Normal Lymphocyte Subpopulation of the Spleen is Altered after Peripheral Nerve Injury in Mice

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Background: Chronic neuropathic pain is often associated with altered immune function and the modulated immune cell response play a role in neuropathic pain by experimental nerve injury. In order to assess the possible changes in lymphocytes function following peripheral mononeuropathy, this study examined the lymphocyte subpopulation of the spleen using the monoclonal antibodies against the membrane surface markers in neuropathic BALB/c mice by a partial transection of sciatic nerve (PST).

Methods: After confirming tactile allodynia by paw withdrawal threshold, the splenic lymphocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD45R/B220 (B cell) and CD4 (helper/inducer T cell) or with phycoerythrin (PE)-conjugated anti-mouse CD90.2 (total T cell) and CD8 (suppressor/cytotoxic T cell). The proportions of subsets were analyzed using a FACScan laser flow cytometry system on postoperative day 5 and day 18 respectively.

Results: PST induced a mechanical allodynia as verified by the von Frey test at both 5 and 8 days postoperatively compared to pre-surgery (P < 0.05). Lymphocyte subpopulation was affected by PST. The proportion of CD4+ subset was significantly larger in the PST group than in the sham operated group on day 5, while the proportion of CD8+ subset was larger on day 18. In the PST group, there were significant changes in the proportion of CD4+ on day 5 and in the proportion of CD8+ on day 18 (P < 0.05) compared to pre-surgery. There were no significant fluctuations in the proportion of total splenic T cell and B cell subsets of PST group compared to sham operated group.

Conclusions: These results suggest that development of mononeuropathy is responsible for the proportional changes in splenic lymphocyte subsets in mice. (Korean J Anesthesiol 2007; 53: S 42~7)

Key Words: immune response, lymphocyte subpopulation, neuropathic pain.

INTRODUCTION

A finely regulated balance of the immune response is essential for the defense mechanism. The complications such as impeded tissue healing, metastasis of malignancy or subsequent infection could be elicited by the changes of immune function which highlights the need for aggressive control of pain to obtain a better prognosis. The immune system is affected by many factors such as stress, surgery and pain. The neurotransmitters which modulate pain and analgesia influence on immune cells. 1-3) Although the results of recent work in the point of neuroendocrine or biochemical views have reported wide variations in the altered immune function under chronic neuropathic pain, 4) little is known as to whether it can be a significant factor affecting the lymphocyte responsiveness. For maintaining the optimum performance of the immune system, normal distribution and proportion of immune cells are important. 5-7) It was known that chronic stress affect on the various immune cell populations. Thus, chronic neuropathic pain as a stressful condition might possibly related to the splenic lymphocytes subpopulation. This study examined the development of mononeuropathy in BALB/c mice caused by a partial sciatic nerve transection (PST) and assessed the proportional changes in mouse splenic lymphocytes such as CD45R/B220+ (B
lymphocyte), CD90.2+ (total T lymphocyte), CD4+ (helper/inducer T cell) and CD8+ (suppressor/cytotoxic T cell) subsets using flow cytometry.

MATERIALS AND METHODS

Animals

After obtaining approval from the Institutional Ethics Committee, male BALB/c mice (specific pathogen-free) aged 4- to 6-weeks were purchased from the Atsugi breeding center at Charles River Japan, (Charles River Japan Inc., Tokyo, Japan). The animals were acclimatized at least 1 week before the experiments and were maintained in an air-conditioned barrier-system room under controlled conditions. They were housed in polysulfone cages (five mouse per cage: Daejeong E&C, Korea) on autoclaved wood-shavings (GLP bedding, B&K, USA) and fed on X-ray irradiated Laboratory Mouse Food A04-10 (UVR Quality Assurance, Orge, France) with acidic water. The animal room temperature was 23°C and the relative humidity was between 50% and 55%. The light/dark cycle was maintained on 12-hour intervals.

Surgery

Before surgery, the mice were placed in clear plastic cages on an elevated mesh floor and allowed to acclimatize for 30 min. The paw withdrawal threshold in response to probing with von Frey filaments was measured for baseline mechanical sensitivity. The animals were randomly allocated into sham operated group (n = 21) and experimental group (n = 21). The experimental group of mice was then anesthetized with ether and the sciatic nerve was partially transected. Briefly, the sciatic nerve was exposed at the mid-thigh level, and the lateral half of the nerve was transected with finely tipped scissors at a point immediately proximal to the branch running to the musculus biceps femoris. The skin was then sutured and the animals were allowed to recover in each cage. In sham operated group of mice, skin incision and dissection of muscle were made and sutured.

Behavioral assessment of tactile allodynia

In order to evaluate the mechanical sensitivity of the injured hind paw, the mice were placed in individual plastic boxes on a mesh floor and allowed 30 min to familiarize. A series of calibrated von Frey filaments (Stoelting Co., Wood Dale, IL) were applied perpendicularly to the plantar surface of the left hind paw with sufficient force to bend the filaments for 6 sec. Brisk withdrawal or paw flinching was considered a positive response. Subsequently, a filament of the next lower force was applied. Each trial was repeated two to three times at approximately 2-min intervals. Unlike the noxious heat or pressure tests in which a cutoff value is often used to avoid causing tissue damage, this study did not use a cutoff value for the Von Frey filament testing.

Cell preparation

For lymphocyte preparation in spleen, mice were killed by cervical dislocation. The spleen of the mice was excised aseptically and stored in a tissue culture medium (RPMI 1640, Gibco BRL NY, USA). A single cell suspension was made by homogenizing the spleens through a 70 μm mesh strainer (BD Falcon, San Diego, CA) using fresh wash media and a 5 mL syringe plunger. After washing and centrifugation, the red blood cells were removed by adding 2 mL of a red cell lysis buffer (0.83% NH₄Cl, 0.01 M Tris-HCl, pH 7.2) and washed twice with PBS. The cells were suspended with complete RPMI medium (RPMI-1640 medium supplemented with 10% fetal bovine serum(FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine). The cell numbers were counted with a hematocytometer and the cell viability was determined using the trypan blue exclusion method in order to exclude the dead cells.

Flow cytometry

For an analysis of the distribution of cells, single cells from the spleen were counted and adjusted 1 × 10⁶ cells/ml. Fluorescein isothiocyanate (FITC)-labeled anti-mouse rat IgG2a kapa CD45R/B220 (B cell marker) and phycoerythrin (PE)-labeled anti-mouse rat IgG2a kapa CD90.2 (T cell marker) were used for staining the B cells and T cells, respectively. FITC- or PE-conjugated antibodies were used as the negative control monoclonal antibodies. All the antibodies and isotype control antibodies were purchased from Pharmigen (San Diego, CA). After incubation, the cells were washed and then fixed with 1% paraformaldehyde in PBS.

Flow cytometric analysis was performed using a FACScan laser flow cytometry system (Becton Dickinson, Mountain View, CA) equipped with a Macintosh PowerMac G3 personal computer.
S 44

**Fig. 1.** Representative data of flow cytometric dot plot for CD4+ FITC and CD8+ PE T cell subsets from spleen of BALB/c mice in response to partial transection of sciatic nerve (PST) injury at pre-surgery (A), 5 days (B) and 18 days (C) postoperatively. After gating on lymphocyte subpopulations, data were displayed as two-color dot plot to measure the proportion of CD4+ and CD8+ subsets. There were increased expression of CD4+ FITC T cell subset (lower right quadrant) in the day 5 sample and increased CD8+ FITC T cell subset (upper left quadrant) in the day 18 sample compared with the pre-surgery.

**Fig. 2.** The proportional changes of helper/inducer T cells defined by CD4+ subset in response to the partial transection of sciatic nerve (PST) injury in mice (n = 21). *: P < 0.05 versus sham operated group at day 5 and day 18, †: P < 0.05 versus pre-surgery (day 0) in each group, by post hoc analysis with Dunnett’s method for multiple comparisons. Data are given as mean ± SD.

computer (Apple Computer, Cupertino, CA) and Cell Quest (Becton Dickinson) software. The gate was set to exclude any cell debris and dead cells, and the results are shown as the percentage of positive cells within a gate identified by flow cytometry.

The data is reported as a mean ± SD. The proportion of lymphocyte subsets in PST and sham operated group were compared using two-way analysis of variance with repeated measures equipped with Sigma-Stat (Version 2.03) from SPSS (USA). The differences of proportional changes in the subpopulation at different the time-courses was analyzed by the Dunnett’s method for multiple comparisons. Statistical significance was accepted at P < 0.05.

**RESULTS**

The mice showed changes in the mechanical sensitivity and in the proportion of each subset of lymphocytes. At both 5 and 18 days postoperatively, the results in this study were obtained in mouse in which PST induced allodynia, as verified by mechanical sensitivity with the von Frey filament. Indeed, the paw withdrawal response to the von Frey filament (gm) were 1.39 ± 0.16 preoperatively, 0.19 ± 0.12 on day 5 and 0.14 ± 0.02 on day 18 (mean ± SD, P < 0.05).

Splenic T cell subpopulation was affected by PST. Fig. 1 (A, B and C) shows representative flow cytometric dot plots for two-color staining of CD4+ FITC and CD8+ PE T cell subsets from spleen of PST mice. PST group showed significant fluctuation in the proportion of CD4+ and CD8+ T cell subsets compared to the sham operated group (P < 0.05). On day 5, the proportion of CD4+ subset was significantly larger in the PST group than in the sham operated group, while the proportion of CD8+ subset was larger in the PST group on day 18. In PST group, CD4+ proportion of day 5 and CD8+ proportion of day 18 were significantly increased compared to pre-surgery (P < 0.05) (Fig. 2, 3). The changes in the proportion of total splenic T cells defined by CD90.2+ subset and total splenic B cells defined by CD 45R/B220+ subset were smaller in the PST group than in the sham operated group (P < 0.05). In PST group, the proportion of 45R/B220+ subset on day 18 was significantly increased compared
to pre-surgery (P < 0.05) (Fig. 4).

**DISCUSSION**

The present study indicates that increased mechanical sensitivity resulting from a partial sciatic nerve transection (PST) is associated with proportional changes in CD4+ or CD8+ T cells of the splenic lymphocytes. Chronic constriction injury (CCI) caused by applying the loose ligatures to the sciatic nerve has been used to examine functional alterations of the immune system in neuropathic pain. However, the disadvantage of this model is the effect of foreign material for ligation, which causes a local inflammatory reaction. For this reason, we considered that PST might be a more suitable model for examining the various immunologic effects of neuropathic pain caused by trauma or an iatrogenic nerve lesion. In fact, PST develops hyperalgesia to the same degree as CCI model without exhibiting perineural inflammation. In this study, the mechanical sensitivity was induced and maintained during the experimental period as explained by the slow regeneration of nerve in mice.

The immune system is particularly sensitive to stress. Acute stress generally has positive effects, while chronic stress typically provokes immunosuppression. The alteration of immune system including lymphoid cell occur both in the bloodstream and in different lymphoid tissues. However, the magnitude of the changes reflects the complex influence on the maturation and mobility of lymphoid cells. Immediately after stress, lymphocyte activation is augmented in favor of CD4+ T cells because all clones of T cells are not affected. In this study, the changes in the total T lymphocyte and B lymphocytes were lower in PST compared to the sham operation, but there were fluctuations in the proportion of CD4+ and CD8+ subsets. The larger proportional change in CD4+ subset on day 5 after PST could be explained by the early phase augmentation of T lymphocytes. The duration of the changes in lymphocyte phenotypic expression was not studied. In general, the extent of immediate local inflammatory reactions depends on the nature and severity of physical trauma and these changes return to normal level within 24 hours or a few days following surgery.

In contrast, CD8+ T cells can be affected more when the trauma severity increased or the condition of stress prolonged. In this study, the findings of day 18 showing significantly
larger proportion of CD8+ subset suggest that PST could cause rather chronic stress than usual inflammatory process. We found out total T cells were well maintained at different point of observation during the study in PSY group. The reflection of the sum of CD4+ and CD8+ subsets consist total T cells, this is responsible for the lower proportional change.

In humoral immunity, B cells have a major role in producing antibodies following the detection of an exogenous antigen. The antibody synthesis of B cells is also controlled by CD4+ or CD8+ T cells through the enhancement or inhibitory effects.19-20) Regarding chronic mononeuropathy, the production of antigen-specific Ig G levels was decreased because of inhibited B cell function.21) Compared with previous reports, the proportion of B cells defined as CD45R/B220+ subset in both of PST and sham groups were increased on day 18, but its proportion was significantly lower in PST group than sham operated group. The discrepancy between our results and those of previous studies might be related to the different immune parameters evaluated such as different monoclonal antibodies and the degree of inflammation or pain according to the method of nerve injury.

One of mechanisms inducing these changes is related to the message of pain transmitting to immune cells. Nociceptive stimuli cause the early phase augmentations of T lymphocyte proliferation. However, it finally results in lymphopenia because of the activation-induced apoptosis or energy of cells.22) At this point, the lymphopenia cause the subsequent changes in T lymphocytes maturation or redistribution. It is also related to the biased lymphocytes proliferation to either side of CD4+ and CD8+ T cells impacting on the normal CD4+/CD8+ ratio.22) The amplitude of such change might be the complex influence of nociception.23) The mechanism for the changes in phenotypic expression are not known and the proportional fluctuations of splenic lymphocytes do not reflect the total lymphocytes population in the body. However, the subtle alterations in the immune function can occur and these changes may contribute to the subsequent infectious complications.

Alternate mechanisms include bioactive substances such as glucocorticoid and catecholamine. In fact, cortisol induce lymphopenia with marked reduction in the number of T lymphocyte subsets, and catecholamine increase the NK-cell activity after major surgery and anesthesia.24) Even though the chronic constriction injury on sciatic nerve failed to show the change in immune function represented by the NK-cell activity, it showed marked modifications of hypothalamic and splenocyte beta-endorphine or substance P concentrations consistent with painful or stressful condition.4) Pain and hyperalgesia activate the endogenous system and induce biochemical modification in central nervous system. Generally, nociceptive stimulation is one of the factors activating the hypothalamic-pituitary-adrenal (neuroendocrine) axis, which are related to lymphocyte proliferation and activation.18,22) Although these immune changes have to be considered as the net result of various factors,1) chronic nociception might cause identifiable alterations in immune system.

In conclusion, mononeuropathy may be responsible for the alteration of the proportional changes of splenic lymphocytes subsets in mice.

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