

## Full-length article

## Effects of complete Freund's adjuvant on immunohistochemical distribution of IL-1 $\beta$ and IL-1R I in neurons and glia cells of dorsal root ganglion<sup>1</sup>

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### Key words

experimental arthritis; interleukin-1; interleukin-1 receptors; spinal ganglia

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### Abstract

**Aim:** To investigate the effects of complete Freund's adjuvant (CFA) on inflammatory hyperalgesia and morphological change of the coexistence of interleukin-1 beta (IL-1 $\beta$ ) and type I IL-1 receptor (IL-1RI) in neurons and glia cells of rat dorsal root ganglion (DRG). **Methods:** The pain-related parameters and the expression of IL-1RI and IL-1 $\beta$  positive neurons and glia cells of DRG in normal saline (NS) and adjuvant-induced arthritic (AA) group were examined with pain behavior assessment methods and immunohistochemical assay, respectively. **Results:** Five hours, 1 d, and 2 d after intra-articular injection of 50  $\mu$ L CFA, tactile hyperalgesia induced by CFA was observed in the foot flexion and extension scores of the ipsilateral hindpaw of AA group. Three days after injection, the distribution of IL-1RI/IL-1 $\beta$  double-stained coexisted neurons and glia cells were observed in ipsilateral DRG of both groups. The number of IL-1 $\beta$  positive neurons, IL-1RI positive neurons, IL-1 $\beta$ /IL-1RI double-stained neurons, and IL-1RI positive glia cells in ipsilateral DRG of the AA group were higher than that of NS group ( $P < 0.05$  or  $P < 0.01$ ). **Conclusion:** The coexistence of IL-1 $\beta$  and IL-1RI in neurons and nonneuronal cells suggests an as yet unknown autocrine and/or paracrine function of IL-1 $\beta$  in the DRG. The function was enhanced in articular arthritis induced by CFA and could play an important role in hyperalgesia under inflammatory conditions.

### Introduction

Interleukin-1 beta (IL-1 $\beta$ ) is a bioactive polypeptide cytokine that is produced by a large variety of cells, including macrophages, fibroblasts, keratinocytes, synovocytes, mast cells, glial cells, and neurons; and has various immune roles responding to antigens, malignant cells, inflammatory stimuli, and tissue injury<sup>[1]</sup>. IL-1 $\beta$  also has proinflammatory cytokine activity and induces hyperalgesia after subcutaneous injection into the rat paw<sup>[2]</sup> or after intrathecal administration in rats and mice<sup>[3,4]</sup>. This means administration of exogenous IL-1 $\beta$  can produce hyperalgesia. However, inflammation can also induce the release of endogenous IL-1 $\beta$  which was responsible for hyperalgesia as well. A previous study showed that intraplantar injection of complete

Freund's adjuvant (CFA) in adult rats resulted in a significant elevation in IL-1 $\beta$  and nerve growth factor (NGF) levels in the inflamed tissue<sup>[5-6]</sup>. IL-1 $\beta$  exerts its effects through high-affinity receptors on target cells, of which two types have been identified: IL-1RI and IL-1RII<sup>[7]</sup>. IL-1 receptor antagonist (IL-1Ra), has been shown conclusively to block the effects of IL-1 $\beta$ <sup>[8]</sup>. Targeted deletion of the IL-1 receptor type I or the IL-1 receptor accessory protein, and transgenic over-expression of IL-1R $\alpha$  within the brain and spinal cord significantly lowered thermal and mechanical pain sensitivity in mice<sup>[9]</sup>.

The major cellular source of IL-1 $\beta$  production in the central nervous system are from glial cells, predominantly microglia. However, several studies have provided evidence for the neuronal expression of IL-1 $\beta$  in the peripheral ner-

vous system: sensory neurons in the dorsal root ganglia (DRG) as well as sympathetic neurons<sup>[10]</sup>. Moreover, cytokines released in the periphery by sensory and by sympathetic neurons have an established role in regulating immune cell activation, connective tissue and vascular responses, and nociception in peripheral inflammatory processes. Among the cytokines produced and released by both sensory and sympathetic neurons are transforming growth factor- $\beta$  (TGF- $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon-gamma (IFN- $\gamma$ )<sup>[11,12]</sup>. Sympathetic neurons have also been demonstrated to express IL-1 $\beta$ <sup>[13,14]</sup>. IL-1 $\beta$  appears to be an important mediator in nociception and has been shown to induce the release of substance P from nociceptive sensory neurons<sup>[15]</sup>. The expression of IL-1 $\beta$  as well as the type I IL-1 receptor in sensory neurons in rat DRG has been investigated using nonradioactive *in situ* hybridization and immunohistochemistry<sup>[16]</sup>. However, there is no morphological evidence for the coexistence of IL-1 $\beta$  and IL-1RI of neurons and glia cells in DRG and the effects of articular injection of CFA.

In the present study, we investigated the effects of CFA on inflammatory hyperalgesia and the morphological changes of IL-1 $\beta$  and IL-1RI coexistence in neurons and glia cells of rat DRG.

## Materials and methods

**Animals** Experiments were carried out on male adult Sprague-Dawley rats (180–200 g), purchased from the Experimental Animals Center of Tongji Medical College of Huazhong University of Science and Technology (Grade II, Certificate No 19-053). The rats were housed individually in cages with a 12-h light/dark cycle, and had free access to food and water for a week before experiments began. They were randomly divided into two groups: (1) normal saline (NS) group ( $n=7$ ), the rats were pretreated with an intra-articular injection of 50  $\mu$ L normal saline into the left (ipsilateral) tibio-tarsal joint under light anesthesia with 10% chlorhydrate (300 mg/kg, ip) injection; (2) adjuvant-induced arthritic (AA) group ( $n=7$ ), the rats were pretreated with an intra-articular injection of 50  $\mu$ L complete Freund's adjuvant (CFA, Sigma) into the left tibio-tarsal joint under the same light anesthesia<sup>[17,18]</sup>.

**Preclinical tests** The following parameters were measured: (1) weight; (2) mobility score<sup>[19]</sup> (0=the rat only lies down; 1=the rat only crawls; 2=the rat walks with difficulty; 3=the rat walks and runs with difficulty; 4=the rat walks and runs normally); (3) diameter of the tibiotarsal joint, detected with vernier caliper.

**Pain behavioral parameters** The following pain parameters were measured using two tests in the following sequence<sup>[20]</sup>: (1) flexion scores: mean scores of rats was evaluated by flexion of one hind paw. Under these conditions a squeak or leg-withdrawal could be elicited. Five such stimuli were repeated at 5-s intervals, and a rating of 1 or 0 was respectively given depending on whether the animal emitted a squeak or leg-withdrawal. Thus for each animal the total scores of squeak and leg-withdrawal ranged from 0 to 10; (2) extension scores: mean scores of rats as evaluated by extension of one hind paw (identical rating used as for flexion score).

Each group animals were tested before injection to obtain baseline value and continued to be detected in 5 h, 1 d, and 2 d post-injection.

**Tissue preparation** On d 3 post-injection, all animals were deeply anesthetized with 20% urethane (1 g/kg) by abdominal injection, cleared with 200 mL of saline solution and perfused with 300 mL of 4% phosphate-buffered paraformaldehyde (pH 7.4) through left ventricle. After perfusion, the L4-L5 ipsilateral DRG was removed and post-fixed for 8–10 h. They were then soaked in 20% sucrose phosphate-buffered solution at 4 °C overnight prior to freezing and subsequent cryosectioning. Sections (10  $\mu$ m) were cut on a cryostat.

The sections were put in 0.01 mol/L PBS buffered solution. Sections were selected randomly to do experiments with double immunofluorescence staining of IL-1RI/IL-1 $\beta$ , or IL-1RI immunohistochemistry single staining procedure.

**Double immunofluorescence staining procedure** We used the following steps: (1) sections were incubated with 0.25% Triton X-100 for 30 min at 37 °C; (2) after being rinsed by 0.01 mol/L PBS, sections were incubated with 15% normal goat serum for 30 min at 37 °C; (3) sections were then incubated for 48 h at 4 °C in a mixture of the two primary antibodies (rabbit anti-rat IL-1RI, 1:100, Santa Cruz; mouse anti-rat IL-1 $\beta$ , 1:100, Serotec); (4) after being rinsed in PBS, sections were incubated for 3 h at room temperature in a mixture of the two secondary antibodies, Cy3-conjugated anti-rabbit IgG (Sigma, 1:200) and FITC-conjugated anti-mouse IgG (Sigma, 1:200); (5) the sections were picked and mounted on slides with glycerin-PBS buffered solution (1:9).

Omission of the primary antibody and incubation in IL-1 $\beta$  or IL-1RI pre-absorbed antibody served as negative controls. Laser scanning confocal microscope (Lesia, German) was used to take photos and fluorescent microscope (Olympus, Japan) was used to capture images and count positive cells.

**IL-1RI immunohistochemistry procedure** We used fol-

lowing steps: steps (1) and (2) were the same as above double immunofluorescence stain procedure; (3) sections were then incubated for 48 h at 4 °C in the primary antibodies (rabbit anti-rat IL-1RI, Santa Cruz, 1:200). (4) after being rinsed in PBS, sections were incubated for 30 min at room temperature of the secondary antibodies, biotinylated goat anti-rabbit IgG (Santa Cruz, 1:200); (5) after being thoroughly washed in PBS, the sections were incubated in a streptavidine-peroxidase conjugate (Santa Cruz, 1:200) for 30 min at room temperature; (6) after being rinsed with 0.05 mol/L Tris-buffered saline, the immunoreaction product was revealed by a freshly prepared solution containing 0.04% 3,3-diamino-benzidine and 0.01% H<sub>2</sub>O<sub>2</sub>. The sections were picked up and mounted on slides with glutin. They were dehydrated with graded alcohol and transparentized with xylene, then covered with neutral gum. Each experiment had set up blank antitheses as described before.

**Statistic analysis** Seven rats per group were used for analysis. Digital images were captured in four sections per animal and 10 squares (38.4 mm<sup>2</sup> each) per section of immunofluorescence or immunohistochemistry respectively, using an Olympus microscope (objective×20; eyepiece×10) and a 3CC immunohistochemistry-D color video camera (Sony). The number of immunofluorescence-stained IL-1RI/IL-1β positive neurons or immunohistochemistry-stained IL-1RI positive glia cells in each image were counted by a blinded experimenter with a computer-assisted image analyzer (Image Pro-plus Kodak, USA). The number of positive DRG large-, medium-, and small-sized neurons were calculated. As reported previously, the primary sensory neurons in the DRG have been classified into large type A neurons (diameter over 30 μm), medium diameter neurons type B, and small type C neurons (diameter under 20 μm)<sup>[21]</sup>. For large neurons were regarded as medium sized ones when they were not cut through their nucleus. In our study we calculated the total of medium and large neurons of immunofluorescence-stained IL-1RI/IL-1β positive neurons. The SPSS software (SPSS 11.0) was used for data analysis. The data was expressed as mean±SD, and analysis of variance (ANOVA) was used to compare the difference among different groups.

## Results

**Preclinical and behavioral studies** After 5 h, 1 d, and 2 d of intra-articular injection of 50 μL CFA, the weight of the rats was unchanged ( $P>0.05$ , data not shown). The rats of the AA group developed inflammation which was confined to the inoculated articular characterized by edema of ipsilateral tibiotarsal joint (Table 1). There was a dramatic decrease observed in the mobility scores of rats of the AA group

**Table 1.** Diameter of the ipsilateral tibiotarsal joint of rats in NS group and AA group.  $n=7$ . Mean±SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs NS group.

Group	Diameter of the ipsilateral tibiotarsal joint/mm			
	Before injection	5 h after injection	1 d after injection	2 d after injection
NS	4.32±0.02	5.33±0.11	5.46±0.40	4.86±0.08
AA	4.52±0.07	6.34±0.15 <sup>c</sup>	6.37±0.24 <sup>b</sup>	5.86±0.40 <sup>b</sup>

compared with the NS group ( $P<0.05$ ,  $P<0.01$ , Table 2). In other words, the inoculated rats are impaired in their ambulatory activities.

**Table 2.** Mobility score of rats in NS group and AA group.  $n=7$ . Mean±SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs NS group.

Group	Mobility score of rats in NS group and AA			
	Before injection	5 h after injection	1 d after injection	2 d after injection
NS	4	3.71±0.18	3.43±0.20	3.71±0.18
AA	4	2.57±0.20 <sup>c</sup>	2.71±0.29 <sup>b</sup>	2.86±0.14 <sup>c</sup>

### Effects of CFA on the pain behavioral parameters of rats

Five hours, 1d, and 2 d after intra-articular injection of 50 μL CFA, tactile hyperalgesia induced by CFA was observed in the foot flexion and extension scores of ipsilateral hindpaw of AA group (Table 3, 4), while the pain scores of the contralateral hindpaw of both groups were close to 0 and had no significant difference ( $P>0.05$ , data not shown).

**Table 3.** Ipsilateral flexion score of rats in NS group and AA group.  $n=7$ . Mean±SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs NS group.

Group	Ipsilateral flexion score			
	Before injection	5 h after injection	1 d after injection	2 d after injection
NS	0	3.86±1.50	3.43±1.70	2.86±1.10
AA	0.43±0.30	7.43±1.34 <sup>b</sup>	7.71±1.41 <sup>b</sup>	7.57±1.32 <sup>c</sup>

### Effects of CFA on double immunofluorescence staining of IL-1β and IL-1RI of ipsilateral DRG

Three days after

**Table 4.** Ipsilateral extension score of rats in NS group and AA group. *n*=7. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs NS group.

Group	Ipsilateral extension score			
	Before injection	5 h after injection	1 d after injection	2 d after injection
NS	0.29±0.18	1.14±0.26	2.57±0.75	2.29±0.97
AA	0.29±0.18	7.57±1.23 <sup>c</sup>	6.14±1.55 <sup>b</sup>	5.86±0.91 <sup>b</sup>

injection, IL-1β and IL-1RI positive products were detected in the same neurons as well as in some glial-like cell types. IL-1β/IL-1RI immunoreactivity positive products were mainly located in the cytoplasm and nucleus of the positive neurons of NS group (Figure 1), while immunoreactivity positive products were distributed in the membrane and nucleus of the positive neurons of the AA group (Figure 2). There were still a few neurons presented no IL-1β or IL-1RI immunoreactivity in the DRG of the NS group, but most neurons in the DRG of AA group presented IL-1β or IL-1RI immunoreactivity (Figure 1C, 2C).

With statistic analysis, it was found that the number of IL-1RI single stained neurons, IL-1β/IL-1RI double-stained

neurons, and the percentages of the number of double-stained neurons to the number of IL-1β positive neurons of small neurons in DRG of AA group were higher than that of NS group (*P*<0.05 or *P*<0.01, Table 5). The number of IL-1RI single stained neurons, IL-1β/IL-1RI double-stained neurons of large and medium neurons in DRG of AA group were higher than that of NS group (*P*<0.05, Table 6). The percentage of double-stained neurons to IL-1β or IL-1RI positive neurons of large and medium neurons in DRG presented no significant difference between the two groups (*P*>0.05, Table 6).

**Effects of CFA on single immunohistochemistry staining of IL-1RI of ipsilateral DRG** Three days after injection, the IL-1RI in DRG was expressed in glial-like cell types as well as in sensory neurons using a microscope under normal light. Some of the glial-like cells were satellite cells ensheathing the large sensory cell bodies, some sparsely distributed in the connective tissues between neurons of DRG. The IL-1RI immunoreactivity positive products were stained as brown and mainly located in the membrane and cytoplasm of glia cells. The stained color of glia cells in DRG of AA group was deeper than that of the NS group (Figure 3, 4). The number of glia cells of DRG of AA group was 13.45±2.35/38.4 mm<sup>2</sup>, which was higher than that of NS group (5.31±1.58/38.4mm<sup>2</sup>) (*P*<0.01).

**Table 5.** The percentage of IL-1β/IL-1RI double-stained small neurons to IL-1β or IL-1RI single-stained small neurons of DRG. *n*=7. Mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs NS group.

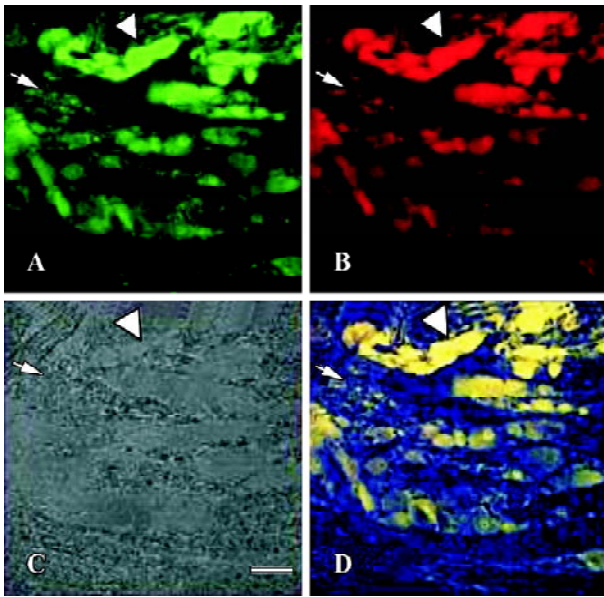
	Double-stained neurons Number/38.4mm <sup>2</sup>	IL-1β positive neurons Number/38.4mm <sup>2</sup>	IL-1RI positive neurons Number/38.4mm <sup>2</sup>	% <sup>1)</sup>	% <sup>2)</sup>
NS group	21.5±2.1	33.8±3.7	29.1±3.2	58.8±6.0	68.2±6.8
AA group	35.9±2.6 <sup>b</sup>	45.1±4.2	45.0±3.1 <sup>b</sup>	82.4±4.0 <sup>b</sup>	81.4±4.3

<sup>1)</sup>The percentages of the number of double-stained neurons to the total number of the IL-1β positive neurons; <sup>2)</sup>The percentages of the number of double-stained neurons to the total number of the IL-1RI positive neurons.

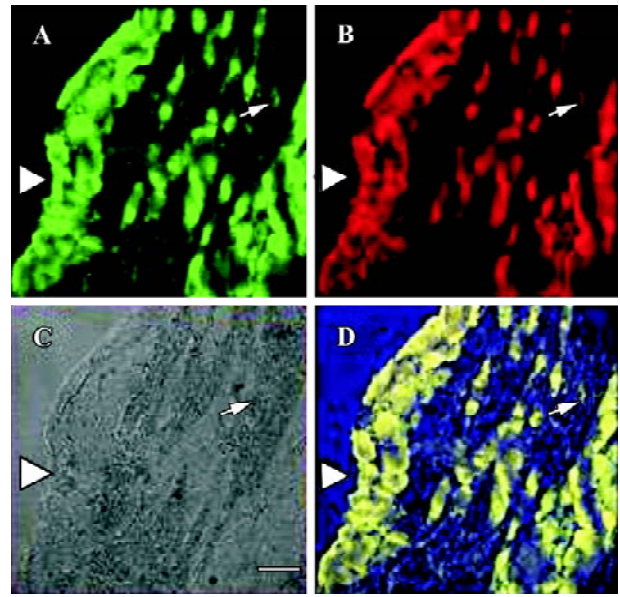
**Table 6.** The percentage of IL-1β/IL-1RI double-stained large, medium neurons to IL-1β or IL-1RI single-stained Large, medium neurons of DRG. *n*=7. Mean±SD. <sup>b</sup>*P*<0.05 vs NS group.

	Double-stained neurons Number/38.4mm <sup>2</sup>	IL-1β positive neurons Number/38.4mm <sup>2</sup>	IL-1RI positive neurons Number/38.4mm <sup>2</sup>	% <sup>1)</sup>	% <sup>2)</sup>
NS group	17.9±1.8	25.9±2.7	21.3±2.3	63.6±6.6	76.1±7.0
AA group	26.2±2.3 <sup>b</sup>	34.8±3.2	30.0±2.4 <sup>b</sup>	76.8±4.8	87.5±2.8

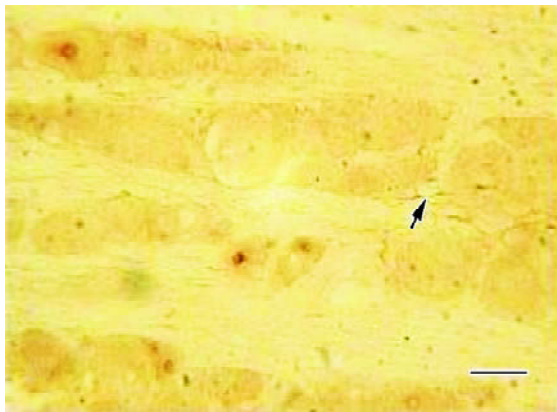
<sup>1)</sup>The percentages of the number of double-stained neurons to the total number of the IL-1β positive neurons; <sup>2)</sup>The percentages of the number of double-stained neurons to the total number of the IL-1RI positive neurons.



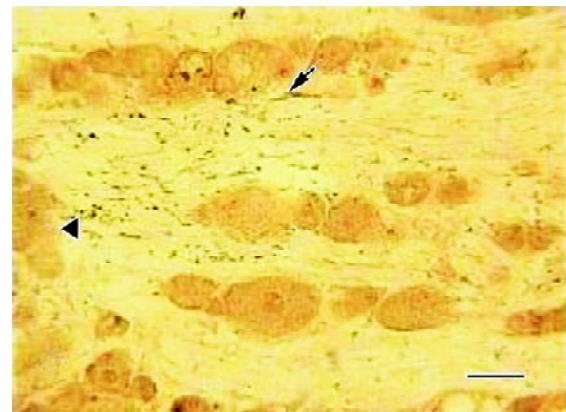
**Figure 1.** The FITC labeled IL-1 $\beta$  immunoreactivity positive cells (A), Cy3 labeled IL-1RI immunoreactivity positive cells (B), cells under normal light (C), and IL-1 $\beta$ /IL-1RI double-stained immunoreactivity positive cells (D) in DRG of NS group. Arrowhead pointed to double-stained neurons, while arrow pointed to double-stained glia cells. The immunoreactivity positive products were mainly located in the cytoplasm and nucleus. Scale bar=100  $\mu$ m.



**Figure 2.** The FITC labeled IL-1 $\beta$  immunoreactivity positive cells (A), Cy3 labeled IL-1RI immunoreactivity positive cells (B), and cells under normal light (C), and IL-1 $\beta$ /IL-1RI double-stained immunoreactivity positive cells (D) in DRG of AA group. Arrowhead pointed to double-stained neurons, while arrow pointed to double-stained glia cells. The immunoreactivity positive products were mainly located in the membrane and nucleus. Scale bar=100  $\mu$ m.



**Figure 3.** The IL-1RI positive glia cells sparsely distributed in the connective tissues between neurons of DRG of NS group, whose cytoplasm was stained as light brown (arrow). Scale bar=25  $\mu$ m.



**Figure 4.** The IL-1RI positive glia cells sparsely distributed in the connective tissues between neurons of DRG (arrowhead) or ensheathen the large sensory cell bodies (arrow) of AA group, whose cytoplasm was stained as deep brown. Scale bar=25  $\mu$ m

## Discussion

IL-1 $\beta$  is one of the cytokines synthesized and released from immunocytes and considered to be an important mediator during inflammation and hyperalgesia. Indeed, the injection of IL-1 $\beta$  into animals has been shown to produce hyperalgesia in response to mechanical and thermal stimuli<sup>[2-4,22]</sup>. There are several lines of evidence indicating that IL-1 $\beta$  al-

ters primary afferent neural activity or sensitivity in mice and rats<sup>[10,23-25]</sup>. Nociceptive primary afferent neurons contain neuropeptides such as SP, neurokinin A, and calcitonin gene-related peptide that are released on stimulation of these afferent fibers. A previous study of cellular culture experiment demonstrated that IL-1 $\beta$  can induce SP to release from cultured DRG by activating the COX-2 system. IL-1R antago-

nist can block this access, which suggests an IL-1 $\beta$  exerted effect by combine with IL-1R on DRG<sup>[15]</sup>. Using RT-PCR with *in situ* hybridization, it was found that type I IL-1R, not type II, expressed on the DRG of normal rats<sup>[26]</sup>. However, there is no study on the coexistence of IL-1RI and IL-1 $\beta$  in neurons and glia cells of DRG and the influence of adjuvant-induced arthritic on it.

In the present study, we demonstrated that both IL-1RI and IL-1 $\beta$  coexisted in the sensory neurons and glia cells of normal saline injected rats. The combined expression of IL-1 $\beta$  and the type I IL-1 receptor suggests autocrine or paracrine IL-1 $\beta$  signaling in sensory neurons.

Meanwhile a significant increase in the IL-1RI and IL-1 $\beta$  double stained neurons in ipsilateral DRG were observed in the AA group than in the NS group, and IL-1RI mainly distributed in the membrane of the AA group than in the cytoplasm of NS group. This morphological change also paralleled to the pain behaviour scores. It indicated that the inflammation resulted in the up-regulation of the expression of IL-1RI on the membrane of neurons in ipsilateral DRG, and the increase of IL-1 $\beta$  sythesis as well.

It has been found that intraplantar injection of CFA increased the concentration of IL-1 $\beta$  and NGF accompanied by elevation of the level of substance P and calcitonin gene-related peptide (CGRP) in the L4 dorsal root ganglion 48 h post CFA injection<sup>[5]</sup>. The author demonstrated that IL-1 $\beta$  could alter neuropeptide expression in primary sensory neurons secondary to an increase of NGF levels as NGF has well known actions on the regulation of both SP and CGRP in adult rat DRG neurons<sup>[6]</sup>. It has been recently proved that IL-1 $\beta$  increases SP synthesis by enhancing the expression of preprotachykinin mRNA encoding for SP and other tachykinins in the DRG<sup>[27]</sup>. Our results showed that IL-1 $\beta$  could be synthesized in neurons of DRG, as wells as in the inflamed tissue from previous study, it indicated that more IL-1 $\beta$  was able to release by neurons of DRG and excite more IL-1RI, which presumably brought about more SP synthesis and release in the central and peripheral terminals of neurons in the DRG, which in turn led to hyperalgesia.

We also found that the number of glia cells in ipsilateral DRG, which express IL-1RI, increased after CFA injection. The expression of type I IL-1 receptor in non-neuronal cells in the DRG might mediate another possible function of IL-1 $\beta$  synthesized by the sensory neurons. It has been reported that IL-1 $\beta$  enhances neurite outgrowth in DRG<sup>[28]</sup>, which would contribute to tactile hypersensitivity of articular arthritis. This effect of IL-1 $\beta$  seems to be indirect and presumably involves an IL-1 $\beta$ -induced increased in release of neurotrophins from nonneuronal cells<sup>[29]</sup>. It could be that,

under inflammatory condition sensory neurons regulate their own maintenance by releasing more IL-1 $\beta$  and thus stimulating their environment to release neurotrophins to induce hyperalgesia.

Our study showed that IL-1 $\beta$ /IL-1RI doubled stained immunoreactivity positive products were located in the nucleus as well as the cytoplasm or membrane of positive neurons in ipsilateral DRG of both groups. This is consistent with the results of immunoelectron microscopy, which found that GSSP-intensified IL-1 $\beta$  immunoreaction products were found dispersed in small aggregates throughout the cytoplasm and a few small immuno-aggregates of IL-1 $\beta$  were also found in the nucleus of neurons in DRG. The functional significance of intranuclear IL-1 $\beta$ /IL-1RI is as yet unclear, but might suggest involvement in transcriptional regulation<sup>[16]</sup>.

In conclusion, we found coexistence of IL-1 $\beta$  and IL-1RI in sensory neurons and glia cells of DRG. The presence of the type I IL-1 receptor in neurons and non-neuronal cells suggests an as yet unknown autocrine and/or paracrine function of IL-1 $\beta$  in the DRG. The function was enhanced in the articular arthritis induced by CFA and might play an important role in hyperalgesia under the inflammatory condition.

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