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Increase in inflammatory cytokines in median nerves in a rat model of repetitive motion injury

Talal Al-Shatti^a, Ann E. Barr^b, Fayez F. Safadi^c, Mamta Amin^b, and Mary F. Barbe^{b,c,*}
aKuwait University, Faculty of Allied Health Sciences, P.O. Box 31470, Sulaibekhat, Kuwait 90805, Kuwait

bDepartment of Physical Therapy, Temple University, 3307 North Broad St., Philadelphia, PA 19140, USA

cDepartment of Anatomy and Cell Biology, Temple University Medical School, 3400 North Broad St., Philadelphia, PA 19140, USA

Abstract

We examined cytokines in rat median nerves following performance of a high repetition reaching and grasping task at a rate of 8 reaches/min for up to 8 weeks. IL-1 α , IL-1 β , TNF- α , IL-6 and IL-10 were analyzed by immunohistochemistry. Double-labeling immunohistochemistry for ED1, a marker of phagocytic macrophages, was also performed. We found increased immunoexpression of IL-6 by week 3, increases in all 5 cytokines by week 5. This response was transient as all cytokines returned to control levels by 8 weeks of performance of a high repetition negligible force task. Cytokine sources included Schwann cells, fibroblasts and phagocytic macrophages (ED1-immunopositive). These findings suggest that cytokines are involved in the pathophysiology of repetitive motion injuries in peripheral nerves.

Keywords

IL-1; IL-6; TNF- α ; IL-10; Work-related musculoskeletal disorder; Cumulative trauma disorder; Median nerve

1. Introduction

Repetitive motion injuries are the result of prolonged repetitive, forceful, or awkward movements (Bernard, 1997; National Research Council, 2001). These disorders are a subset of work-related musculoskeletal disorders. Repetitive motion injuries of the wrist and hand pose a significant source of worker pain and discomfort, potential long-term disability and high economic toll (National Research Council, 2001).

Our laboratory has investigated the pathophysiology of repetitive motion injuries of the upper limb caused by voluntary high repetition tasks, with or without force (Barbe et al., 2003; Barr et al., 2003, 2004; Clark et al., 2003, 2004; Barr and Barbe, 2004). Using a novel rat model, we found that repetitive reaching and grasping causes a marked and widespread increase in recruited macrophages in musculotendinous and skeletal tissues as well as in the median nerve at the level of the carpal tunnel. We observed further signs of trauma to the median nerve in

*Corresponding author. Department of Physical Therapy, College of Health Professions, Department of Anatomy and Cell Biology, Temple Medical School, Temple University, 3307 North Broad St., Philadelphia, PA 19140, USA. Tel.: +1 215 707 4896; fax: +1 215 707 7500. E-mail address: mary.barbe@temple.edu (M.F. Barbe).

the form of decreased nerve conduction velocity, the onset of myelin degradation and epineural fibrosis following performance of these tasks for 9-12 weeks.

Neural trauma has been shown to result in endoneurial production of cytokines and their mRNA, including IL-1 α , IL-1 β , TNF- α , IL-10 and IL-6 (Bolin et al., 1995; Reichert et al., 1996; Be'eri et al., 1998; Sommer and Schäfers, 1998; George et al., 1999; Shubayev and Myers, 2000; Taskinen et al., 2000; Deprez et al., 2001; Shamash et al., 2002). Several of these cytokines are present in intact, normal peripheral nerves but are upregulated after injury or irritation (Bourde et al., 1996; Reichert et al., 1996). The production of TNF- α and IL-1 following nerve injury is a critical determinant of later sequelae of injury and inflammation in peripheral nerves, particularly because of the key roles of these cytokines in Wallerian degeneration and mediation of inflammatory pain (Sommer and Schäfers, 1998; George et al., 1999; Okamoto et al., 2001; Milligan et al., 2003). IL-6 is a neuropoietic cytokine that plays a role in peripheral nerve regeneration (Hirota et al., 1996) and neuronal survival (Thier et al., 1999; Otten et al., 2000), as well as opposing roles such as peripheral nerve degeneration and neuronal cell death (Gadient and Otten, 1997), and the initiation and maintenance of mechanical allodynia and chronic pain (Arruda et al., 2000; Milligan et al., 2003). These myriad functions are mediated by complexes formed by IL-6, either its membrane-bound or soluble receptor (IL-6R or sIL-6R), and a signal-transducing receptor component, glycoprotein 130 (gp130), that then activate components of the JAK/STAT pathway (Otten et al., 2000; Rose-John, 2003). IL-10 is a multifunctional cytokine with diverse effects on a variety of cell types. In general, it serves to down-modulate inflammatory responses by inhibiting cytokine production by activated macrophages (including intraneural macrophages), monocytes and other cell types (de Waal Malefyt et al., 1991; Wagner et al., 1998; Taskinen et al., 2000; Moore et al., 2001).

In the present study, we wanted to examine both pro-inflammatory and putatively immunodown-modulatory cytokines in peripheral nerves in our model. Therefore, we have examined cytokine production in median nerves following performance of a voluntary high repetition reaching and grasping task with negligible force (HRNF). We found increased immunoexpression of a number of pro-inflammatory cytokines (IL-1 α , IL-1 β , TNF- α , and IL-6) in median nerves from HRNF rats, with the response peaking at 5 weeks of task performance. We also examined the median nerves for immunoexpression of IL-10, a key anti-inflammatory protein. Our observed increase in pro-inflammatory cytokines returned to control levels by 8 weeks of task performance. These results show that repetitive tasks result in a transient inflammatory response in peripheral neural tissues.

2. Material and methods

2.1. Experimental animals

Studies were conducted on young, female Sprague-Dawley rats (285-310 g), which were housed in the central animal facility in separate cages in a 12-h light: dark cycle with free access to water. In order to motivate them to perform a task for a food reward, these animals were food-restricted and maintained at 80-90% of the body weight of age-matched, non food-restricted rats. Rats were weighed weekly and food adjusted accordingly. Water was given ad libitum. All experiments were approved by the Temple University Institutional Animal Care and Use Committee in compliance with NIH guidelines for the humane care and use of laboratory animals.

A total of 33 female, 12-14 week old Sprague-Dawley rats were used. Nineteen of them were trained to perform a high repetition, negligible force (HRNF) reaching and grasping task with the forelimb for 3 weeks ($n = 5$), 5 weeks ($n = 6$), or 8 weeks ($n = 8$). Fourteen additional rats that did not participate in the task were used as an aged-matched control group. Four of these

control rats went through the shaping steps (described below), but did not proceed to the task regimen. These latter rats are considered shaped-only control rats. The remaining control rats are considered normal, control rats.

2.2. Behavioral apparatus and repetitive movement task

The rats were placed in operant test chambers for rodents (Med. Associates, VT) with a portal located in one end. The portal was fitted with a 1.5 cm wide tube that sloped downward 10° with respect to the chamber floor and was located at the animal's shoulder height. The tube was 2.5 cm in length so that the elbow had to be fully extended in order for the animal to reach pellets of food. Food pellets (45 mg; Biosource) were dispensed (Pellet dispenser, Med. Associates) every 15 s (high repetition) during the reach task session. An auditory indicator (Stimulus clicker, Med. Associates) signaled that a pellet had been dispensed, thereby cueing the animal to attempt a reach. Animals reached with imperfect accuracy, and so made repeated attempts to retrieve a pellet. This resulted in an actual reach rate of 8.27 reaches/min $\pm$ 4.11 SD (Barbe et al., 2003). Further details of the training apparatus are described in Barbe et al. (2003), Barr et al. (2003) and Clark et al. (2003).

Briefly, the experimental rats learned to reach for the food during an initial 10-12 day shaping period. During this period, the rats were first encouraged to reach through open bars for food pellets placed on an elevated platform for 5 min/day. When they began to reach freely for the food, they were transferred to the test chamber until they could reach into the tube dispenser with no specified reach rate for 10-20 min/day. When they were able to perform the task consistently, the experimental animals were begun on the task regimen for 2 h/day, 3 days/week for 3-8 weeks. The daily task was divided into 4, 0.5-h training sessions separated by 1.5 h. Rats were allowed to use their preferred limb to reach, thereafter referred to as the "reach limb".

The non-reach limb was not examined in this study. Also, 1 of the 9 rats that had performed the task for 5 weeks and 3 of the 11 rats that had performed the task for 8 weeks were not included in the tissue analyses of the reach limb median nerves because they demonstrated ambidexterity either by using both limbs from the onset of the experiments, or by switching to more frequent use of the non-reach limb after several weeks of task performance (perhaps due to increased discomfort). Nerves from these latter limbs were not examined due to the difficulty in interpreting their findings.

2.3. Immunohistochemical analyses

In order to examine median nerves for the cytokine changes, immunohistochemical analysis was performed on tissues collected from 10 normal, control rats and 4 shaped-only control rats, as well as 14 rats that had performed the HRNF task for 3 weeks ($n = 5$), 5 weeks ($n = 5$), and 8 weeks ($n = 4$). Following euthanasia, animals were perfused transcardially with 4% paraformaldehyde in PO₄ buffer. The forearms were post-fixed en bloc in the same fixative for 2-3 days. The flexor forelimb mass with median nerve intact was dissected from the forearm, equilibrated for 3 days in 30% sucrose in phosphate buffered saline (PBS) for cryoprotection, and then stored at -80 °C.

The forelimb tissues were sectioned into 16 μ m longitudinal slices, and mounted onto coated slides (Ultra-stick, Corning). Tissue sections, on slides, containing median nerve at the level of the carpal tunnel were treated with 3% H₂O₂ in methanol for 30 min (omitted for fluorescent staining), digested with 0.5% pepsin in 0.01 N HCl for 20 min at room temperature and then incubated with goat serum (4%) in PBS for 30 min at room temperature to prevent nonspecific staining. Sections were incubated over-night at room temperature with one of the following primary antibodies from Chemicon diluted with 4% goat serum in phosphate buffered saline

(PBS): anti-IL-1 α (Chemicon, #AB1415, 1:250), anti-IL-1 β (Chemicon, #AB1832P, 1:250), anti-TNF- α (Chemicon, #AB1837P, 1:300), anti-IL-6 (Chemicon, #AB1833P, 1:250), and anti-IL-10 (Chemicon, #AB1492, 1:250). Negative controls testing specific immunoreactivity included omission of the primary anti-bodies. After washing, all sections were incubated for 2 h at room temperature in the appropriate secondary antibodies conjugated to HRP, Cy2 (green), or Cy3 (red) (Jackson Immuno) that had been diluted 1:100 in PBS. HRP, when used as the conjugate, was visualized as a brown immunoreactive stain using FAST Sigma diaminobenzidine (DAB).

The sections for fluorescence double-labeling were further probed following cytokine staining for the presence of phagocytic macrophages using anti-ED1 (marker for a lysosomal membrane protein in monocytes/macrophages, Chemicon, #MAB1435, 1:250). After washing, the sections were incubated with anti-ED1 overnight at room temperature. The sections were again washed and then incubated with the appropriate secondary antibody diluted 1:100 in PBS for 2 h, washed and coverslipped with 80% glycerol in PBS before visualization using an epi-fluorescent microscope.

2.4. Bioquantification of immunohistochemistry

To quantify the changes in IL-1 α , IL-1 β , TNF- α and IL-6 immunoreactive product in the median nerve with task performance, HRP-DAB stained slides were quantified using a microscope (Nikon) interfaced with an analog camera and a bioquantification software system (Bioquant TCW 98, Nashville, TN). The assessment and analysis of the data was carried out in a blinded fashion. Prior to acquisition, the camera was white-balanced to ensure that uniform back-ground color was maintained. The microscope's light intensity was maintained at a constant level to ensure the background values were similar for each acquired image. Likewise, the f-stop for the camera was maintained at a constant level for each acquired image. While 3-5 sections per rat forelimb that contained the median nerve at or near the level of the wrist were stained for each cytokine, only sections that actually contained the median nerve at the point that it crossed the carpal tunnel were chosen for analysis, as described in Clark et al. (2003,2004). Measurements of the median nerve were made at the point that it spanned the carpal tunnel, as well as immediately distal and proximal to the tunnel (<1 mm distal or proximal), as described in Clark et al. (2003,2004). Three different microscope fields were measured per nerve, in 2-3 sections per nerve and per cytokine. While these criteria limited the number of nerve sections that could be analyzed for each cytokine and rat, and were particularly limiting due to the extremely small height of the rat median nerve (forelimb tissues were sectioned longitudinally), these criteria ensured consistency in the region of nerve measured in each rat. Measurements were made only within the boundaries of the sectioned median nerve and surrounding epineurium in a 0.0768 mm² or smaller field (as shown in Clark et al., 2003, and in Fig. 1F) using the Irregular Region of Interest Option (ROI) of the Bioquant software. A field size was chosen that excluded tendons and synovial tissues also present in the tunnel (See Fig. 1F). The Videocount Area Array option of the Bioquant software was also utilized for these measurements. Videocount area is defined as the number of pixels in a field that meet a user-defined color threshold of staining multiplied by the area of a pixel at the selected magnification ($\times 40$ in our analyses). In this case, color threshold values were selected based on mean levels of brown HRP-DAB immunostaining for each cytokine. The threshold values for each cytokine were stored in the computer program for consistent auto-measurement of the immunostained slides. Percent area fractions of immunoreaction product for each cytokine were calculated by dividing the videocount area containing pixels at or above the defined background threshold by the videocount area of the total number of pixels in the chosen field (ROI), and multiplying by 100. Again, this determination was made at three locations along the median nerve immediately proximal, distal and within the carpal tunnel. Group means

plus standard error of the mean (SEM) of the percent area fraction ($n = 3-9/\text{group}$) were plotted against week of task exposure.

2.5. Statistical analyses

Univariate ANOVAs were used to determine whether week of task performance had any effect on the area fraction of IL-1 α , IL-1 β , TNF- α and IL-6 immunoreactive product in the median nerve at the wrist. A p value ≤ 0.05 was considered significant. Microscope field (3 observations/nerve) was used as a blocking factor in the ANOVA. Post hoc analyses were carried out using the Bonferroni method for multiple comparisons in which all HRNF weeks were compared to week 0, and the p values were adjusted accordingly.

3. Results

3.1. Cytokine immunoexpression increases in the median nerve with task exposure

3.1.1. IL-1 α —Immunoexpression of IL-1 α increased with performance of the HRNF task (Figs. 1A, 2A,B and 4A). The ANOVA results of the immunohistochemical quantification of median nerves at the level of the carpal tunnel showed a significant difference in the percent area fraction of IL-1 α immunoreactivity across weeks ($p = 0.0407$). As shown in Fig. 1A, Bonferroni post-hoc analyses showed that the percent area fraction of IL-1 α in nerves at week 5 ($n = 3$) was significantly increased when compared to controls ($p = 0.0055$; $n = 3$), but not in week 3 ($p = 0.3342$; $n = 3$) or week 8 ($p = 0.1388$; $n = 4$) when compared to control rats.

3.1.2. IL-1 β —Immunoexpression of IL-1 β increased with performance of the HRNF task (Figs. 1B, 2C,D and 4D). The ANOVA results of the immunohistochemical analyses of median nerves at the level of the carpal tunnel showed a significant difference in the percent area fraction of IL-1 β immunoreactivity across weeks ($p = 0.0091$). As shown in Fig. 1B, Bonferroni post-hoc analysis showed that the percent area fraction of IL-1 β in week 5 nerves ($n = 5$) was significantly increased when compared to control nerves ($p = 0.0021$; $n = 9$), but not in week 3 ($p = 0.0676$; $n = 5$) or week 8 ($p = 0.9581$; $n = 4$) when compared to control nerves.

No differences in IL-1 β immunohistochemical expression were observed when comparing normal control rats to shaped-only control rats ($p = 0.6189$).

3.1.3. TNF- α —Immunoexpression of TNF- α increased with performance of the HRNF task (Figs. 1C, 2E,F and 4G). The ANOVA results of the immunohistochemical quantification of median nerves at the level of the carpal tunnel showed a significant difference in the percent area fraction of TNF- α immunoreactivity across weeks ($p = 0.0061$). As shown in Fig. 1C, post-hoc analysis showed that the percent area fraction of TNF- α in week 5 ($n = 4$) was significantly increased when compared to control nerves ($p = 0.0012$; $n = 6$), but not in week 3 ($p = 0.5453$; $n = 4$) or week 8 ($p = 0.9369$; $n = 4$) when compared to control nerves. No differences in TNF- α immunohistochemical expression were observed when comparing normal control rats to shaped-only control rats ($p = 0.3845$).

3.1.4. IL-6—As with the other cytokines examined, immunoexpression of IL-6 increased with performance of the HRNF task (Figs. 1D and 2G,H). The ANOVA results of the immunohistochemical analyses of median nerves at the level of the carpal tunnel showed a significant difference in the percent area fraction of IL-6 immunoreactivity across weeks ($p = 0.0005$). As shown in Fig. 1D, post hoc analysis shows that the percent area fraction in week 3 ($n = 4$) and week 5 ($n = 3$) were significantly increased when compared to control nerves ($p = 0.0007$ and 0.0006 , respectively), but not in week 8 ($p = 0.4096$; $n = 3$) when compared to control nerves ($n = 8$).

3.1.5. IL-10—Immunohistochemical staining of nerves showed that IL-10 expression by macrophages increased in median nerves at the level of the carpal tunnel at 5 weeks of task performance (Fig. 3). Unfortunately, the immunohistochemistry for IL-10 could not be quantified due to insufficient amounts of median nerve tissue that matched the criteria for quantification described in the methods section. The increased IL-10 immunohistochemistry was observed in the median nerves of three 5-week HRNF rats, at the level of the wrist. Two of these rats are shown in Fig. 3B-E.

3.2. Macrophages, Schwann cells and intraneural fibroblasts are primary producers of cytokines in this nerve injury model

To identify the cell types that were immunoreactive for cytokines, single-labeling immunoperoxidase immunohistochemistry and double-labeling immunofluorescence was performed on median nerves collected from the carpal tunnel regions of control rats and from the reach limbs of rats that had performed the HRNF task for 5 weeks. All controls had greatly diminished or absent cytokine immunoreactivity and contained no cells resembling macrophages (Fig. 2A,C,E,G; Fig. 3A). In the reach limb median nerves collected from rats that had performed the HRNF task for 5 weeks, many (but not all) cells that appeared morphologically to be Schwann cells were immunoreactive for IL-1 α , IL-1 β , TNF- α and IL-6 (examples indicated by large dark blue arrows in Fig. 2B, D, F and H). IL-10 was not immunoexpressed by Schwann cells (Fig. 3B-E). Fig. 4A, D and G also shows IL-1 α , IL-1 β and TNF- α immunostained cells that appear morphologically to be Schwann cells (all cells not indicated by white arrowheads).

Cells that appeared morphologically to be macrophages were located in the median nerves of the 5-week HRNF rats. These macrophage-like cells were immunoreactive for IL-1 α , IL-1 β , TNF- α , IL-6 and IL-10 (examples indicated by black arrowheads and an “m” in Fig. 2B, D, F, and in Fig. 3B). Immunostaining with ED1 antibody, which recognizes phagocytic macrophages, showed immunoexpression of IL-10 as well as IL-1 α , IL- β and IL-6 by ED1-immunostained cells (Fig. 3C-E; and as indicated by small white arrow-heads in Fig. 4). In contrast to Fig. 2D, IL-1 β immunoexpression in Fig. 4D appeared to be expressed more intensely by macrophages than by Schwann cells.

It also appeared from the micrographs that IL-1 β was immunoexpressed within axonal profiles (indicated by black arrows in Fig. 2D and its inset). In contrast, IL-1 α does not appear to be immunoexpressed within axonal profiles (indicated by black arrows in Fig. 2B and its inset). Finally, TNF- α and IL-6 were also immunoexpressed by thin, elongated cells resembling epineural fibroblasts (examples indicated by black arrowheads and an “f” in Fig. 2F and H).

4. Discussion

Our results show that continued performance of a HRNF task results in significant increases in IL-6 in week 3, and significant increases in IL-1 α , IL-1 β , TNF- α , and IL-6 in week 5 in the reach limb median nerve at the level of the wrist and forearm. These increases are followed by declines in all cytokines by week 8 of task performance. A similar result was also observed for IL-10, in which increased IL-10 immunoreactivity was observed in macrophages in week 5, although full confirmation of that result was hindered by limited amounts of forelimb tissue containing the median nerve at the level of the carpal tunnel.

This transient pro-inflammatory response is associated temporally with past behavioral and physiological results examining the effects of this same HRNF task on musculoskeletal and neural tissues using our model (Barbe et al., 2003; Clark et al., 2003). In those studies, we observed transient declines in behavioral outcomes, such as reach rate and task duration, in weeks 3-6 of performance of a HRNF task. Peak cytokine production in the current study is

also temporally associated with the initial influx of recruited macrophages into the median nerve as a consequence of performing this repetitive task (Clark et al., 2003).

Many studies have shown that IL-1 α , IL-1 β , TNF- α and IL-6 are produced by many neural sources including endoneurial macrophages (both resident and recruited), Schwann cells and fibroblasts (Bolin et al., 1995; Reichert et al., 1996; Murwani and Armati, 1998; Sommer and Schäfers, 1998; Deprez et al., 2001; Shamash et al., 2002). Our results concerning the cellular producers of IL-1 α , IL-1 β , TNF- α and IL-6 are consistent with the above studies. Schwann cells appear immunohistochemically to be the primary source of IL-1 α , IL-6 and TNF- α in our study. Some regional variation (within, proximal, or distal to the carpal tunnel region) in cellular localization of cytokines, IL-1 β for example, was observed in this study. We hypothesize that this variation results from differences in the specific site(s) of nerve injury in each rat.

Concerning IL-10, Be'eri et al. (1998) found that recruited macrophages and monocytes contribute the most IL-10 in neural tissues after neural injury, followed by endoneurial fibroblasts and then Schwann cells. A study by Jander et al. (1996) found that the mode of nerve injury influences the cellular location of endoneurial IL-10 production. Low levels of IL-10 mRNA were expressed by most of the Schwann cells near the site of injury immediately after nerve transection (with a decrease to control levels by 14 days), but not by ED-1 positive macrophages. In contrast, ED-1 positive macrophages rather than Schwann cells were the primary sources of IL-10 mRNA in experimental autoimmune neuritis (EAN) in that same study. In our study, we observed IL-10 immunoreactivity only in ED-1 positive macrophages after performance of the repetitive task for 5 weeks. We hope to explore further IL-10 expression in our model in future studies.

The mode of peripheral nerve injury has also been shown to alter the profile of the network of cytokines expressed as well as the kinetics of the production of those cytokines (Deprez et al., 2001; Kleinschnitz et al., 2004). Other studies have examined cytokine expression following nerve crush, chronic constriction injury, nerve transection, or in association with autoimmune inflammatory diseases (Bolin et al., 1995; Ito et al., 1998; Sommer and Schäfers, 1998; Taskinen et al., 2000; Shamash et al., 2002; Siebert and Brück, 2003). Our study is the first to examine cytokine expression in peripheral nerves that are injured as a result of performing repetitive tasks. Our previous findings of a build-up of fibrotic tissue around the median nerve with an associated reduction in nerve conduction velocity of that nerve (Clark et al., 2003) suggest that nerve compression is one likely mode of nerve injury in our model. Therefore, our findings should resemble those from chronic constriction injury (CCI) studies. CCI often leads to immediate and concomitant increases in mRNA expression of IL-1 β , TNF- α and IL-10 as well as protein levels of TNF- α within 6-24 h after placement of loose constrictive ligatures around a peripheral nerve (George et al., 1999; Shubayev and Myers, 2000; Kleinschnitz et al., 2004). These immediate increases are linked to the onset of neural injury, are usually transient (1-14 days), and are associated with increased immunoreexpression of inflammatory cytokines by resident neural cells such as Schwann cells, fibroblasts, endothelial cells and resident macrophages. A delayed or second cytokine peak often occurs within 5-28 days following CCI, presumably when the blood-nerve barrier breaks down and cytokine-producing macrophages infiltrate the injured nerve (Shubayev and Myers, 2000; Kleinschnitz et al., 2004). However, a study by Okamoto et al. (2001) found different kinetics of cytokine increases following CCI than those described above. They found a multi-phasic and prolonged cytokine response in which they observed increased expression of IL-6 and IL-1 β mRNA at 7 days following placement of loose ligatures around sciatic nerves. Over the next 2 weeks, this initial cytokine expression declined as TNF- α mRNA expression increased (peaking by 14 days). Lastly, they observed a gradual and progressive increase in IL-10 mRNA expression, peaking by 45 days post-ligation. Sommer and Schäfers (1998) also observed delayed production of

TNF (4-12 days) and IL-1 β proteins (12-95 days) following CCI. In their study, the timing of TNF production, but not IL-1 β production, temporally matched macrophage infiltration into the nerve. They hypothesized that Schwann cells were the key producers of IL-1 β in their CCI model. Thus, even in studies using a similar mode of injury, there are differences in the kinetics and sources of cytokine production. One cause of these differences may be the “tightness” of the ligatures used to elicit the constriction injury. A loose ligature would delay the onset of the nerve injury for days to weeks compared to a tight ligature.

Cytokine production in our model of repetitive strain injury is delayed until weeks 3-5 of performance of a HRNF task. If, as others have proposed, IL-6 is an immediate cytokine responding to nerve damage and inflammation (Ito et al., 1998; Bourde et al., 1996), then week 3 marks the onset of peripheral nerve injury in our model. Macrophages are just beginning to infiltrate the median nerve by week 3, and the rats are just beginning to refuse to participate in the task (task duration) at this same time point (Clark et al., 2003). By 5 weeks, the neuro-inflammatory response is in full swing. All of the cytokines examined are increased to a level detectable by immunohistochemical analyses. Schwann cells, fibroblast-like cells, and phagocytic macrophages are all participating in producing the various cytokines. By week 8, there is a resolution of the cytokine neuroinflammatory response. However, the number of ED-1 macrophages continued to increase in the median nerve with continued performance of the HRNF task (Clark et al., 2003). These results combined suggest that Schwann cells are the main source of cytokines in peripheral nerves injured by performing repetitive tasks. Schwann cells play key roles in neuroimmune responses such as cytokine production in peripheral nerves (Berg-steinsdottir et al., 1991; Hirota et al., 1996; Wagner and Myers, 1996; Murwani and Armati, 1998). While macrophages are certainly needed for successful regeneration (Brown et al., 1991), Stoll et al. (1993) report that only pre-phagocytic macrophages in nerves undergoing Wallerian degeneration are immunoreactive for TNF- α .

So, why do we see a transient production in cytokines despite continued performance of the task, and, therefore continued use of the reach limb? In peripheral nerves of other studies, increased levels of either exogenous or endogenous IL-10 have been found to inhibit the endoneurial production of several cytokines including TNF- α , IL-1 α , IL-1 β and IL-6 by macrophages and monocytes after injury (de Waal Malefyt et al., 1991; Wagner et al., 1998; Taskinen et al., 2000). Administration of exogenous IL-10 to a chronic constriction nerve injury site down regulates the inflammatory response in peripheral nerves following injury (Wagner et al., 1998). We surmise that the onset of production of IL-10 protein is associated with the resolution of the inflammatory cytokine response in our nerves, despite continued use of the limb. Our observed declines in pro-inflammatory cytokines, inflammatory mediators known to be involved in hyperalgesia and mechanical allodynia (Sommer and Schäfers, 1998; George et al., 1999; Okamoto et al., 2001; Milligan et al., 2003), may allow the behavioral recovery seen in our HRNF paradigm by 8 weeks of performance (Clark et al., 2003). The preceding behavioral decline observed by week 5 (Clark et al., 2003) is no doubt also providing rest to tissues, further aiding the processes of healing to occur. Unfortunately, full recovery of tissue morphology is not occurring in our HRNF paradigm, as evidenced by increases in collagen type I and CTGF expression around the median nerve, as well as continued increases in ED1-positive macrophages (Clark et al., 2003). Their continued presence is intriguing despite the drop in cytokine production. It would be interesting to perform studies examining the nerves after longer periods of task performance.

In summary, the results of this study confirm that cytokines known to be expressed after axonal injury are expressed in peripheral nerves in our model of repetitive strain injury, and are thus supportive of the notion that performance of low force, highly repetitive tasks results in peripheral neuropathies. The timing of the attenuation of the cytokine response in the HRNF exposure group seen in this study corresponds to a recovery of motor performance, an increased

in IL-10 immunoexpression by macrophages, but also to the onset of a fibrotic response (Clark et al., 2003). Is the fibrosis that occurs in spite of a natural resolution of the inflammation a result of the severity of the inflammation or of the original insult? If the former is true, anti-inflammatory treatment could be effective. If the latter, then a reduction in exposure to the task would be more effective. An understanding of the pathophysiology of nerve inflammation in association with performance of repetitive tasks can lead to treatment strategies to prevent or ameliorate the development of neuroinflammation and perhaps even axonal degeneration or fibrotic scar formation.

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Fig. 1. Histograms showing quantification of immunohistochemical staining of IL-1 α (A), IL1 β (B), TNF- α (C) and IL-6 (D). The percent area fraction of digitized nerve sections containing cytokine immunostaining was quantified in median nerves at the point that they spanned the carpal tunnel, in control rats and in rats that had performed a HRNF task for 3, 5 or 8 weeks. The region of nerve measured lay within the boundaries of the sectioned median nerve (N) and surrounding epineurium (E), excluding surrounding tissues such as loose connective tissue (CT), as shown in panels E and F. The green line demarcates the region of interest (ROI) measured in this nerve section (panel F). The blue color in panel F indicates the pixels in the ROI that contained brown immunoreaction product (shown in panel E) that meet the defined threshold of immunostaining. The percent area fractions of the area containing thresholded pixels versus the area of the entire ROI are shown in A - D and are expressed as mean + SEM. Data from normal control rats and shaped-only control rats are combined and are referred to as C. The number of reach limb nerves quantified for controls, or per task week, are: $n = 3 - 9$ for control rats, $n = 3 - 5$ for week 3, $n = 3 - 5$ for week 5, and $n = 3 - 4$ for week 8, depending on the cytokine. *Significantly different from week 0 ($p \leq 0.001$). Scale bar in panel E = 50 μm .

Fig. 2.

Immunohistochemical localization of IL-1 α , IL-1 β , TNF- α and IL-6 in the median nerves from normal control rats (C) or from reach limbs of rats that performed a HRNF task for 5 weeks. No immunostaining for these cytokines is visible in the normal, control rat nerves (A, C, E, G). In nerves collected from the HRNF week 5 rats (B, D, F, H), cytokine protein immunoexpression is increased in a variety of cell types. The large dark-blue arrows in panels B, D, F and H indicate examples of immunoreactive Schwann cells. Black arrowheads indicate examples of immunoreactive cells resembling macrophages (m) or fibroblasts (f). The small black arrows in panel B indicate examples of axons located within Nodes of Ranvier, all of which are immuno-negative for IL-1 α . The inset of panel B, a higher power photo, also shows that IL-1 α is not expressed in the axon. In contrast, the small black arrows in panel D and its

inset indicate examples of axons at sites of Nodes of Ranvier that are immunoreactive for IL-1 β . The asterisk in panel D indicates an area that is further enlarged in the inset of panel D. Scale bars = 50 μ m.

Fig. 3.

Immunohistochemical localization of IL-10 in median nerves of either a normal control rat (A) or in the reach limb of rats that performed a HRNF task for 5 weeks. No immunostaining for IL-10 is visible in the normal, control rat nerve (A). In nerves collected from HRNF week 5 rats (B - E), IL-10 immunoexpression is localized only to cells resembling macrophages (m). IL-10 immunoreactive cell shown in panel C also expresses ED1, a marker of infiltrating, phagocytic macrophages (D). Panel E shows a merger of photos C and D. Scale bars in A and B = 50 μ m; scale bar in panel C = 25 μ m.

Fig. 4.

Immunofluorescent photomicrographs showing co-localization of cytokines with ED1-positive cells (macrophages), in reach limb median nerve from rats that performed a HRNF task for 5 weeks. Panels in A, D, and G show nerve cells immunoreactive for IL-1 α , IL-1 β and TNF- α . Small white arrowheads in panels A and D indicate cells immunoreactive for IL-1 α , IL-1 β and TNF- α , respectively, that are also immunoreactive for ED1, as shown in panels B, E and I. Panels C, F, and I show co-localization in merged photos. The larger white arrowhead in panel H indicates an ED1-positive macrophage that is not double-labeled for TNF- α (G and I). Scale bars in each panel = 50 μ m.