A Comparison of the Changes in the Non-Neuronal Cell Populations of the Superior Cervical Ganglia following Decentralization and Axotomy

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ABSTRACT: **Transecting the axons of neurons in the adult superior cervical ganglion (SCG; axotomy) results in the survival of most postganglionic neurons, the influx of circulating monocytes, proliferation of satellite cells, and changes in neuronal gene expression. In contrast, transecting the afferent input to the SCG (decentralization) results in nerve terminal degeneration and elicits a different pattern of gene expression. We examined the effects of decentralization on macrophages in the SCG and compared the results to those previously obtained after axotomy. Monoclonal antibodies were used to identify infiltrating (ED1+) and resident (ED2+) macrophages, as well as macrophages expressing MHC class II molecules (OX6+). Normal ganglia contained ED2+ cells and OX6+ cells, but few infiltrating macrophages. After decentralization, the number of infiltrating ED1+ cells increased in the SCG to a density about twofold greater than that previously seen after axotomy. Both the densities of ED2+ and OX6+ cells were essentially unchanged after decentralization, though a large increase in OX6+ cells occurred after axotomy. Proliferation among the ganglion's total non-neuronal cell population was examined and found to increase about twofold after decentralization and about fourfold after axotomy. Double-labeling experiments indicated that some of these proliferating cells were macrophages. After both surgical procedures, the percentage of proliferating ED2+ macrophages increased, while neither procedure altered the proliferation of ED1+ macrophages. Axotomy, though not decentralization, increased the proliferation of OX6+ cells. Future studies must address what role(s) infiltrating and/or resident macrophages play in regions of decentralized and axotomized neurons and, if both are involved, whether they play distinct roles.** © 2002 Wiley Periodicals, Inc. J Neurobiol 53: 68–79, 2002

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INTRODUCTION

After a peripheral nerve lesion (e.g., resulting from nerve transection, ligation, crushing, or freezing), a series of responses occur both in the injured neurons themselves and in nearby non-neuronal cells. For example, following lesioning of the sciatic nerve (a mixed nerve containing axons of motor, sensory and sympathetic neurons), a number of changes occur in the nerve trunk distal to the site of lesion. These changes include degeneration of the severed axons (Stoll and Muller, 1999), infiltration of circulating monocytes that differentiate into macrophages (Olsson and Sjostrand, 1969; Stoll and Muller, 1986; Perry et al., 1987; Clemence et al., 1989), and proliferation of both myelin-forming and nonmyelin-forming Schwann cells (Clemence et al., 1989). The main function attributed to macrophages in this context is

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the phagocytosis of myelin and degenerating axonal debris (Perry and Gordon, 1991; Griffin et al., 1992). In addition, it has been proposed that macrophages can release cytokines that can affect both neurons and non-neuronal cells. An example of this is the hypothesis that infiltrating macrophages release IL-1, which increases levels of NGF in Schwann cells (Lindholm et al., 1987; Brown et al., 1991). In addition to the macrophages that infiltrate into degenerating nerve tracts, normal (unlesioned) nerve contains a population of "resident" macrophages (Perry et al., 1987; Monaco et al., 1992), as do many, if not all, tissues. Whether infiltrating and/or resident macrophages play roles after nerve injury and, if both are involved, whether they play distinct roles is not presently known.

Interestingly, many of the changes found in the distal degenerating nerve stump also occur in the region of the cell bodies of the axotomized neurons, at some distance proximal to the lesion site (Zigmond, 1996). For example, 2 days after axotomy of the postganglionic neurons of the superior cervical ganglion (SCG), there is an infiltration of ED1+ macrophages, and the level of these cells remains elevated for at least 2 weeks after surgery (Schreiber et al., 1995a). An influx of macrophages into the vicinity of axotomized neuronal cell bodies has also been observed in sensory ganglia (Smith and Adrian, 1972; Lu and Richardson, 1993). By 5 days after nerve transection, there is a large increase in immunoreactivity for MHC class II molecules within the SCG, and this increase is still observed 14 days after the lesion. Only minor changes are found in the number of ED2+ macrophages under these conditions.

In addition to changes in the number of macrophages, axotomy results in a striking increase in the proliferation of satellite cells and Schwann cells in the SCG (Bachoo et al., 1992) and a dramatic change in the immunoreactivity of these cells for glial fibrillary acidic protein (GFAP; Vaidyanathan et al., 1992). Whether there is also a change in proliferation of macrophages in response to lesions to pre- or postganglionic sympathetic nerve tracts is something that we address in the current article. This question is particularly relevant given the proliferation of microglia (the resident macrophage of the central nervous system) in the facial and hypoglossal nuclei after transection of their respective peripheral nerve tracts (Graeber et al., 1988; Svensson et al., 1994).

The function of macrophages in the vicinity of axotomized cell bodies is less evident than within the degenerating portion of a nerve trunk, because at least initially, there is little degeneration of these cell bodies. The axotomized neurons, however, undergo rapid and dramatic biochemical changes, including changes in gene expression (Hökfelt et al., 1994: Zigmond et al., 1996; Zigmond, 1997). The possibility exists that some of these changes in neuronal gene expression might be triggered by release of cytokines from non-neuronal cells, including macrophages. For instance, by analogy to what happens in the distal stump of the sciatic nerve, secretion of IL-1 by macrophages might produce changes in gene expression in axotomized neurons and surrounding glial cells, such as the increase in NGF that occurs in satellite cells in sensory ganglia after sciatic nerve injury (Zhou et al., 1999). Interestingly, hemocytes, which behave in many ways like vertebrate macrophages, have been implicated in the hyperexcitability that occurs in axotomized nociceptive neurons of *Aplysia* (Farr et al., 2001). Transforming growth factor β, which is produced by hemocytes, can produce electrophysiological changes that are similar to those produced by axotomy (Farr et al., 1999). Finally, it is worth noting that macrophages contain receptors for several neuropeptides (e.g., vasoactive intestinal peptide and pituitary adenylate cyclase activating peptide) and that such proteins are induced following axotomy as discussed below. Thus, the interaction between neurons and macrophages after nerve injury may involve bidirectional communication (Leceta et al., 2000; Ganea and Delgado, 2001).

Another common procedure used for looking at changes in the SCG following nerve injury is transection of the cervical sympathetic trunk (CST), a procedure often referred to as decentralization. The CST contains the preganglionic axons that innervate the SCG. These axons arise from cell bodies located in the spinal cord (Rando et al., 1981). The mRNA levels for a variety of proteins in the SCG are changed by axotomy (e.g., galanin, vasoactive intestinal peptide, pituitary adenylate cyclase activating peptide, and various nicotinic receptor subunits). Decentralization, in contrast, has little, if any, effect (see discussion in Mohney et al., 1994) on the level of these particular mRNAs (Hyatt-Sachs et al., 1993; Rao et al., 1993; Mohney et al., 1994; Schreiber et al., 1994; Zhang et al., 1994; Moller et al., 1997; Zhou et al., 1998, 2001). Nevertheless, decentralization does result in biochemical changes in the SCG. It is of interest, therefore, to look for changes in non-neuronal cells in the SCG after CSTX given that these cells may be a source of cytokines and growth factors that regulate gene expression in the ganglion.

Changes in postganglionic neurons after decentralization have usually been attributed to a decrease in synaptic stimulation of these neurons (e.g., Hendry et al., 1973; Kessler and Black, 1982). It is noteworthy, however, that the axons in the CST fire at very low rates in animals housed under normal (nonstressed) housing conditions, and, therefore, any effect of deaf-

ferentation on the firing rate of SCG neurons is likely to be small (see discussion in Zigmond and Chalazonitis, 1979). Perhaps a more important trigger for the changes in postganglionic neurons after CSTX is the extensive preganglionic nerve terminal degeneration, which could be a trigger for, among other things, macrophage infiltration. A characterization of the changes in non-neuronal cells in the SCG after CSTX should serve to aid in our understanding of the causes of some of the biochemical changes that occur in the SCG after this procedure. Previous ultrastructural studies on degenerative changes in the SCG after decentralization did not detect the presence of macrophages (Hamori et al., 1968; Lakos, 1970; Joo et al., 1971; Raisman, 1974).

The present study examines the macrophage response to preganglionic nerve transection and compares the response to that of our previously reported study on macrophage changes after postganglionic nerve transection (Schreiber et al., 1995a). In addition, we have investigated whether there are proliferative changes in macrophages in the SCG after decentralization and axotomy. A preliminary report of our results has been presented (Schreiber et al., 1995b).

MATERIALS AND METHODS

Animal Surgery and BrdU Treatment

Male Sprague-Dawley rats (200 g; Zivic Miller Laboratories, Zelienople, PA) were anesthetized with chloral hydrate (770 mg/kg subcutaneously). Neurons in the SCG were decentralized (CSTX) by bilaterally transecting the cervical sympathetic trunk (CST) 2–3 mm from its point of entry into the ganglion. SCG neurons were axotomized, by bilaterally transecting the internal and external carotid nerves 2–3 mm from where these nerve tracts exit the SCG. Ganglia from sham-operated animals served as controls. In these animals, the SCG was exposed, but the nerve trunks were not cut. A second control group consisted of ganglia from normal (unoperated) animals (a group we refer to as time zero or $T = 0$). Certain animals were also injected with 5′-bromodeoxyuridine (BrdU, 0.1 mg/kg, intraperitoneally) every 6 h for 2 days following surgery.

Fixation and Immunohistochemistry

After postoperative recovery periods of 2–14 days, the animals were reanesthetized and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 *M* phosphate buffer, pH 7.4. After excision, the SCG were desheathed and postfixed in 4% paraformaldehyde for an additional hour and then immersed overnight in graded concentrations of sucrose up to 30%. SCG were then placed in plastic embedding trays filled with O.C.T. Compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan) and flash frozen with liquid nitrogen. Ten micron-thick cryostat sections of SCG were incubated with one of three monoclonal antibodies that recognize rat macrophages: ED1, ED2, and OX6. ED1 recognizes a cytoplasmic antigen in monocytes and infiltrating macrophages (Dijkstra et al., 1985). ED2 recognizes a membrane-bound antigen that is specific for resident macrophages (Barbe et al., 1990). OX6 recognizes MHC class II glycoproteins, which are expressed on various antigen-presenting cells, including macrophages (McMaster and Williams, 1979). Attempts to see if any of these antigens were colocalized in individual macrophages were made difficult due to the fact that all of the antibodies were mouse monoclonals. Primary antibodies that were directly labeled with a fluorophore were tried but the intensity of staining produced was not great enough to allow the analysis to be done.

The mouse monoclonal antibodies ED1, ED2 or OX6 (1:100, overnight, Harlan Bioproducts, Oxford, England) were detected using a donkey antimouse antiserum conjugated to biotin (1:200, 1 h, Jackson ImmunoResearch Labs, West Grove, PA) and subsequently visualized using Texas Red conjugated to streptavidin (1:500, 30 min, Jackson ImmunoResearch Labs).

BrdU is incorporated into DNA during the S-phase of the mitotic cycle. Cells that have undergone S-phase during the period of BrdU injections were identified with a rat anti-BrdU antibody (1:6, 18–24 h, Sera-Labs, Oxford, England; Gratzner, 1982; Nowakowski et al., 1989). BrdU+ cells are referred to here as proliferating cells. Some sections were single labeled for BrdU, and some sections were processed for double labeling of macrophage antigens and BrdU by using one of the macrophage antibodies and a rat anti-BrdU (same dilution as for single labels). After the initial macrophage immunolabeling, sections were fixed with 4% paraformaldehyde, rinsed in PBS, immersed in 2 *N* HCl to denature the DNA, rinsed in PBS, and incubated for 18–24 h at room temperature with rat anti-BrdU (1:6). BrdU+ nuclei were detected using a goat anti-rat antiserum conjugated to fluorescein isothiocyanate (1:250, 2 h, Organon-Teknika Cappel Corp., Durham, NC). Omission of the primary antibodies resulted in the absence of immunofluorescence for both macrophages and BrdU.

Image Analysis of Macrophage Time Course

Fluorescent images labeled with ED1, ED2, and OX6 and taken from different regions of the SCG were captured using a SIT camera and analyzed using Image 1 software (Universal Imaging Corp., West Chester, PA). The final data are expressed as mean number of fluorescent objects detected per 10,000 $\mu m^2 \pm$ S.E.M (five fields/SCG, 2–5 SCG from two to four rats per surgical condition). These objects are referred to as cells, although they include both whole cell profiles and parts of cells. Greater than 95% of the macrophages fell within the size range of 5–150 μm^2, and objects outside of this range were not analyzed.

Density of BrdU Nuclei and Colocalization of Macrophage Markers with BrdU by Confocal Microscopy

The density of BrdU-labeled nuclei was obtained by counting such nuclei in a 10,000 μm^2 area at 10× magnification from an average of 10 fields from 3–7 SCG per surgical condition. Colocalization of BrdU+ nuclei and macrophages was analyzed with a confocal scanning laser microscope LSM410 (Zeiss, Oberkochen, Germany). The number of BrdU nuclei and macrophages were each counted at 40× in an average of 20 fields from 3–5 SCG per condition. Images of BrdU+ nuclei (detected by FITC) and macrophage-positive cells (detected by Texas Red) were captured in the RGB mode. The BrdU+ nuclei and labeled macrophages in the individual channels were examined, and their relative positions were marked on sheets of transparency film. A macrophage was considered to be double-labeled cells if it had a BrdU+ nucleus that was surrounded or in direct apposition to the cytoplasmic Texas Red label. To confirm colocalization, the red and green images were superimposed in the RGB mode, and the extent of colocalization was noted by the color change to yellow and quantified.

Statistics

Time course data after decentralization and sham operation and analysis of data from the double-label experiments after sham operation, decentralization, and axotomy were analyzed by one-way ANOVA. The significance between groups was determined by Scheffe's or Bonferroni's post hoc test. Comparisons between decentralized and sham-operated SCG at particular time points were made using an unpaired Student's t test. Differences were considered significant if $p \leq .05$.

RESULTS

ED1, ED2, and OX6 Immunostaining in the Normal SCG

In the normal rat SCG, immunostaining was seen with all three antibodies used (e.g., see $T = 0$ in all the panels of Figs. 1 and 2), although only a rare ED1+ cell was seen. Many ED2+ cells and OX6+ cells were distributed throughout the SCG, near neurons and blood vessels, within the connective sheath that surrounds the ganglion, and near the entry or exit regions of the preganglionic and postganglionic nerve trunks, especially the external carotid nerve.

ED1 Immunostaining in the SCG after Decentralization

Two days after decentralization, there was a small but significant increase in the number of ED1+ cells over sham-operated ganglia (1.6-fold increase, $p \leq .02$), though at that time point, the difference compared to unoperated controls was not significant ($p \leq .25$). Four days after decentralization, maximal levels of ED1+ immunoreactivity were reached and were 6.5- and 5.9-fold higher compared to unoperated or sham controls, respectively [Fig. 1(A)]. These levels were maintained significantly above both unoperated and sham-operated controls for up to 14 days. While the ED1+ response 2 days after decentralization ap-

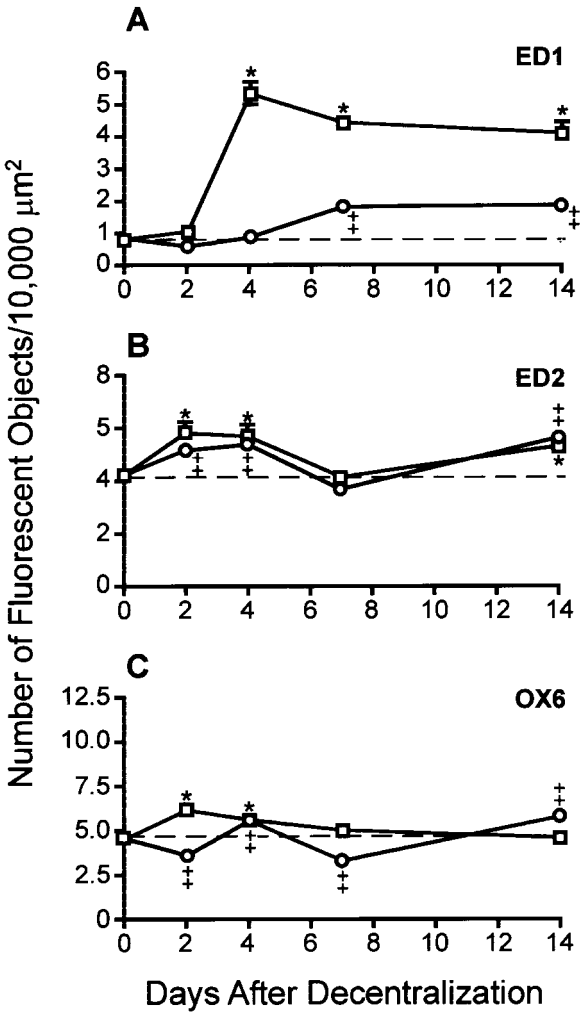

Figure 1 Quantification of ED1 (a), ED2 (b), and OX6 (c) immunostaining after decentralization. Immunostaining was quantified by image analysis on SCG sections taken from normal animals ($T = 0$) and from animals at various times after decentralization (squares) or a sham operation (circles). The data are expressed as numbers of fluorescent objects (5 to 150 μm^2) counted per 100 by 100 μm area of tissue examined. The dashed line indicates the value for ganglia taken from animals at $T = 0$. Each value represents the mean ± SEM for 5 field/SCG, $n = 2$–5 ganglia. *‡indicate time points at which the values for decentralized and sham-operated ganglia, respectively, differed significantly form those in unoperated animals ($T = 0$).

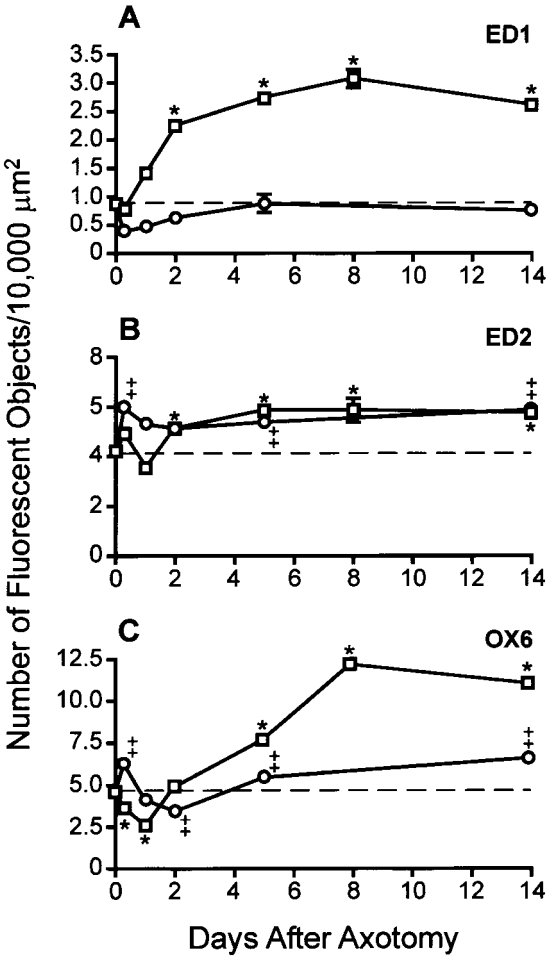

Figure 2 Quantification of ED1 (a), ED2 (b), and OX6 (c) immunostaining after axotomy. Other conditions used and the method for data analysis were the same as those reported in the legend to Figure 1 . The squares are data from ganglia after axotomy and the circles, after sham operation. The dashed line indicates the value for ganglia taken from animals at $T = 0$. *‡indicate time points at which the values for axotomized and sham-operated ganglia, respectively, differed significantly form those in unoperated animals ($T = 0$). These data are reproduced from Schreiber et al. (1995a), by permission from John Wiley & Sons.

peared to be reduced in comparison to axotomy, the peak ED1 response after decentralization appeared to be enhanced over axotomy [Figs. 1(A) and 2(A)].

ED2 Immunostaining in the SCG after Decentralization

When the density of ED2+ cells was determined, there were small but statistically significant increases in the number of ED2+ cells 2, 4, and 14 days after decentralization and after sham operation compared to the unoperated control (less than 1.4-fold). A similar trend was noted in the time course of ED2+ cells after axotomy, although the time points where differences were seen varied somewhat [Figs. 1(B) and 2(B)]. Two days after decentralization, there was a small but significant increase in the number of ED2+ cells compared to 2- day sham-operated SCG [Fig 1(B), $p \leq .0045$]. Nevertheless, this was the only time point at which a significant difference was found between the sham-operated and decentralized SCG.

OX6 Immunostaining in the SCG after Decentralization

Similar to the ED2 immunostaining, OX6+ cells were present in all unoperated and sham-operated SCG. Small but statistically significant increases or decreases (less than 1.4-fold) were seen 2, 4, 7, and 14 days after sham operation compared to unoperated controls [Fig. 1(C)]. Two days after decentralization was the only time point at which the lesioned animals showed a small but statistically significant increase in the number of OX6+ cells compared to both unoperated and sham-operated controls [1.4 and 1.7- fold, respectively, Fig. 1(C); $p \leq .0001$]. The maximum increase in OX6+ cells following decentralization was considerably smaller than after axotomy [Figs. 1(C) and 2(C)]. Eight days after axotomy the density of OX6 reached maximal levels, levels 2.7-fold higher than those found in ganglia from unoperated animals. As mentioned in the Discussion section, at least some of these OX6+ cells would probably be double labeled with ED1+ and ED2.

Macrophages Comprise a Portion of the Proliferating Cells in the SCG after Decentralization and Axotomy

The density of all cells that were BrdU+ was significantly elevated in the ganglion after decentralization (1.9-fold) compared to a sham operation (Fig. 3). Axotomy produced a significantly greater density of such BrdU+ cells (3.5-fold) than did decentralization. Although the density of the entire BrdU population was evenly distributed throughout the SCG after axotomy, there was a somewhat higher density of BrdU labeled cells after decentralization in the third of the ganglion closest to the CST than in the third near the internal carotid nerve. Given that we have already shown that many of the BrdU+ cells after axotomy are satellite/Schwann cells (Bachoo et al., 1992), we examined whether any of the proliferating cells in the SCG were macrophages.

The percentage of macrophages that was proliferating was determined after sham operation, decentralization, and axotomy, as shown by double labeling of

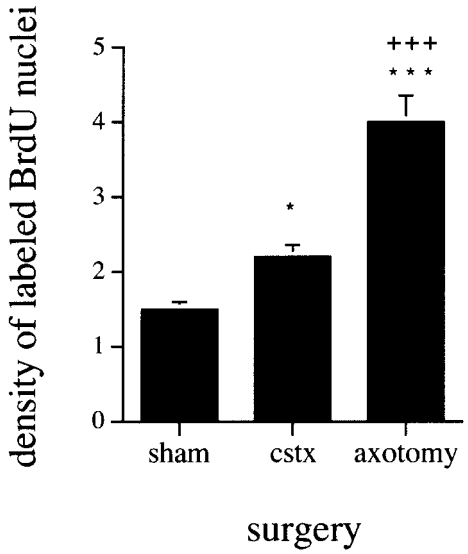

Figure 3 The density of BrdU+ nuclei in the SCG is significantly increased 2 days after decentralization (CSTX; 1.9-fold) and axotomy (3.5-fold) as compared to sham operation (*$p < .05$; ***$p < .00001$) and the density of BrdU+ nuclei after axotomy is significantly increased over that of decentralization (two-fold; +++$p < .00001$). The data are expressed as the mean density of BrdU+ nuclei counted per 100 by 100 μm area of an SCG section. Each value represents the mean ± SEM for an average of 10 fields/SCG, $n = 3$–7 ganglia.

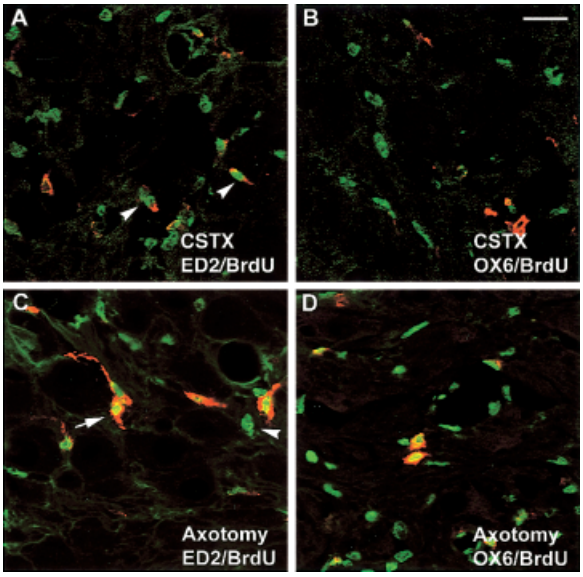

Figure 4 BrdU and macrophage immunostaining in the SCG 2 days after decentralization (CSTX; A,B) and postganglionic axotomy (C,D). ED2+ (A,C) and OX6+ (B,D) macrophages are labeled with Texas Red, while the BrdU+ nuclei are labeled with FITC (green). Colocalization was noted when the fluorophores overlapped [yellow, e.g., see arrow in (C)] or when the BrdU was in direct contact with the macrophage marker [see arrowheads in (A)]. A cell labeled only with BrdU is indicated by an arrowhead (C). The latter cell may be a satellite cell (Bachoo et al., 1992). Quantitative double-labeling analyses are shown in Tables 1 and Figure 5. Scale bar, 25 μm.

the cells with the anti-BrdU antibody (Table 1). Although 16–24% of the ED1+ macrophages were colabeled with BrdU, no significant differences were found in the percentage colocalized under the different experimental conditions. The percentage of ED2+ cells colabeled with BrdU was lower in each experimental group than that seen with the ED1+ cells; however, an eight- and sevenfold increase in this percentage was seen in decentralized and axotomized ganglia, respectively, compared to sham-operated ganglia (Table 1). With respect to the percentage of OX6+ cells that were also BrdU positive, there was almost a threefold increase after axotomy, but no change after decentralization. Examples of such cells colabeled with ED2 or OX6 and BrdU are shown in Figure 4.

Table 1 Percentage of Each Macrophage Population That Are Proliferating as Shown by Colocalization with BrdU

Antibody	Sham	CSTX	Axotomy
ED1	20%	16%	24%
ED2	1%	8%**	7%**
OX6	15%	15%	42%*·***

Sections from superior cervical ganglia were examined 2 days after a sham operation, decentralization or axotomy. CSTX refers to cutting the preganglionic cervical sympathetic trunk. See Materials and Methods for detailed procedures. The data are shown as the percentage of cells labeled with one of three macrophage markers (ED1, ED2, or OX6) that were colabeled with BrdU.

Statistical comparison between lesioned group and sham-operated group: **$p = .00001$; *$p = .002$. Comparison between axotomized and decentralized ganglia, ***$p = 002$.

We also expressed our macrophage data in the context of the change in the total population of BrdU+ cells (Fig. 5). The areas of each circle shown in Figure 5 are proportional to the total number of BrdU+ cells in a standard area of the ganglion. The total area of each circle is then subdivided into regions representing the percentage of BrdU+ nuclei associated with (dotted area) and not associated with (darkly hatched area) each of the three antimacrophage specific antibodies. The percentage of colabeled cells increased after decentralization but not after axotomy for the ED1+ cells and after both operations for the ED2+ cells. The percentage of BrdU+ cells that were also OX6+ did not change after either type of lesion. As represented in Fig. 5, a significant percentage of the BrdU+ cells were colabeled. Based on our previous work, a significant percentage of the noncolabeled

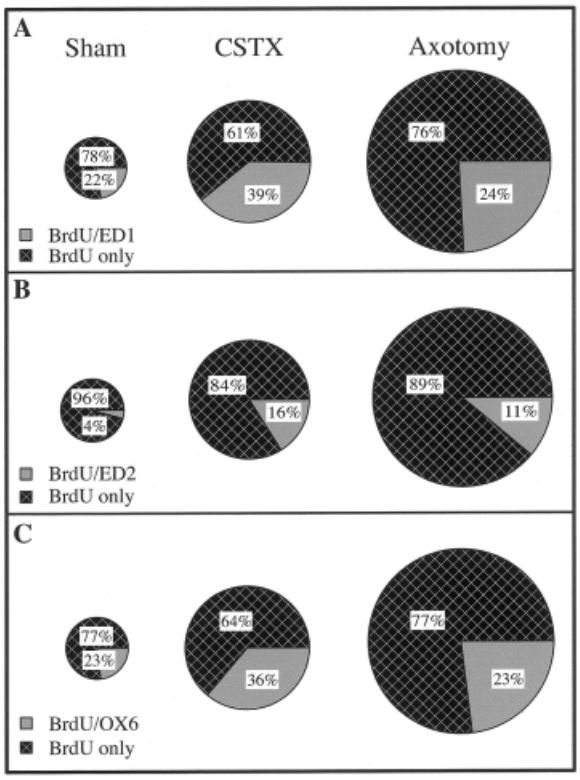

Figure 5 Diagrams showing the percentage of BrdU+ nuclei in the SCG that were double labeled with one of three macrophage antibodies, ED1, ED2, or OX6. The area of the circle represents the total number of BrdU+ nuclei 2 days after sham operation, decentralization (CSTX), or axotomy. The gray areas represent the percentages of these BrdU+ cells that were colabeled with the particular macrophage antibody, while the hatched areas indicate the percentage of BrdU+ nuclei that was not colabeled with a macrophage marker.

BrdU-positive cells are probably satellite/Schwann cells (Bachoo et al., 1992).

DISCUSSION

Our results establish a number of points. First of all, following decentralization of the SCG, there are changes in the macrophage population of the ganglion. Second, these changes differ significantly from those we previously reported after axotomy. Third, after both decentralization and axotomy, macrophages comprise a significant percentage of the proliferating non-neuronal cells that are seen in the SCG. Finally, after both surgical procedures there is a significant increase in the percentage of resident macrophages that are proliferating.

Between 2 and 4 days after the CST is transected, there is a significant influx in infiltrating macrophages (ED1+ cells). These ED1+ cells remain elevated in the ganglion for at least 14 days after the lesion. The maximum increase in ED1+ macrophage density seen after decentralization is about twice that seen after postganglionic axotomy. Only minor changes in the resident macrophage (ED2+) and the macrophages expressing MHC class II molecules (OX6+) are seen after CSTX. Similarly, only minor changes in the density of ED2+ cells were seen after axotomy. There was, however, a large increase in the number of OX6+ positive cells in the SCG beginning 5 days after axotomy and increasing to a peak at 8 days (Schreiber et al., 1995a). The density of macrophages that were seen after either type of surgery was evenly distributed throughout the SCG with all three antibodies studied (data not shown).

Our results on the influx of ED1+ macrophages, both in the current article and in our previous article (Schreiber et al., 1995a), differ from those reported by Magnusson and Kanje (1998). These authors looked at ED1+ macrophages in SCG 3, 6, 24, 48, and 72 h after decentralization or axotomy. After decentralization, they found an increase in ED1+ macrophages only at 24 h and, after axotomy, they found increases in ED+1 macrophages at 6, 24, and 48 but not at 3 or 72 h. Thus, these time courses differ from ours both in the rapidity of the increases in invading macrophages and in the length of the time these ED1+ cells are present in the SCG. These differences could result from methodological differences between the two studies. For example, in our experiments, bilateral transections and sham operations were done on a separate group of animals to minimize the possibility of changes on the contralateral side after a unilateral lesion (e.g., Joh et al., 1973; Lu and Richardson, 1993; Zhang et al., 1994). On the other hand, Magnusson and Kanje performed unilateral surgeries and compared their operated ganglia to unoperated contralateral ganglia.

Nevertheless, the functional significance of macrophage infiltration within the SCG is unknown at this point, although, following decentralization, they presumably participate in clearing the debris from the degenerating nerve terminals. Only a few studies have reported on the anatomical changes that occur in the SCG after lesioning the CST. Hamori et al. (1968), Lakos (1970), and Joo et al. (1971) used the electron microscope to examine synaptic contacts in the rat and/or cat SCG. Complete disappearance of these contacts was observed in the cat with the persistence of very few contacts in the rat. Raisman et al. (1974) did a quantitative study of morphological changes in the SCG over a more extensive time course. The authors examined degenerating nerve terminals and surviving synapses. The total density of synapses in

the SCG decreased by 90% within the first 24 h after the lesion and remained at that low level, if regeneration was prevented, for up to 191 days. This loss of synaptic contacts was accompanied by the appearance of many "vacated synaptic thickenings," i.e., thickened plasma membrane of the postganglionic neurons that looks identical to the postsynaptic plasma membrane at normal synapses but is not opposed by a presynaptic nerve terminal.

In addition to these changes in presynaptic contacts, some morphologic, histochemical, and biochemical changes have been reported in the postganglionic neurons after CSTX. These include a dramatic increase in neurofilament positive staining (Elfvin et al., 1987; Roivainen et al., 1993), a long-term increase in the expression of a number of immediate early genes (Koistinahoo et al., 1993), an increase in immunoreactivity for protein kinase C-β (Roivainen et al., 1993), a decrease in tyrosine hydroxylase activity and mRNA (Hendry et al., 1973; Hanze et al., 1994; Kroesen et al., 1997), a decrease in the exocytotic responses to muscarine (Zaida and Matthews, 1999) and a change in NPY mRNA (Hanze et al., 1994 reported a decrease and Kroesen et al., 1997 reported an increase). In addition, decentralization and axotomy elicit different responses in the satellite/Schwann cell population in the SCG. Specifically, Nacimiento and Kreutzberg (1990) reported that histochemical staining for a prominent nucleoside-producing ectoenzyme, 5′ nucleotidase, increased in these cells after axotomy but not after decentralization. In contrast, Vaidyanathan et al. (1992) reported an increase in GFAP staining in satellite/Schwann cells in the SCG after both decentralization and axotomy. Finally, it should be noted that following CSTX, a small number of neurons in the caudal SCG ($<1\%$) are axotomized (Bowers and Zigmond, 1979).

In the vast majority of published studies on biochemical changes after decentralization of the SCG, the changes are attributed to a decrease in afferent stimulation of the ganglion. While such an effect certainly takes place, it is likely to be small in size given the low firing rates of these neurons under nonstressful conditions (Polosa, 1968; Janig and Schmidt, 1970). Additional effects of decentralization must also be taken into account as possibly producing stimuli that affect neurons and satellite/Schwann cells in the SCG. These include the degeneration of preganglionic nerve terminals and the resultant release of axonal and nerve terminal products, the influx of macrophages, and the proliferation of ganglion nonneuronal cells. As we speculated in our study on macrophage influx after axotomy, macrophages might release cytokines within the SCG that either directly or indirectly signal changes in the postganglionic neurons.

Unlike in the case of ED1, the density of ED2+ cells did not differ between sham-operated and axotomized ganglia from 2 days to 2 weeks after the surgery, except at 2 days after CSTX. The striking change we observed with the ED2+ population was a seven- and eightfold increase in proliferation of these cells 2 days after decentralization and axotomy respectively compared to sham-operated animals. In most tissues, resident macrophages do not divide (e.g., Furth, 1989; Westermann et al., 1989), although as already noted microglia proliferation in the facial nucleus after axotomy is an exception (Graeber et al., 1988). Although the percentage of the total ED2+ cells that proliferate is small even after these lesions, this change is our first clear indication that the resident macrophages are altered after sympathetic nerve lesions. Given these large increases in proliferation of the ED2+ cells, one might ask why there is not a consistent increase in the total number of ED2+ cells after axotomy and decentralization. This is probably because even though the increases in proliferation are substantial, they still only represent slightly less than 10% of the ED2+ population. An important avenue for future studies is to determine what signals trigger the change in proliferation of ED2+ after both decentralization and axotomy.

In contrast, no significant change was found in the percentage of ED1+ cells that were proliferating 2 days after either lesion (Table 1). It is likely that the ED1+ cells that were also BrdU+ represent cells that were proliferating in the bone marrow prior to invading the SCG during the 48 h of BrdU injections. The fact that the percentage of the total population of proliferating cells that were ED1 positive was greater after decentralization than after axotomy might be more a reflection of the lower state of proliferation of other non-neuronal cells after the former than the latter.

In any study with "cell markers," it is important to determine how specific a particular antibody is to the cell type of interest. In particular, there is a potential problem with regard to OX6 staining, because under special circumstances Schwann cells can express MHC class II molecules. For example, injection of bacterial antigens into sciatic nerve of adult rats *in vivo* caused the induction of MHC class II molecules on some myelin-forming Schwann cells in the vicinity of the injection site (Bergsteindottir et al., 1992). On the other hand, MHC class II molecules were expressed on very few nonmyelin-forming cells, which comprise the population of Schwann cells present in pre- and postganglionic rat sympathetic axons. Furthermore, double-labeling experiments detected no

OX6+ satellite/Schwann cells in the SCG in control or axotomized ganglia (Schreiber et al., 1995a), in the dorsal root ganglia after a peripheral nerve lesion (Lu and Richardson, 1993), or in the distal portion of the lesioned sciatic nerve (Stoll et al., 1989). We, therefore, think that most, if not all, of these OX6+ cells in the rat SCG are macrophages in normal and sham-operated ganglia and in ganglia after decentralization or axotomy.

A second point to be considered is whether the three antibodies used label distinct or overlapping populations of macrophages. Because there is slow continuous renewal of the ED2+ resident macrophages in tissues throughout life (Vass et al., 1993), there is a possibility that ED2+ cells could also be ED1+ at least for some period of time. If such cells exist, their number must be very small given that in unoperated ganglia there are very few ED1+ cells but many ED2+ cells. In addition, it has been found in other tissues that ED1+ macrophages, both *in vivo* and in culture, require 1 to 2 weeks before they start to express ED2 (Barbe et al., 1990).

In normal (unoperated) animals the number of OX6+ cells is very similar to the number of ED2+ cells, suggesting that the resident macrophages express MHC class II molecules. Interestingly, there is little change in OX6+ staining after decentralization, particularly compared to the large increase seen after axotomy. It is known that the level of these antigens expressed by macrophages can be modulated under a number of conditions. Blood monocytes, for example, have low levels of these antigens, but activated macrophages express high levels (Adams and Hamilton, 1992). The relative lack in upregulation of OX6+ after decentralization suggests that the state of activation of macrophages is different after decentralization than after axotomy. It needs to be stressed, however, that the function of the increase in MHC class II molecules in the axotomized SCG or, in fact, in the distal stump of degenerating peripheral nerves (e.g., Stoll et al., 1989; Monaco et al., 1992) is not currently known. Barclay (1981) suggested that these surface molecules might be involved in cell–cell interactions in addition to antigen presentation to T lymphocytes.

It must be acknowledged that sympathetic neurons from the SCG innervate both primary and secondary lymphoid organs and that interruption of sympathetic function, for example, by decentralization and presumably by axotomy, affects immune function (Felten et al., 1987). Therefore, the surgical manipulations used in the present study are likely to produce a systemic alteration in the immune system. Nevertheless, the differences we have found between decentralization and axotomy in the macrophage population of the SCG argues against such a systemic effect playing a major role in the macrophage population in the SCG. In addition, recent studies have demonstrated that monocyte chemoattractant protein (MCP-1) is increased within the SCG after axotomy (Schreiber et al., 1997; 2001), as it is within the degenerating stump of the transected sciatic nerve (Carroll and Frohnert, 1998; Toews et al., 1998: Subang and Richardson, 2001). We are currently examining whether this peptide plays a necessary role in the infiltration of macrophages into the SC−/−, using MCP-1 null mutant mice. If macrophage infiltration is substantially reduced in the MCP-1 −/− mice, these animals will serve as a preparation for examining what effect(s) macrophage induction plays in the ganglionic response to postganglionic nerve axotomy.

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