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**STRAIN DIFFERENCES IN CONCANAVALIN A-INDUCED PAW EDEMA IN THE RAT:
INVOLVEMENT OF HISTAMINE H₁ AND H₂ RECEPTORS**

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The present study tests the hypothesis that the difference in the intensity of paw edema found between the Dark Agouti (DA) and Albino Oxford (AO) rat strains originates from the distinct participation of histamine, serotonin and their corresponding receptors in Concanavalin A (Con A)-induced inflammation. DA and AO male rats were intraplantarly injected with specific receptor antagonists prior to Con A, and the intensity of inflammation was determined by measuring the paw diameter.

Our results have showed that histamine H₁ and H₂ receptor antagonists reduced the Con A-induced paw edema in DA rats, while serotonin 5HT₃ receptor antagonist diminished the inflammation in both DA and AO rat strains. The calcium channel blocker did not change Con A-induced inflammation.

Strain differences in the intensity and kinetics of inflammation observed between the DA and AO rats are most likely defined by the diversity of mediators released and their receptors activated upon Con A injection.

Key words: Albino Oxford (AO) rat, antagonists, Concanavalin A (Con A), Dark Agouti (DA) rat, paw edema

INTRODUCTION

T cell mitogen concanavalin A (Con A), a glycan-binding protein isolated from the seeds of jack beans (*Canavalia ensiformis*), is widely used for *in vitro* studies of the mechanisms involved in T cells activation (Di Sabato *et al.*, 1987). Con A *in vivo* stimulates T cell proliferation and activates macrophages (Deng *et al.*, 1996), either via direct binding to macrophage surface (Barral-Netto and Barral, 1986) or by the activation of lymphocytes. Furthermore, Con A recruits cells to the peritoneal cavity (Rodriguez *et al.*, 1992) and stimulates macrophage nitric oxide (NO) production (Andrade *et al.*, 1999). Intravenous or retro-orbital application of Con A at substantially high doses (10-30 mg/kg) causes severe hepatitis in mice, characterized by the inflammatory infiltration (neutrophils,

macrophages, and T cells) into the liver and the release of various cytokines, including tumor necrosis factor alpha (TNF α) (Tiegs *et al.*, 1992). In contrast, a non-hepatotoxic dose of Con A (3 mg/kg) induces high levels of interleukin (IL)-6 and prevents liver damage provoked by the high dose of Con A (Xu *et al.*, 2006). Accordingly, Con A seems to be an activator of the immune system at multiple levels.

It has been reported that Con A injected in the footpads of mice and rats induces an intense and reproducible inflammatory response characterized by edema, erythema and induration at the site of the injection (Shier *et al.*, 1974; Vujić-Redžić *et al.*, 2000). The Con A-induced local inflammatory response is a valuable tool for rapid testing of potential anti-inflammatory drugs. However, we have observed significant strain differences in the intensity of Con A-induced paw edema in rats. Depending on the rat strain, the increase of paw diameter varies from 28% to 82% and reaches its maximum at different time points following the injection of Con A. Intraplantar, intraperitoneal or intracerebroventricular application of the opioid peptides methionine-enkephalin and β -endorphin, as well as anti-methionine-enkephalin antibodies and peptides belonging to the neuropeptide Y family, also modulated the Con A-induced paw inflammation in rats, in a receptor- and strain-specific manner (Vujić-Redžić *et al.*, 2000; Stanojević *et al.*, 2006a; Stanojević *et al.*, 2006b). Although several mechanisms underlying inflammatory action of Con A have been proposed, including histamine and serotonin release, mediators involved in Con A inflammation have not been studied extensively.

Histamine is an early mediator of inflammation released from basophiles, mast cells and platelets, mainly responsible for formation of edema. Histamine induces various cellular responses via H₁ receptors on vascular endothelial cells including changes in vascular permeability as a result of cell contraction, secretion and/or surface expression of mediators of inflammation (prostacyclin, platelet-activating factor and leukotiene B4) and NO release (Leurs *et al.*, 2002). Histamine stimulates endothelial cells to express the adhesion molecules by activating both the H₁ and H₂ receptors (Kubes and Kanwar, 1994), and consequently stimulates endothelial-neutrophil adhesion. Delayed effects of histamine on endothelial cells comprise H₁ receptor-mediated increase in the mRNA expression and secretion of neutrophil-attracting chemokine IL-8 (Jeannin *et al.*, 1994). Furthermore, H₁ receptors mediate the histamine-induced stimulation of respiratory burst in neutrophils (Benbarek *et al.*, 1999) and the increase in production of proinflammatory cytokine IL-6 in macrophages (Triggiani *et al.*, 2001). In addition, histamine exerts its proinflammatory effects via histamine H₄ receptors expressed on mast cells, dendritic cells, monocytes, eosinophils and lymphocytes (Huang and Thurmond, 2008). Due to their high affinity to histamine, (i.e. 1000 fold greater than the H₁ receptors), the H₄ receptors respond to lower histamine doses released by both immunological and non-immunological stimuli. In contrast, the activation of H₁ receptors requires high concentration of histamine, like those typically found in inflammation immediately upon mast cell degranulation. It has been shown that histamine exerts anti-inflammatory activities, as well. Namely, histamine stimulates the synthesis of anti-

inflammatory cytokine IL-10 via H₂ receptors (Sirois *et al.*, 2000) and inhibits the synthesis of pro-inflammatory IL-12 (van der Pouw Kraan *et al.*, 1998), IL-18 and TNF α (Yokoyama *et al.*, 2004). Furthermore, H₂ receptors have been shown to negatively regulate the release of histamine from basophils and mast cells (Lichtenstein *et al.*, 1975).

Serotonin is a major neurotransmitter component of the inflammatory chemical milieu, and may be released from platelets, mast cells, lymphocytes, monocytes, macrophages and basophils that infiltrate areas of tissue damage; in fact, platelets and mast cells can be viewed as mobile and stationary storage cells for serotonin, respectively (Dray, 1995). Serotonin exerts similar proinflammatory effects as histamine. A number of serotonin (5-hydroxytryptamine) receptor classes (5HT₁- 5HT₇) have been identified in peripheral neuronal and non-neuronal tissues. Serotonin 5HT₃ receptor takes a special place as phylogenetically much older, and as the only ligand-gated cation channel receptor among the other serotonin receptors (Färber *et al.*, 2004). It was proposed that serotonin 5HT₃ receptors are exclusively neuronal, both centrally and peripherally (Fozard, 1984). However, 5HT₃ receptors were also detected on immune cells, especially upon Con A stimulation (Fiebich *et al.*, 2004a; Stefulj *et al.*, 2000). Additionally, some specific 5HT₃ receptor antagonists displayed anti-inflammatory activity (Fiebich *et al.*, 2004b).

In view of these facts, the present study was set up to test the hypothesis that the difference in the intensity of paw edema in Dark Agouti (DA) and Albino Oxford (AO) rat strains originates from the distinct participation of histamine, serotonin and their corresponding receptors in Con A-induced inflammation. The involvement of histamine and serotonin in Con A-induced paw edema was investigated by the use of antagonists specific for histamine H₁ and H₂ and serotonin 5-HT₃ receptors. Since Ca²⁺ ions participate in the release of inflammatory mediators (De Vries *et al.*, 1995; Rodler *et al.*, 1995) and the release of peripheral nerves neurotransmitters involved in the control of local inflammatory milieu (Augustin, 2001), a specific calcium channel blocker was also utilized.

MATERIALS AND METHODS

Animals

Seven months old DA and AO male rats were obtained from our breeding colony at Immunology Research Center "Branislav Janković", Belgrade. Animals were housed individually in perspex-walled cages with free access to food and water. Colony conditions were maintained using 12 h light and 12 h dark cycles (lights on at 08:00). All procedures involving animals were approved by the Institutional Animal Care and Use Committee and followed the guidelines described in the European Community's Council Directive dated 24th November 1986 (86/609/EEC).

Drugs

Specific antagonists for histamine H₁ receptors (Chloropyramine hydrochloride; N-p-Chlorobenzyl-N', N'-dimethyl-N-(2-pyridyl) ethylenediamine;

Chl) and antagonist for serotonin 5HT₃ receptors (Granisetron hydrochloride®; Gran) were purchased from Pliva, Croatia. Histamine H₂ receptor antagonist (Ranitidine hydrochloride®; Ran) was obtained from Remevita, Serbia. Calcium channel antagonist verapamil hydrochloride (5-(N-(3,4-Dimethoxyphenylethyl) methylamino)-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile hydrochloride; Ver) was obtained from Galenika, Serbia (Izopamil®). Concanavalin A (isolated from *Canavalia ensiformis*, type III) was acquired from Sigma (St. Louis, Mo., USA). All the drugs were dissolved in sterile, apyrogenic saline prepared from purified water (with endotoxin units below the level of detection according to limulus amoebocyte lysate (LAL) assay, Torlak Institute in Belgrade).

Induction of inflammation and treatments

Inflammatory edema was induced by a single intraplantar (i.pl.) injection (i.e. into the dorsal part of the metatarsal region of hind paws) of Con A pH 7 (8 mg/mL saline, 400 µg Con A in 50 µL per paw).

Antagonists specific for histamine H₁ (chloropyramine) and H₂ (ranitidine), receptors, and calcium channel blocker (verapamil) were intraplantarly injected at the dose of 100 µg per paw (2 mg/mL saline, 50 µL per paw) 30 minutes prior to Con A, while the control animals were injected with 50 µL of saline per paw 30 minutes prior to Con A. The antagonist specific for serotonin 5HT₃ receptors (granisetron) was injected at 50 µg per paw (1 mg/mL saline, 50 µL per paw) in the same manner. Separate groups of rats were i.pl. treated with 50 µL of the specific receptor antagonists or saline in the absence of inflammation.

To minimize animal discomfort during handling and i.pl. treatments, the rats were briefly (1-3 min) exposed to isoflurane vapor and closely monitored for the occurrence of early signs of sedation observed as behavioral tranquility and calmness. This procedure permitted full recovery within a minute after the treatment.

As maximal increase of paw diameter occurred 12 h and 9 h after induction of inflammation in DA and AO rats, respectively, the intensity of inflammation was determined by measuring the diameter of the tarso-metatarsal joints using a nonius before, and 3, 6, 12, and 24 h after induction in DA rats and before, and 3, 6, 9 and 24 h after induction in AO rats. The number of rats per group was 6-8.

The results are expressed as relative increase of paw diameter (%) according to the formula:

$$d \text{ rel} = ((d \text{ after injection} - d \text{ before injection}) / d \text{ before injection}) \times 100$$

Statistical analysis

Statistical analysis of the data was performed using the SPSS 10.0 computer programme. The results were analyzed by one-way ANOVA and Student's independent samples t-test. LSD and Scheffe tests were used for *post hoc* analysis for the evaluation of the differences between independent groups. In the figures, all data are displayed as mean ± (S.E.M.).

RESULTS

Development of Con A-induced paw inflammation

Both rat strains developed inflammatory edema upon Con A injection that was observed 24 h post-induction (Fig. 1). Local Con A injection did not cause a spontaneous pain behavior in either of the strains, as judged by the absence of limping or guarding of the affected hindpaws. As in our previous study (Stanojević *et al.*, 2006a), a maximal increase of paw diameter was observed 12 h after the Con A injection in DA rats, reaching the levels of 70% increase over the controls injected only with saline (Fig. 1, upper panel). AO rats exhibited substantially a lower degree of paw inflammation reaching 40% of increase in paw diameter 9 h after the induction with Con A (Fig. 1, lower panel). Some degree of tissue swelling was observed in rats of AO strain during the first hours following the injection with saline.

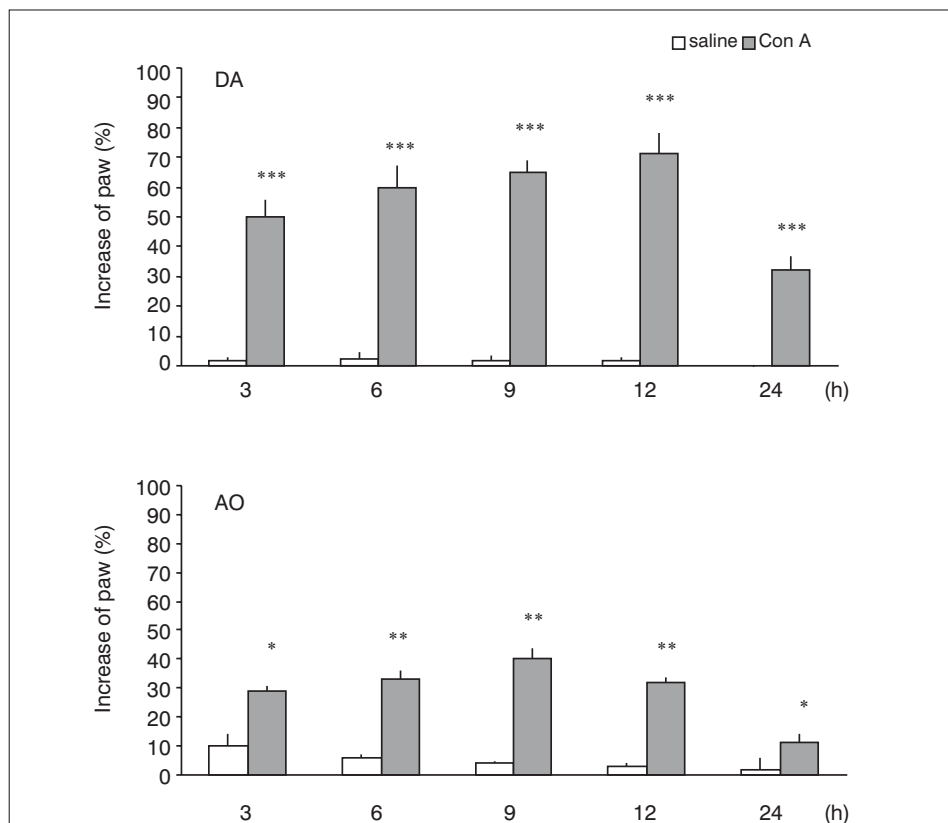


Figure 1. Development of Concanavalin A (Con A)-induced paw edema in rats of DA (upper panel) and AO (lower panel) strains. Values represent mean \pm SE. Statistically significant differences: * $p < 0.01$; ** $p < 0.001$; and ***, $p < 0.0001$ vs. saline

The effect of histamine receptor antagonists on Con A-induced paw inflammation

In DA rats, the H₁ receptor antagonist chloropyramine decreased paw diameter up to 12 h following the Con A injection, while the H₂ receptor antagonist ranitidine decreased it at all time points (Fig. 2, upper panel). In rats of the AO strain, the i.pl. injection of chloropyramine gradually increased inflammation following the pattern of Con A induced paw swelling, but with much higher intensity (more than 80% of increase over the control 9 h post-injection, compared to 40% in Con A-treated paws) and remained effective even 24 h after injection (Fig. 2, lower panel). Histamine H₂ receptor antagonist ranitidine exerted proinflammatory activity as early as 3 h after the Con A injection in AO rats that remained relatively unchanged for the next 6 hours and returned to the control values within 24 h.

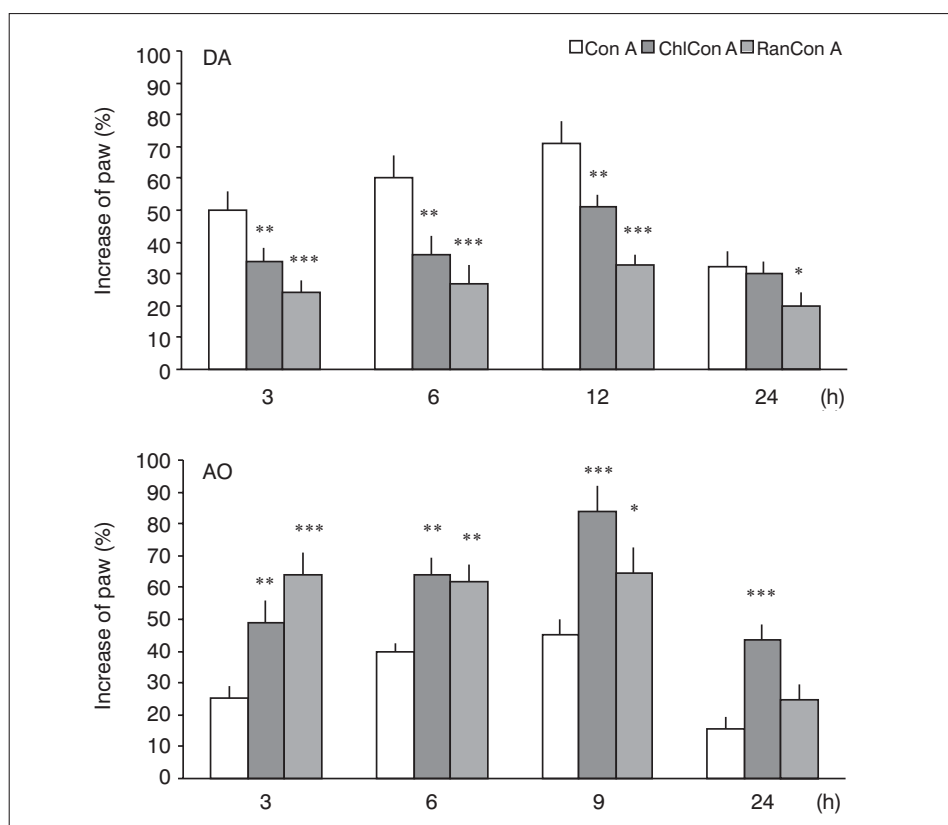


Figure 2. The effect of i.pl. injection of 100 µg of chloropyramine (Chl) and ranitidine (Ran) on development of paw edema induced by Con A in rats of DA (upper panel) and AO (lower panel) strains. Values represent mean ± SE. Statistically significant differences: * p < 0.05; ** p < 0.01; and *** p < 0.0001 vs. Con A

The effect of Ca²⁺ channel blocker and serotonin 5HT₃ receptor antagonist on Con A-induced paw inflammation

Ca²⁺ channel blocker verapamil enlarged the Con A-induced paw edema in DA rats 6 h after inflammation induction, while serotonin 5HT₃ receptor antagonist granisetron decreased paw diameter 12 and 24 hours after Con A injection (Fig. 3, upper panel). In AO rats, verapamil suppressed inflammation only 24 h after Con A injection, while granisetron decreased Con A-induced inflammation 6, 9 and 24 h post-induction (Fig. 3, lower panel).

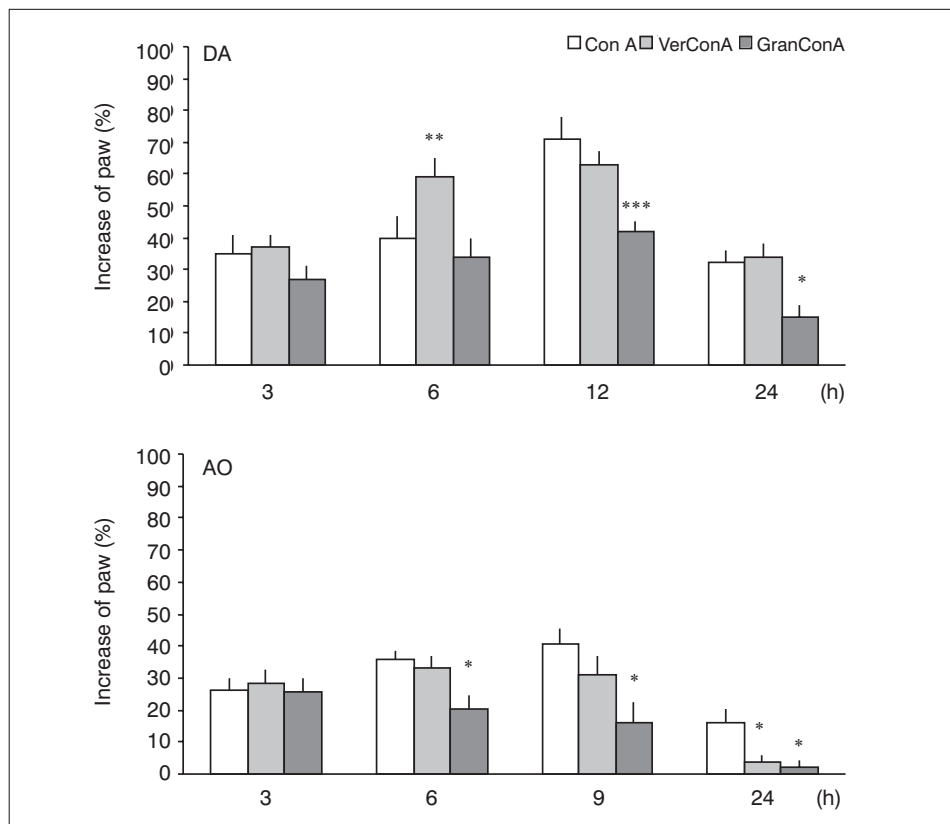


Figure 3. The effect of i.pl. injection of 100 µg of verapamil (Ver) and 50 µg of granisetron (Gran) on development of paw edema induced by Con A in rats of DA (upper panel) and AO (lower panel) strains. Values represent mean ± SE. Statistically significant differences: * p < 0.01; ** p < 0.001; and *** p < 0.0001 vs. Con A

The effect of histamine receptor antagonists, Ca²⁺ channel blocker and serotonin 5HT₃ receptor antagonist on the diameter of non-inflamed paws

In DA rats all the antagonists exerted an edema-promoting effect. In AO rats, chloropyramine and ranitidine increased paw diameter relative to saline-injected

paws, while verapamil and granisetron did not have any effect on the non-inflamed paws. Actually, the level of the increase of the paw diameter induced by verapamil and granisetron was comparable in two rat strains, but the increase induced by saline was higher in AO rats resulting in the absence of the statistical differences in verapamil and granisetron versus saline in this strain. Figure 4 shows representative results, i.e. 12 h post injection in DA and 9 h post injection in AO rats.

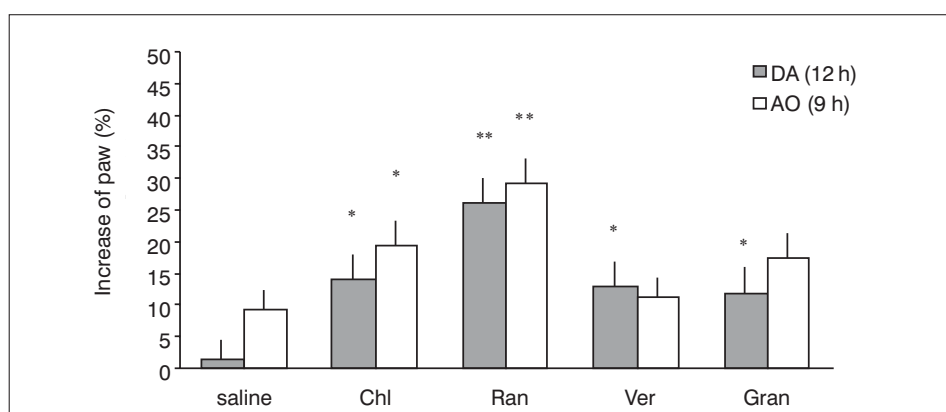


Figure 4. The effect of i.pl. injection of 100 µg of chloropyramine (Chl), ranitidine (Ran) and verapamil (Ver), and 50 µg of granisetron (Gran) on the diameter of the non-inflamed paws of DA and AO rat strains. Values represent mean ± SE. Statistically significant differences: * $p < 0.05$ and ** $p < 0.001$ vs. saline

DISCUSSION

The present study reflects the results from our previous experiments, in which the i.pl. injected Con A exerted the edema-promoting effects in both rat strains, with a substantially stronger intensity of inflammation in DA than in rats of the AO strain (Stanojević *et al.*, 2006a). Con A increases microvascular permeability directly by binding to endothelial cells (Northover and Northover, 1994), but also indirectly by inducing histamine release from mast cells (Lopes *et al.*, 2005), which induces plasma extravasation at the post-capillary venules. This is supported by the suppression of paw edema with antagonists of histamine H₁ and H₂ receptors in DA rats. It is in line with previous reports that H₁ and H₂ histamine receptor antagonists partially blocked zymosan-induced plasma exudation, and H₁ receptor antagonists reduced neutrophil influx (Kolaczowska *et al.*, 2001).

Histamine effects are mostly related to a rapid increase in vascular permeability, but in our model histamine antagonists were effective in blocking the paw edema development up to 24 h after the Con A injection. Accordingly, some histamine-liberating substances induce maximal paw edema at the moment when

histamine content already returned to the basal levels (Guo *et al.*, 1997). This could be explained by the fact that gap formation in endothelial cells elicited by histamine may persist longer than the metabolism of histamine. In addition, some histamine effects might be related to a continuous histamine release. Although the richest source of endogenous histamine are mast cells located around vascularized tissues and nerves, in which granule-stored histamine is rapidly and transiently liberated upon activation, it was shown that macrophages, T cells and neutrophils, cells that are readily found in the inflammatory foci, can also produce and release histamine, but in a rather continuous manner (Yokoyama *et al.*, 2004; Tanaka and Ichikawa, 2006). Along with many other inflammatory and immune mediators, histamine and serotonin can also stimulate peptidergic afferent nerve fibers or enhance their excitability and release vasoactive peptides, and in that sense indirectly prolong inflammation (i.e. calcitonin gene related peptide induces neurogenic dilatation of arterioles, substance P and neurokinins increase venular permeability) (Holzer, 1998).

Since the differentiation of monocytes into macrophages enhances H₁ and suppresses H₂ receptors expression (Wang *et al.*, 2000), the more prominent involvement of H₂ histamine receptors during the Con A-induced inflammation might point to a greater contribution of less mature (i.e. inflammatory) monocytes in rats of the DA strain.

In contrast, histamine was not the primary inflammatory agent implicated in the response induced by Con A in AO strain rats. Rather, it served to constrain inflammation development as judged by aggravation of inflammation following the pretreatment with both of the specific antagonists. As histamine inhibits the synthesis and/or LPS-induced release of TNF α in monocytes, while H₂ receptor antagonist ranitidine increases the release of TNF α from mast cells (Bissonnette, 1996), it has been suggested that histamine acts as an autocrine regulator of TNF α release, through histamine H₂ receptors. Furthermore, histamine also suppresses recruitment of neutrophils and macrophages upon ischemia/reperfusion injury, air-pouch and pleural inflammation (Hiraga *et al.*, 2007; Hirasawa *et al.*, 1991), acting via H₂ receptors. The administration of H₂ receptor antagonists induces an increase of neutrophil infiltration (Hirasawa *et al.*, 2002). In spite of general inflammation-promoting effects of H₁ receptors, it has been also shown that histamine via H₁ receptors downregulates IL-12p70 production in stimulated dendritic cells (Gutzmer *et al.*, 2002). In that sense, a local application of histamine H₁ and H₂ receptor antagonists might counteract antiinflammatory effects of histamine in AO rats and aggravate inflammation. H₁ and H₂ antagonists might nonspecifically liberate a variety of inflammatory mediators from mast cells and thus indirectly augment lectin-induced inflammation.

We have shown that serotonin was involved in the Con A-induced paw edema in both rat strains via 5HT₃ receptors, since their blockade significantly decreased paw diameter in the peak and late phases of inflammation in DA rats, and in early, peak and late phase of inflammation in AO rats. Specific 5HT₃ receptors were detected on immune cells, especially upon Con A stimulation (Fiebich *et al.*, 2004a; Stefulj *et al.*, 2000). In addition, 5HT₃ receptors are present on capsaicin-sensitive sensory nerves and following their activation with serotonin

they increase the release of inflammatory neuropeptides and in that way promote inflammation (Hua and Yaksh, 1993). Nevertheless, the 5HT₃ receptor antagonists exerted an antiinflammatory effect by inhibition of TNF α and IL-1 β release from human monocytes (Fiebich *et al.*, 2004b). It seems that in Con A-induced inflammation model resident immune cells in concert with those recruited to the inflammation site can release serotonin, which binds to 5HT₃ receptors and in autocrine/paracrine way regulates and increases cytokine production *in situ*. The effect of serotonin on 5HT₃ receptors present on primary afferents in the inflammatory focus can not be excluded.

The release of histamine and serotonin from mast cells is a Ca²⁺ dependant process (Suzuki-Nishimura *et al.*, 1989). Besides, it has been reported that development of carrageenan-induced edema (Khaksari *et al.*, 1999) and skin inflammation (De Vries *et al.*, 1995) in rats, as well as serotonin-induced paw edema in mice (Cole *et al.*, 1995) were blunted by Ca²⁺ antagonist. Con A induces a rapid initial increase in intracellular concentration of Ca²⁺ and successive Ca²⁺ influx through Ca²⁺ channels (Ikegami *et al.*, 1991). Accordingly, we have expected that Ca²⁺ channel blocker verapamil could inhibit the increase in paw diameter in Con A induced-inflammation. The finding that verapamil did not affect paw inflammation in AO rats supports the notion that only intracellular Ca²⁺ is required for mast cell histamine/serotonin release (Truneh and Pearce, 1981). In contrast, the aggravation of paw swelling in DA rats following a treatment with verapamil suggests its proinflammatory action. Several proinflammatory effects of verapamil were described, such as stimulation of NO production (Sirmagul *et al.*, 2004) and rise in IL-6 production and successive increase in vascular permeability (Rodler *et al.*, 1995).

Although saline should be devoid of any inflammatory activity, the finding that saline induced a slight paw edema in AO, but not in DA rats most likely revealed strain differences in mast cell membrane stability. It has been reported that trauma induced by needle penetration can activate small fiber sensory neurons to release proinflammatory and proanalgesic substance P, neurokinins and calcitonin gene-related peptide and consequently cause a discharge of mast cell inflammatory mediators (Guo *et al.*, 1997). Saline injection induced a significant increase in histamine release, but the level of histamine returned to control values during the next 2 hours (Guo *et al.*, 1997). In addition, the saline injected into the upper surface of the rat paw in our experimental model could disturb the organization of the subcutaneous tissue, e.g. by inducing an increase in the thickness of connective tissue, separation of collagen fibers, and dilatation of blood and lymphatic vessels (Daitoku *et al.*, 2007), therefore contributing to a transient edema formation in AO rats.

All antagonists used in our study increased the paw diameter in both rat strains if injected in the non-inflamed paws. Such a uniform edema-promoting behavior of antagonists is probably not due to the blockage of specific receptors and prevention of binding of endogenous ligands, but rather to some unspecific activity. As antagonists were injected at the dose of 100 μ g per paw, except granistreon (50 μ g per paw), and their actual concentrations ranged from 3 to 7 x 10⁻³ M in tissue, it is most likely that their high concentration although safe for

systemic application, exerted a damaging effect if injected locally in the paws. In presence of inflammation, the primary proinflammatory effect of antagonists was probably shifted towards the receptor-specific antagonistic activity against mediators released following Con-A injection.

In general, our results showed that histamine mediated Con A-induced paw edema in DA rats via H₁ and H₂ receptors, while serotonin participated in inflammation in both DA and AO strains via 5HT₃ receptors. Since histamine and serotonin are co-stored and co-released from mast cells, it seems that strain differences in Con A-induced inflammation may originate from different receptor subtypes expressed on vascular endothelial cells in AO and DA rats. Extracellular calcium ions were not involved in Con A-induced inflammation.

Strain differences found in the intensity and kinetics of inflammation between DA and AO rats are most likely defined by the diversity of mediators released and their receptors activated upon Con A injection. While still bearing in mind the individual variations in building the inflammatory response, the model of Con A-induced rat paw edema can be quite useful for determining the anti-inflammatory potency of different pharmacological substances.

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**RAZLIKE U EDEMU ŠAPE PACOVA INDUKOVANOM KONKANAVALINOM A
U ZAVISNOSTI OD SOJA: UTICAJ HISTAMINSKIH H₁ I H₂ RECEPTORA**

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SADRŽAJ

Testirana je hipoteza da razlike u intenzitetu inflamatornog edema šape indukovano konkanavalinom A u pacova Dark Agouti (DA) i Albino Oxford (AO) soja potiču od različitog doprinosa histamina i serotonina i njihovih odgovarajućih receptora. Mužjaci pacova DA i AO soja su intraplantarno tretirani antagonistima specifičnih receptora pre izazivanja inflamacije konkanavalinom A i intenzitet inflamacije je praćen merenjem dijametra šape.

Naši rezultati su ukazali da antagonisti histaminskih H₁ i H₂ receptora smanjuju edem šape indukovano konkanavalinom A u DA pacova, dok antagonist serotoninskih 5HT₃ receptora smanjuje edem šape u oba soja pacova. Blokator kalcijumskih kanala ne utiče na inflamaciju izazvanu konkanavalinom A.

Razlike u intenzitetu i kinetici inflamatornog odgovora indukovano konkanavalinom A između DA i AO sojeva su najverovatnije posledica razlika u oslobođenim medijatorima i aktivaciji odgovarajućih receptora nakon injekcije konkanavalina A.