice (data not shown).

Discussion

In the current study, we demonstrated that the magnitude and rate of T cell accumulation in the injured FMN were influenced by differences in the level of neuronal death induced by facial nerve crush and resection, two forms of mechanical injury to the facial nerve that result in mild and severe neuronal loss, respectively. Interestingly, T cell and neuronal death responses were comparable in magnitude and followed a similar time course between 1-7 days post-injury, regardless of injury severity. Consistent with our hypothesis, greater T cell trafficking to the injured FMN was associated with higher levels of neuronal death, as demonstrated by the accumulation of microglial phagocytic clusters, between 14-21 days following facial nerve resection. By contrast, facial nerve crush induced minimal neuronal death that was accompanied by fewer numbers of T cells. Although Raivich et al. (1998) found an elevated plateau in the T cell response between 2-4 days that was followed by a more prominent peak at 14 days following facial nerve transection, we were unable to detect an early plateau in the T cell response in either nerve crush or resection injuries, possibly due to the limited number of early time points assessed.

Greater T cell trafficking to the injured FMN was associated with increased neuronal death and cumulative neuronal loss in mice that received facial nerve resection. Consistent with this relationship, in a previous study comparing the T cell and neuronal death response following facial nerve transection in two inbred congenic strains of mice, we showed that greater T cell trafficking to the injured FMN was associated with the strain exhibiting greater neuronal death at 14 days post-injury (Ha et al., 2006). That greater T cell infiltration to the injured FMN might exacerbate neuronal cell death is unlikely, as we and others have shown that differences in the magnitude of the T cell response in the injured FMN were not associated with altered levels of neuronal death or long-term neuronal loss (Galiano et al., 2001; Ha et al., 2007b; Huang et al., 2007; Raivich et al., 2003; Raivich et al., 2002). Here, we found that the number of T cells correlated with the number of microglial phagocytic clusters, suggesting that the T cell response to neuronal death is a graded event. The mechanisms that underlie this site-specific trafficking of T cells to the injured brain appear to involve the time-dependent expression of cytokines

and cell adhesion molecules by injured neurons and microglial phagocytic clusters (Raivich et al., 1998). The increased presence of microglial phagocytic clusters following facial nerve resection may facilitate the robust T cell response in the injured FMN. However, the correlation between the levels of neuronal death and T cells is reduced by the second increase in the T cell response observed at 28 days post-crush, which occurs in the absence of notable neuronal cell death. Interestingly, a comparison of findings from the current study with that of Raivich et al. (1998) revealed that the number of T cells in the injured FMN following facial nerve transection in C57BL/6 mice, while higher than the response following nerve crush, was more comparable to levels induced by resection. Facial nerve transection results in nerve severing with a potential for reconnection to occur and produces neuronal loss that is considered intermediate to that induced by facial nerve crush and resection (Moran and Graeber, 2004; Raivich et al., 2004). While it would be expected that facial nerve transection would induce a T cell response that is also intermediate in magnitude to that induced by facial nerve crush and resection, it is plausible that the two injuries would induce T cell responses of similar magnitude, as nerve resection and transection involve mechanical shearing of the neural sheath. Future comparisons which include graded degrees of injury may be useful to further characterize the T cell response to neuronal injury.

The differences in the timing of the peak T cell response in relation to the peak of neuronal death between facial nerve crush and resection suggest that parameters of the T cell response may be directed by factors in addition to the level of neuronal cell death. The maximal T cell response coincided with peak levels of neuronal death at 14 days post-crush and is consistent with the time course previously described following nerve transection (Raivich et al., 1998). By contrast, for facial nerve resection, we found that the maximal T cell response occurred at 21 days post-injury, one week following the peak of neuronal death. Moreover, we observed significant T cell infiltration in the injured FMN following facial nerve crush despite the presence of few microglial phagocytic clusters, which is consistent with other findings where T cells have been found to traffic to the injured FMN despite the absence of neuronal death (Raivich et al., 2003). It is interesting to speculate that the time course of the T cell response in the injured FMN may partly be dependent on the ability of the nerve to reconnect. Previously, it was shown that the decreased expression of NeuN in the FMN following facial nerve crush was transient in mice, where the return of NeuN expression coincides with the time when mice generally show a return of whisker function (McPhail et al., 2004b). That this loss of neuronal phenotype failed to return in mice that received facial nerve resection was suggested to be related to the inability of the nerve to reconnect. In the current study, the decline in the T cell response in the injured FMN between 14-21 days post-crush appears to be associated with the time when mice gain functional recovery of the whisker response. By contrast, the persistence of T cells in the injured FMN, as observed in this study for resection and in the transection studies by Raivich et al. (Raivich et al., 2004; Raivich et al., 1998), may be related to the absence of recovery due to permanent nerve disconnection or a prolonged recovery period, as in the case with nerve transection.

It is important to note that overall neuronal death (as assessed by the number of microglial phagocytic clusters across all time points) correlated with cumulative neuronal loss that occurred long-term, as more cell death observed following facial nerve resection was associated with greater neuronal loss at 49 days post-resection, compared to nerve crush. Interestingly, the percentage of neurons taken up by phagocytic microglia could not account for the larger percentage of neurons lost at 49 days post-injury. With an average of 1700 neurons counted in the uninjured contralateral FMN of our mice, cumulative neuronal loss by phagocytic microglia was approximately 4% and 16% following nerve crush and resection, respectively, while neuronal loss quantified by Nissl stain at 49 days post-injury was approximately 8% and 58% following nerve crush and nerve resection, respectively. The disparity between the two measures of neuronal loss may be due to insufficient sampling of

the number of microglial phagocytic clusters, as all post-injury time points were not accounted for. Alternatively, studies by McPhail et al. (2004a) suggest that following facial nerve resection, the majority of injured neurons may reside in a shrunken and atrophic state, rendering them undetectable by Nissl staining. Re-injuring the facial nerve resulted in a reversal of neuronal atrophy such that the number of countable neurons increased to 79% of the contralateral side. It is noteworthy that their finding translates to an actual loss of only 21%, which more closely matches the loss accounted for by phagocytic microglia in our study and provides further evidence that microglial phagocytic clusters may serve as an informative and conservative marker of neuronal death.

In conclusion, our data demonstrate that the rate of accumulation and magnitude of T cells in the injured FMN is related to the level of neuronal death. Further understanding of the mechanisms by which T cells respond to different forms of neuronal injury may be useful in elucidating their role in CNS degeneration and injury.

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Figure 1.

Temporal relationship between infiltrating CD3⁺ T cells (solid lines) and the number of CD11b⁺ microglial phagocytic clusters (dashed lines) in the FMN following facial nerve resection (●) and crush (○). For the T cell curve, each point represents the mean±S.E.M. of 4 mice/treatment group except for days 5, 7, and 21 in the resection group, where n=3/time point. For the neuronal death curve, each point represents the mean±S.E.M. of 4 mice/treatment group except for day 21 in the crush group (n=2) and days 1, 5, 7, and 21 in the resection group, where n=3/time point.

Figure 2.

Photomicrographs of the T cell and neuronal death response, as demonstrated by the presence of microglial phagocytic clusters, at their respective peak levels in the FMN following facial nerve crush (A&C) or resection (B&D). Scale bar = 50 μ m (A&B), 10 μ m (C&D).

Figure 3.

Spearman's rank correlation analysis between the number of CD3⁺ T cells and CD11b⁺ microglial phagocytic clusters for both treatment groups combined (A), crush only (B), and resection only (C). Each graph represents data pooled across all time points. Spearman's rank correlation coefficients (p) are indicated and are significant in all cases ($p < 0.01$).

Figure 4. Photomicrographs of the uninjured and injured FMN following facial nerve crush (A-B) or resection (C-D) at 49 days post-injury. Quantitative cell counts are shown in E (n=3/group, *p<0.05). Scale bar = 100μm.