Acta Biologica Hungarica 54 (3–4), pp. 275–283 (2003)

IMMUNOHISTOCHEMICAL ANALYSIS OF SUBSTANCE P CONTAINING NERVE FIBRES AND THEIR CONTACTS WITH MAST CELLS IN THE DIABETIC RAT'S TONGUE

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(Received: February 2, 2003; accepted: September 10, 2003)

Sensory neuropathy is common symptom of the diabetes mellitus and the prevalence of oral lesions is higher in diabetic patients. The distribution of substance P was studied immunohistochemically in streptozotocin induced diabetic rat's tongue. The morphological association of sensory nerves (substance P immunoreactive) with mast cells (nerve fibre–mast cell contact) was monitored. The substance P nerve fibre–mast cell contacts were very scanty in control tongue. The number of substance P nerve terminals and mast cells was significantly increased ($p < 0.05$) in diabetes mellitus after 4 weeks of the treatment compared with the control tongue. The number of mast cell–nerve contacts was even more significantly increased ($p < 0.001$) in diabetes. The distance between nerve fibres and mast cells was about 1 mm and very often less than 200 nm. In some instances, the mast cells were degranulated in the vicinity to nerve fibres. Increased number of mast cell–nerve contacts in neurogenic inflammation might cause vasoconstriction and lesions of the oral mucosa, so some disorders such lichen planus, leukoplakia and cancer might frequently develop in diabetes mellitus.

Keywords: Substance P – inflamed tongue – diabetic rat – streptozotocin – immunohistochemistry

INTRODUCTION

Diabetes mellitus affects 5% of the population of Hungary [20]. Its complications in the oral cavity include parodontosis, leukoplakia, lichen oris and glossitis. The prevalence and occurrence of oral leukoplakia and lichen planus in patients with diabetes mellitus was found much higher (6.2%) compared to the control group (2.2%) [1], where occurrence was the highest in the second year of established diabetes. It was also demonstrated that the incidence of diabetes mellitus in patients with malignant tumours in the oral cavity was more than 3 times higher than that for the normal population [30].

Neuropeptides have been shown to stimulate and activate mast cells isolated from different tissues of many species [10, 25, 27], suggesting a modulatory effect on mast

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0236-5383/2003/$ 20.00

cell activity. In oral lichen planus the number of mast cells, as well as the mast cell–nerve interactions increased, suggesting a controlling role over the lesioned cell populations and a secondary role in the immune response [35]. Several studies suggest that substance P (SP), vasoactive intestinal polypeptide (VIP) and mast cells play a role in inflammatory processes of the gastrointestinal tract [11, 17, 31]. It is now clear that mast cells can synthesise and release a large number of biologically active substances, including arachidonic acid metabolites, biogenic amines, cytokines, enzymes and glycosaminoglycans. Neuropeptides such as SP are released from nerve terminals following the stimulation of sensory fibres, and are thought to participate in neurogenic inflammation in the skin and mucous membrane. SP induced neurogenic inflammation due to its effects on neurokinin 1 receptors as well as stimulate mast cells to release histamine, nitric oxide, serotonin and prostaglandins [16].

In the last century, pain has been recognised as a symptom of a variety of acute and chronic diabetic neurological syndromes, including truncal, proximal motor, acute sensory and chronic distal sensorimotor neuropathies [5, 6, 18, 29]. Abnormal sensations and pain are features of approximately 10% of all cases of diabetic neuropathy and can decrease the quality of life of these patients.

Therefore, the aim of this study was to investigate and quantify the SP nerve fibres which are known as sensory nerve terminals, the mast cells as well as the changes of the mast cell–nerve contacts in diabetes mellitus using immunohisto- and immunocytochemical methods.

MATERIALS AND METHODS

Male Wistar rats (n = 15) received a single dose of streptozotocin (65 mg kg^{-1}, Zanosar; Upjohn, Kalamazoo, MI, USA) injected into the tail vein. Controls (n=10) received 0.25 ml saline. The effect of streptozotocin was studied after 2, 4 weeks. Induction of diabetes was assessed by the presence of rapid weight loss (rats were weighed weekly and at the time of death), polyurea and glycosuria. Untreated controls were of the same initial weight range (330–450 g) as the diabetic group. Both groups were maintained under identical conditions and sacrificed after 2, 4 weeks. The animals were terminally anaesthetised with intraperitoneal injections of sodium pentobarbitone (60 mg/kg, Sanofi Phylaxia, Budapest, Hungary) and perfused via the aorta with saline, followed by the fixative solution containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.19% picric acid in 0.1 M phosphate-buffer (pH 7.3). Small pieces of the root of the tongue (which lacked lingual tonsils and had classical tubulo-acinar mixed glands [23]) were placed overnight in glutaraldehyde-free fixative containing 10% sucrose (at 4 °C). Sections (30 μm thick) were treated for 15 min with 0.3% hydrogen peroxide in order to remove endogenous peroxidase activity.

Light microscopic immunohistochemistry

For visualising morphological associations between mast cells and SP a double immunostaining was applied. Each specimen had its own control. The first primary antibody was used to detect substance P (anti-SP, rabbit; 1 : 10,000, Amersham, Buckinghamshire, UK), the second was anti-histamine (rabbit; 1 : 2,000, Sigma, Saint Louis, MO, USA), where the immunoreactivity (IR) was revealed using nickel-intensified diamino-benzidine (DAB) reaction. The primary antibodies were used for 48 h. For the final immunostaining an Avidin-Biotin immunoperoxidase technique was employed using a commercially available kit (Vectastain Elite ABC, Vector Laboratories, Peterborough, UK). All manipulations were performed at room temperature. The IR was visualised with DAB chromogen reaction (0.025% 3,3-diamino-benzidine, 0.0015% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.5) for 10 min at room temperature. Using quantitative analysis, the number of IR nerve fibres, mast cells as well as the mast cell–nerve fibre contacts was counted in a 15–20 mm^2 tissue area and calculated for 1 mm^2 tissue area as the average. For analysis, 40× magnification was used with a graduated eyepiece graticule and the entire section was assessed. In some serial sections the mast cells were also visualised through the use of both 0.1% toluidine blue (Sigma) and immunostained for histamine. Ten photomicrographs were taken from the tunica mucosa and the number of mast cells, IR nerve fibres as well as the mast cell–nerve contacts were again counted.

SP immunostaining for electron microscopy

The rats were perfused as described above. Small pieces of the tongue root were placed overnight in glutaraldehyde-free fixative, then in 10% sucrose for 24 h at 4 °C. 30 mm sections were cut by Vibroslices and plunged rapidly into liquid nitrogen, thawed in 0.1 M PBS. From this point on, the immunocytochemistry was performed as described above with the exceptions that Triton X-100 was omitted from all solutions, and the sections were incubated for 48 h at 4 °C in the antibody. Furthermore, the sections were osmicated in 0.5% OsO_4 containing PBS solution for 1 h and dehydrated by ascending alcohols and propylene oxide. The sections were then flat embedded in Epon. The selected sections were reembedded, and ultrathin sections were obtained with an ultramicrotome using diamond knife. The sections were counterstained using uranyl acetate and lead citrate. The materials were observed using a Jeol 100 electron microscope.

All experimental procedures used conformed to the "Principles of laboratory animal care" (NIH publication No. 86-23, revised 1985) as well as specific national laws (e.g. the current version of the Hungarian Law on the Protection of Animals, No. 243/1998).

Controls

Specificity of the immunoreaction was examined by omission of the primary antiserum or replacing the antiserum with normal rabbit serum, or when the sections were incubated in antisera preabsorbed with 10^{-6} M of the corresponding peptide, where no immunostaining appeared.

Statistical analysis

Analysis of variance (ANOVA) was employed to determine overall differences in the various nerve parameters and mast cells. In order to evaluate the statistical significance of differences between control and treated animals, data were compared with Student's two-sample *t*-test. A *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

The SP IR nerve fibres were found in all layers of the tongue, but their density was higher in the mucosa beneath the epithelium. Some IR nerve fibres were also found in the epithelium and around the acini of both mucous and serous glands (Weber's

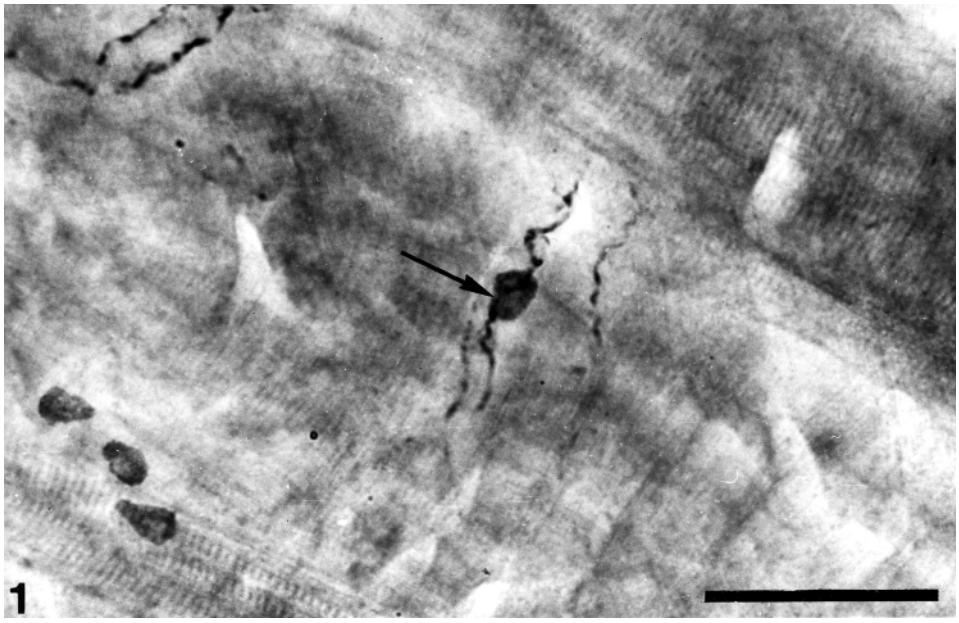

Fig. 1. A part of the tunica muscularis of the tongue from control rat. Arrow indicates a SP IR nerve fibre in contact with the mast cell. Bar scale = 100 μm

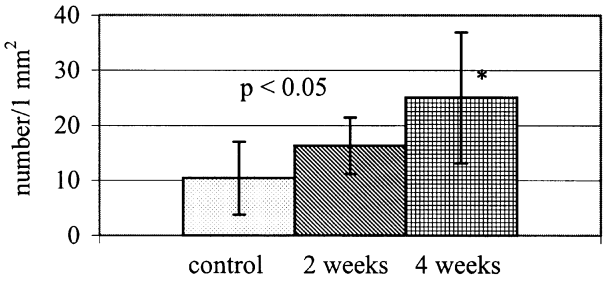

Fig. 2. The effect of streptozotocin treatment on the quantity of SP IR nerve fibres in the rat's tongue

glands), however their number was moderate. Some SP IR nerve cell bodies were also observed in the mucosa near the muscle layer in both groups. Mast cell–nerve fibre (SP IR) contacts (within 3 μm) were rarely observed in the controls (Fig. 1).

After streptozotocin treatment the oral mucosa seemed normal, however the light- and electronmicroscopic examination revealed that the lamina propria was infiltrated diffusely by inflammatory cells (lymphocytes, plasma cells and mast cells). The distribution pattern of SP IR nerve fibres in the tongue was similar to that of the controls. After four weeks of the treatment significantly increased number of SP IR nerve fibres and mast cells were observed in all layers of the diabetic tongue compared with that of control tissue (Fig. 2), especially around the blood vessels and in the lamina propria below the epithelial lining (Fig. 4). The number of SP positive nerve fibres and their contacts with mast cells increased even more significantly ($p < 0.01$) in diabetes (Fig. 3) in all layers of the tongue.

Under the electronmicroscope the varicosity of SP IR nerve terminals had a large number of large granulated and small clear synaptic vesicles; electron-dense immunoreactive material outlined the membranes of small and large granulated vesi-

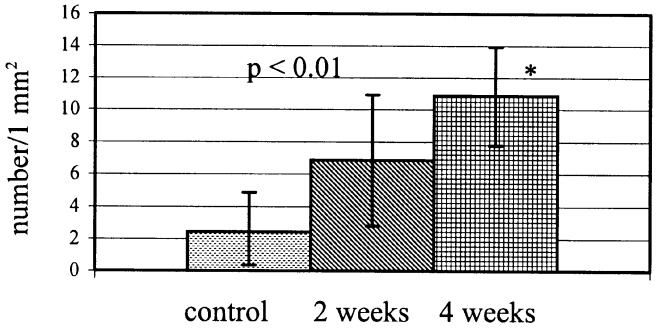

Fig. 3. The effect of streptozotocin treatment on the quantity of mast cell–SP IR nerve fibre contacts in the rat's tongue

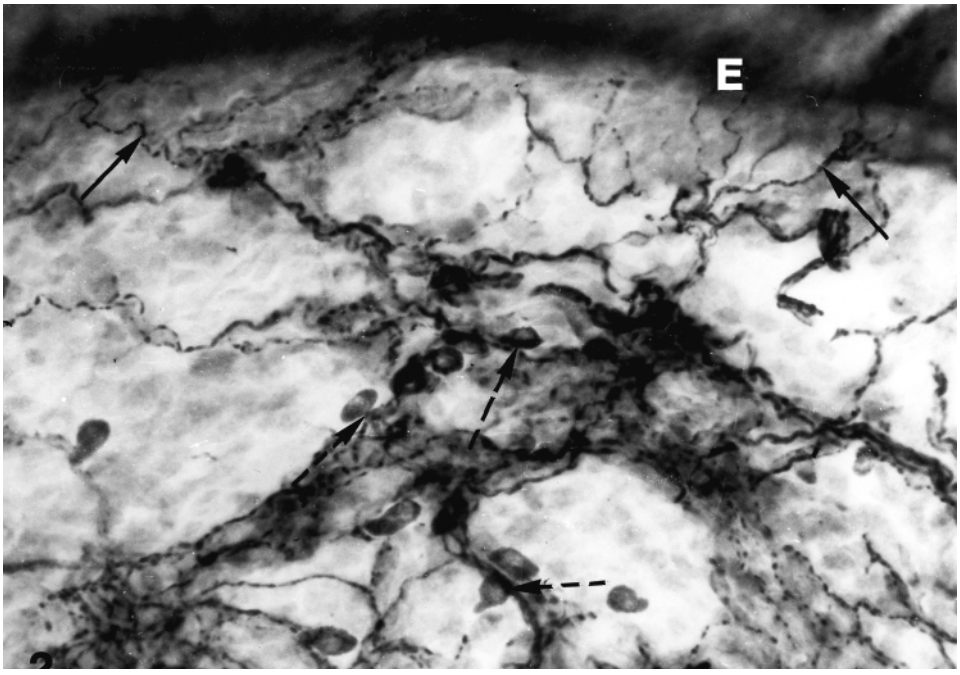

Fig. 4. A part of the lamina propria mucosae of the tongue from diabetic rat. Arrows indicate the SP IR nerve fibre beneath the epithelium (E). Dashed arrows show the mast cell–SP IR nerve fibre contacts. Bar scale = 100 μm

cles with immunolabelling of their central core were seen. These varicosities were in a very close situation (20 to 200 nm) to the membrane of the mast cell (Fig. 5).

Staining with histamine and toluidine blue indicates the presence of mast cells in all tissue layers in both groups. In the diabetic rats, a significantly increased number of them were seen in the lamina propria and around the blood vessels compared with those in the control group. Some immunocompetent cells (plasma cells and lymphocytes) were also IR for SP in the inflamed area of the diabetic rats (Fig. 5).

DISCUSSION

Close contacts between mast cells and nerve fibres have been demonstrated previously in normal and pathological conditions in several organs [4, 7, 8, 23, 32, 34]. Neuropeptides such as SP are released from nerve terminals following the stimulation of sensory fibres, and are thought to participate in neurogenic inflammation in the skin and mucous membrane [10].

Morphological contacts between mast cells and SP containing nerves provide further evidence to the view that SP is capable of amplifying the inflammatory reaction

also through the axon-reflex mechanism [24]. SP is known to enhance endocytosis by macrophages, and to stimulate their secretion of Il-1 and tumour necrosis factor [15]. SP also enhances T lymphocyte proliferation, mainly affecting the helper/inducer subset appearing in the lesions [3]. SP is also chemotactic for T cells [12], and induces the expression molecule, by the postcapillary venules in skin [21]. In the present study, a clear increase of SP positive nerves was detected in the diabetic tongue, as well as SP positive nerve fibres were seen in morphological contact with the mast cells indicate that SP is able to amplify the inflammation through the axon-reflex mechanism. Neurogenic inflammation caused by diabetes induce mast cell degranulation [2, 8, 26] and further the activated mast cells release an array of vasoactive compounds, such as histamine and leukotrienes, which are capable of causing vasoconstriction, lesions of the mucosa. When the oral mucosa homeostasis is not main-

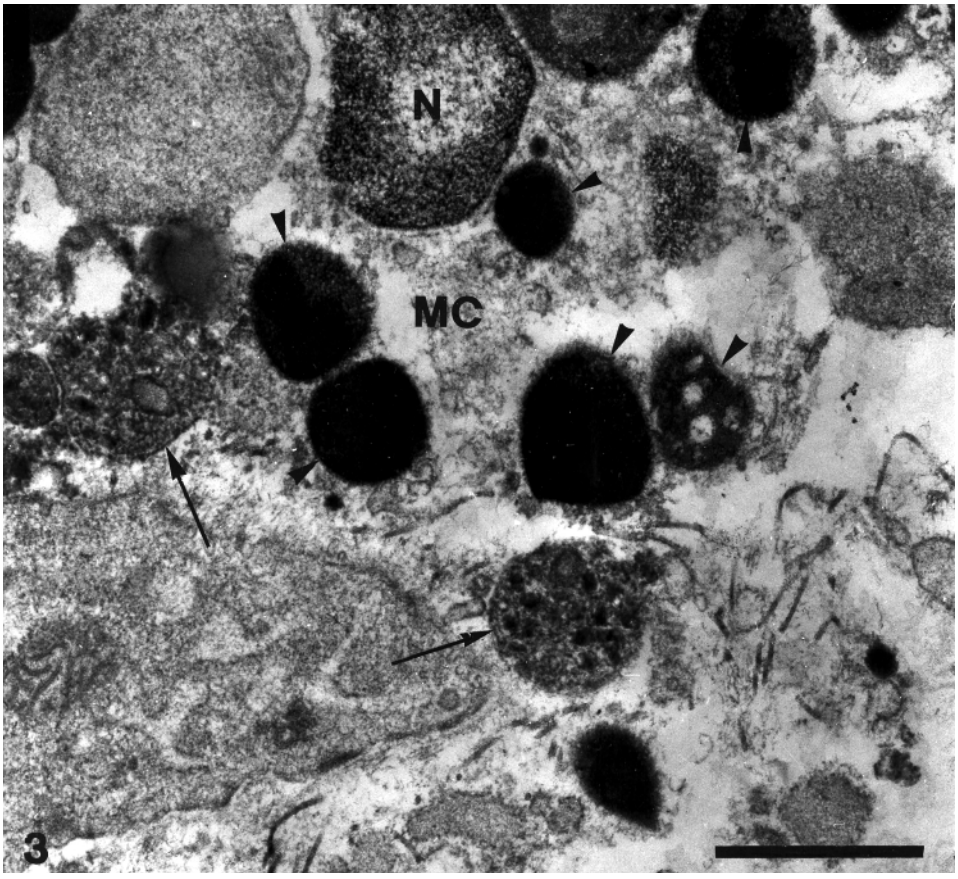

Fig. 5. Electronmicrograph from a part of the lamina propria mucosae of the diabetic rat's tongue. Arrows indicate the SP IR nerve fibres in a close situation (within 1 m) to the mast cell (MC). Arrowheads show the granules in the vicinity to the nucleus (N) of mast cell. Bar scale = 1 μm

tained, disorders such lichen planus, leukoplakia, oral cancer may frequently develop in diabetes mellitus [19, 30], might be due to hyperreactive peptidergic neurons [14].

Acute axonal degeneration [9] and/or regeneration have also been suggested to be involved in pain generation [15], it is also possible that pain may be associated with a particular phase of nerve regeneration [9]. Damage to primary sensory afferents in laboratory rats leads to the development of axonal sprouts [13]. A recent meta-analysis of four trials [28, 34] reported an overall favourable effect of capsaicin which deplete the SP level of sensory nerves, compared with placebo in diabetic neuropathy. In chronic neuropathic pain states, amount of basal SP release into the peripheral tissues is increased. Such enhanced SP release may lead to neurogenic inflammatory responses, and then finally cause desenzitization of SP receptors existing in small blood vessels and/or inflammatory cells such as mast cells [33]. Changes of SP IR nerve fibres, mast cells and mast cell–nerve fibre contacts in the diabetes mellitus in time may contribute to the formation of oral lesions, so the cancer.

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