Original Article

Influence of the intensity, level and phase of spinal cord injury on the proliferation of T cells and T-cell-dependent antibody reactions in rats

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Study design: Three independent experiments in a rat model of contusive spinal cord (SC) injury were performed. Two studied the alterations induced by SC injury on some immunological aspects of the T-cell response. The third one evaluated the motor recovery of rats with low-thoracic injuries.

Objective: To examine the effect of level, intensity and phase of SC injury on T-cell proliferation and T-cell-dependent antibody response.

Setting: Neuroimmunology Department, UIMEN, IMSS-CAMINA Research Center.

Methods: Lymphocyte proliferation and hemagglutination assays were performed. Animals were injured either moderately or severely at T1 or T12 SC segments. Analysis of peripheral T-cell proliferation in response to mitogens and to myelin basic protein (MBP), as well as of antibody production against a T-dependent antigen, was performed at acute, subacute and chronic phases.

Results: A significant decrease of both response to mitogens and antibody production was found especially during the acute phase and in animals with severe and high (T1)-level injury. Animals with low (T12) and moderate contusions recovered to control levels at the chronic phase. An autoimmune reaction against MBP was observed only in animals with severe contusion at low level.

Conclusions: The intensity, level and phase of SC injury differentially alter the function of T cells. These results will allow a better interpretation of studies directed to elucidate the role of T lymphocytes in various processes developed after SC injury.

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Introduction

Lately, the function of the immunological system after spinal cord (SC) trauma has become an essential issue. The immune system protects the body from infectious diseases that are the most important causes of morbidity in individuals with SC injuries.^{1–4} Besides, recent studies have provided substantial evidence about the important role of the immune system in destructive, protective or restorative phenomena after SC injury.^{5–14} Thus, alterations of the immunological function after an insult upon the SC is an important issue to study.

Previous studies conducted by Campagnolo *et al*^{15,16} suggested that individuals with complete cervical SC

injury presented more immunological alterations than those with lower lesions. Currently, no data from well-controlled studies about this issue exist. Similarly, the intensity of injury and the phase after damage have not been investigated in relation to their effect upon immune competence.

In this work, we evaluated the influence of the level, intensity and phase after lesion on T-cell proliferation and on T-cell-dependent antibody production in a controlled model of SC contusion.

Methods

Study design and animal care

Three independent experiments were performed. In the first study the proliferative response of T cells was

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evaluated. For this purpose 96 Fischer 344 female adult rats, weighing 200–220 g, were allocated into four groups ($n=24$): group 1, rats submitted to moderate SC contusion at T1 SC-segment (moderate-T1); group 2, rats with moderate SC contusion at T12 SC-segment (moderate-T12); group 3, rats with severe SC contusion at T1 (severe-T1) and group 4, rats with severe SC contusion at T12 (severe-T12). The rats of each group were killed to be studied 1 day (acute phase of lesion, $n=8$), 10 days (subacute, $n=8$) or 30 days (chronic, $n=8$) after SC contusion. In a second independent experiment, T-dependent antibody reactions were evaluated. Thirty-two rats were subjected to an SC contusion and then allocated into four groups (as described for the first experiment, $n=8$ per group). Serum samples from each rat were obtained at 7, 14, 21, 28, 35, 60 and 90 days after SC injury. Finally in the third experiment, 14 rats were subjected to a moderate ($n=7$) or a severe ($n=7$) contusion at T12 level to assess motor recovery.

Animals that were only anesthetized (with no surgical procedure, ie naïve) or subjected to laminectomy at T1 level (sham injured) were used as controls of immune function assessment ($n=8$ per group). The animals were matched for age and weight in each experiment and housed in a light- and temperature-controlled room. To minimize stress, all animals were handled at least once or twice a day, starting 7 days before surgical procedure; likewise, they were housed in groups of two animals. Efforts were made to minimize both animal suffering and number of rats used. All applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research. All procedures were in accordance with the National Institutes of Health (US) Guide for the Care and Use of Laboratory Animals. Sterile beds and filtered water were replaced daily. Bladder draining was assisted by massage at least twice a day until normal function was attained. During the first week after contusion, the animals received a course of gentamicin (1 mg/kg, subcutaneously) per day, and were carefully monitored for evidence of urinary tract infections or any other sign of systemic disease. Animals with overt signs of bladder or respiratory infections (eg cloudy or bloody urine, stertors or breathing difficulty) were excluded from the study.

Spinal cord injury

Rats were anesthetized with ketamine at 77.5 mg/kg and xylazine at 12.5 mg/kg. Thirty minutes later, they were subjected to laminectomy at T1 or T12 segment, a 10-g rod was dropped onto the laminectomized cord from a height of 50 (severe) or 25 mm (moderate), using the NYU impactor (NYU, New York, USA), a device shown to inflict a well-calibrated contusive injury of the SC.¹⁷

Immunoassays

Lymphocyte proliferation After humane euthanasia and under aseptic conditions, the cervical lymph nodes

(LNs) and the spleen of each rat were removed and separately placed in RPMI-1640 medium (GIBCO), washed and gently compressed to obtain total cells. Erythrocytes present in the spleen-cell suspension were lysed by incubation with 0.15 M NH_4Cl , 0.01 M KHCO_3 and 0.0001 M ethylene diaminetetra acetic acid for 5 min at room temperature. After three washes with a medium containing 20% fetal bovine serum (FBS), cell viability from both LN and spleen suspensions was assessed separately by 0.04% Trypan blue dye exclusion. Only lots with >93% viable cells were used. Cell suspensions (250 000 LN or spleen cells/100 μl) were added to 96-well culture plates (COSTAR). For nonspecific stimulation, Con A (5 $\mu\text{g}/\text{ml}$) was added. Antigen-specific stimulation was studied by adding guinea-pig myelin basic protein (MBP) as antigen (15 $\mu\text{g}/\text{ml}$, Sigma). Control wells contained only medium. Plates were incubated at 37°C in a 95% air–5% CO_2 atmosphere. After 72 h, 1.0 $\mu\text{Ci}/\text{well}$ of ^3H -thymidine was added and, after 18 h of culture, the cells were harvested on glass fiber filters. The incorporated radioactivity was measured by scintillation counting. The stimulation index (SI) was calculated by dividing the mean cpm of experimental wells by the mean cpm of the cells cultured in medium alone.

Hemagglutination Seven days before the surgical procedure and to every antibody testing, rats were intraperitoneally immunized with 300 μl of sheep erythrocytes in 0.75% saline solution. Antibody titers were analyzed by hemagglutination, as follows: 3 ml of 50% sheep erythrocytes was diluted 1:50 in phosphate-buffered saline and then 25 μl of the suspension were added to round bottom wells of polystyrene plates (Corning, New York, USA). Twenty-five microliters of serially diluted serum samples were then added to each well. After 30 min of incubation, the hemagglutination titer was read.

Motor recovery assessment

Motor recovery assessment was performed by the use of the Basso, Beattie and Bresnahan (BBB) open-field test of locomotor ability.¹⁸ Recovery was scored from 0 (complete paralysis) to 21 (complete mobility) by two independent and experienced observers blinded to the type of contusion inflicted to each rat. The locomotor activities of the trunk, tail and hind limbs were evaluated in an open field by placing the animals during 4 min in the center of a square enclosure made of wood with a smooth floor. Each hind limb was assessed individually and the average for both scores was obtained. The results were shared by the observers until the end of evaluations, and then averaged to obtain the final score for each animal. Before each evaluation, each rat was examined carefully for perineal infection, wounds in the hind limbs and tail and foot autophagia.

Statistical analysis

Data were analyzed by the GraphPad Prism 3.0 software. Results obtained from proliferation studies were compared by Kruskal–Wallis test followed by the Mann–Whitney *U*-test, whereas those obtained from hemagglutination were analyzed by one-way analysis of variance (ANOVA) for repeated measures followed by the Bonferroni test. The BBB rating score was analyzed by two-factor ANOVA for repeated measures. Statistical significance was considered relevant when $P \leq 0.05$.

Results

Effect of SC injury on the mitogen-induced T-cell proliferative response

At the first and tenth days of injury, LN lymphocytes from sham-injured animals showed a significantly lower reaction to concanavalin-A (ConA) than that presented by cells obtained from naïve rats (Figure 1, $P < 0.001$). Regardless of the level or severity of the lesion, SC injury induced a significant reduction in LN T-cell response to ConA, 24 h after procedure (Figure 1). After 10 days of lesion (subacute phase), a significant recovery was observed in animals with moderate-T12 contusions; this was maintained until the end of the study. The splenocytes of SC-injured groups presented a significant decrease of T-cell proliferative response to ConA 24 h after injury (Figure 2). This inhibitory effect was significantly stronger 10 and 30 days after the injury in rats subjected to severe contusion than in those with moderate lesion. In fact, by the end of the study, the animals with moderate injury (especially at T12 level) presented values near the ones observed in naïve animals and presented an important recovery from day 10 (approximately 85% compared to naïve animals).

Figure 1 LN cells proliferation in response to ConA in naïve, sham-injured or moderately and severely SC-injured rats. Data are presented as the mean $\pm$ standard deviation of eight animals. ^aDifferent from all groups ($P < 0.001$), ^bdifferent from SC-injured groups ($P < 0.01$), ^cdifferent from moderately (T1) and severely (T1 and T12) contused rats ($P = 0.01$)

Conversely, and regardless of the level, animals with severe contusion remained with a significantly low proliferative function (approximately 46% of naïve).

Effect of SC injury on the central nervous system auto-reactive T-cell proliferative response

No auto-reactive response against MBP was mounted by LN cells at any time neither in moderate T1 or T12, nor in severe-T1 (Figure 3), whereas a significant MBP-reactive response was detected 1 day ($SI = 1.84 \pm 0.3$ versus 0.69 ± 0.2 of naïve rats) and 10 days after contusion (2.12 ± 0.3 versus 0.95 ± 0.02) in the group of animals with severe T12 injury. In none of the groups, splenocytes reacted to MBP at any time (Figure 4).

Figure 2 Splenocyte proliferation in response to ConA in naïve, sham-injured or moderately and severely SC-injured rats. Each bar represents the mean $\pm$ standard deviation of eight animals. ^aDifferent from SC-injured groups ($P < 0.01$), ^bdifferent from moderately (T1) and severely (T1 and T12) contused rats ($P = 0.01$), ^cdifferent from severely contused rats ($P = 0.01$)

Figure 3 LN cells proliferation in response to MBP. Data are presented as the mean $\pm$ standard deviation of eight animals. ^aDifferent from all groups ($P = 0.02$)

Figure 4 Splenocytes proliferation in response to MBP in naïve, sham-injured or moderately and severely SC-injured rats. Each bar represents the mean $\pm$ standard deviation of eight animals

Figure 5 Antibody reaction against a thymus-dependent antigen (sheep erythrocytes). Data are presented as the mean $\pm$ standard deviation of eight animals. a, Different from b ($P=0.04$), c, d, e and f ($P<0.001$); b, different from c, d, e and f ($P<0.001$); d, different from c, e and f ($P=0.02$); f different from c and e ($P=0.03$)

Effect of SC injury on T-cell-dependent antibody reaction

As no differences among SC-injured groups were observed at day 35, this evaluation was prolonged until day 90 after injury (Figure 5). From the beginning of the study sham-injured animals presented a 50% decrease in antibody titers as compared to naïve animals ($P=0.04$).

Spinal cord injury induced a strong reduction of antibody response against this T-dependent antigen (Figure 5). From day 7 to day 21, antibody titers were barely detectable in SC-injured animals (10–12% from naïve). From this time until the end of the study, the titers significantly rose in animals with moderate-T12 injury (23% from naïve), whereas they barely changed in animals with moderate-T1 (8% from naïve), severe-T1

Figure 6 Motor recovery of rats subjected to moderate or severe SC injury at T12 level, as assessed by the BBB test. Each point represents the mean $\pm$ standard deviation of seven rats. ^aDifferent from severely contused animals ($P<0.0001$)

(7% from naïve) or severe-T12 damage (13% from naïve). At the end of the study, a significant difference between groups with severe contusion was also observed: rats with injury at T12 showed higher antibody titers than those with T1 contusion.

Motor recovery of rats with T12 injury

As the improvement of T-cell function in severely T12 contused animals did not parallel the one observed in moderate T12 group, we hypothesized that a lower motor recovery in those with the severe lesion could induce a stress-related immune depression. To verify this, we performed the third experiment in which the motor recovery of the two groups with severe and moderate T12 contusions was evaluated. From the first week (Figure 6), the animals with moderate T12 injury presented a significantly better motor recovery than the rats with severe T12 contusion ($P<0.0001$). At the end of the follow-up, severely T12 contused rats presented a BBB score of 3.42 ± 0.34 (mean $\pm$ SD) whereas it was 9.28 ± 0.60 for the moderately T12 injured animals.

Discussion

The role of the immunological system in processes developed after SC injury has not been clarified. This is the reason why numerous studies have been conducted to describe and evaluate the role of immune cells after SC damage.^{19–26} In order to better interpret the available data, it must be considered that the function of the immune system depends on the nervous system. Therefore, lesions caused in some specific areas of the neural tissue (hypothalamus, limbic forebrain, brainstem autonomic nuclei, cerebral cortex or SC) could alter immune functions.²⁷

Previous reports have demonstrated the influence of the nervous system on the immunological function.²⁸

The presence of noradrenergic sympathetic nerve fibers in the parenchyma of lymphoid and hematopoietic tissues,²⁹ as well as of adrenaline and neuropeptide receptors on lymphocytes, strongly supports an intimate interaction between the two systems.³⁰ In fact, diverse alterations on T- and B-cell functions after a chemical sympathectomy are observed.^{31–34} Similar alterations have also been reported in individuals with SC injuries at levels above the emergence of the preganglionic sympathetic fibers.^{35–39}

The extent and consequences of immune alterations depend not only on the level but also on the intensity of the lesion. Likewise, there could be different various phases after injury. In this work, these variables were evaluated in a controlled animal model. ConA-induced T-cell proliferation and the production of a thymus-dependent humoral response were significantly diminished as a consequence of SC injury, especially during the acute phase. T-cell proliferation was decreased both in spleen and LN, even though it was more apparent in the latter, probably as a reflection of differences between peripheral LNs and spleen innervations, microenvironments or lymphocyte trafficking patterns.⁴⁰ The immunosuppressive effect on both T-cell and antibody reactions was stronger and lasted longer in the case of high (T1) and severe contusions.

The immunodepression induced after a T1 lesion could be the result of a disconnection of the sympathetic nervous system from the superior centers, as any damage at T1 or above, partially or totally disrupts supraspinal influences upon the preganglionic sympathetic neurons located within the intermediolateral gray matter of the thoracolumbar SC. Immediately after SC trauma, there is a period of increased autonomic discharge that causes a catecholamine and neuropeptide-dependent stimulation, which in turn may act on the immune system disturbing its function.⁴¹ Catecholamines, for instance, are capable of inhibiting lymphocyte proliferation, antibody secretion and production of proinflammatory factors.⁴² Thus, autonomic dysregulation could be partially responsible of the reduction of immune function, especially during the acute phase of the lesion in animals with high SC injuries. During chronic phases, the autonomic deregulation may also be altering the immune function of animals with lesions at high levels. Individuals with injuries above T6 may present autonomic dysreflexia, a phenomenon characterized by widespread reflex sympathetic discharges, as a result of diverse stimuli produced below the level of the injury.^{43–45} The autonomic deregulation effect is supported by the fact that animals with moderate T12 contusions presented a significantly better lymphocyte function recovery.

Noteworthy, rats with severe T12 lesions did not present immunological recovery (T-cell proliferation) or it was very low and delayed (antibody reaction), as compared to the one observed in those with moderate T12 contusions. It is not plausible that autonomic deregulation was the cause of immunodepression in these animals, because the lesion was inflicted in a level

that almost all sympathetic fibers remain in connection with the superior centers. Thus, the immune depression could be caused by other factors, like stress. The lack or decrease in mobility, contributes to depression of the immune system in individuals with SC injury.^{4,27,46} In agreement, previous studies have suggested that immobilization of the paralyzed muscles (as a stress factor) sustains the impairment of the immune function, whereas mobilization may contribute to immunological recovery: individuals undergoing physical rehabilitation therapy, regained much of their natural and adaptive immune function following injury; this improvement generally paralleled that of the Functional Independence Measurement Score.²⁷ Moreover, light or moderate exercise has demonstrated to improve the immunological function after SC injury.^{47,48} In contrast, individuals not receiving these therapies or not presenting functional improvement, showed continued impairment of immune function.^{27,49} In the model studied herein, the motor recovery of animals with severe T12 contusions was significantly lower than that observed in rats with moderate T12 lesions, which immune function improved faster. This observation may suggest that one of the main factors contributing to the diminished immune function of the latter could be a lower mobility, and as a consequence, an enhanced action of this stressor on rats with severe contusions. The stress mediated by corticosteroids, catecholamines or endorphins exerts a suppressive effect on the immune system.^{27,50}

Autoreactivity against MBP was observed only in rats subjected to severe–low (T12) contusion. In this work, the decreased proliferative response of T cells when cultured with ConA discloses the important impairment of cellular function after T1-contusions. Disturbance in T-cell function, probably caused by central nervous system (CNS) disconnection, could be the main reason why in these cases (T1-lesions) autoreactive T cells did not proliferate adequately after the MBP challenge. On the other hand, in animals subjected to moderate T12 contusions (with sympathetic outflow better connected to the CNS), the absence of this anti-MBP reaction could be explained by other mechanisms. Availability, concentration and biochemical features of the antigen are known to be some of the most important factors for the development of an immunological response. After a moderate SC contusion, both the availability and concentration of the antigen may be lower than those observed in animals subjected to severe lesions. Thus, the development of immune reactions against MBP is less probable in moderate than in severe contusions. In fact, the reaction was only observed in lymphocytes from the severe T12 contusion group and, even though significant, it was very low. The amount and features of the neural antigens released after an injury of the CNS seem to be important factors to achieve immune activation and also to modulate its quality, as lymphocytes from transgenic animals overexpressing a T-cell receptor for MBP, are capable of protecting the neural tissue after optic nerve damage (low quantity of antigen)

or to destroy it after SC injury (large amounts of antigen).^{51,52}

The absence of an anti-MBP reaction in animals with T1 and moderate T12 contusions must be an important issue to be considered in studies directed to evaluate the impact of the autoimmune reaction on neural tissue after injury.

Conclusions

In this work, it was shown that the level, the intensity and the phase of injury influence the functions of T cells. A diminished T-cell response gives rise to a high risk to develop infectious diseases. This is the case of individuals with SC injury in whom an altered function of T lymphocytes and other immune cells may be predisposing to severe urinary or respiratory infections, which are in many cases, the main cause of morbi-mortality, as it is in experimental models of SC injury.^{35,53} Immunological alterations like the ones observed in the present work should be considered when studying the role of the immune system on SC injury.

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