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INVESTIGATIVE REPORT

Serotonergic Mechanisms in Human Allergic Contact Dermatitis

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Expression of serotonin (5-hydroxytryptamine; 5-HT), 5-HT receptors 1A (5-HT1AR) and 2A, and serotonin transporter protein (SERT) was studied in positive epicutaneous reactions to nickel sulphate in nickel-allergic patients, at 72 h post-challenge with the antigen. In addition, the effects of 5-HT2AR agonist 2,5-dimethoxy-4-iodoamphetamine (DOI), and the selective serotonin reuptake inhibitors (SSRIs) citalopram and fluoxetine, were tested on nickel-stimulated peripheral blood mononuclear cells from nickel-allergic patients, regarding their proliferation and interleukin (IL)-2 production, as well as the effect of these SSRIs on a murine Langerhans' cell-like line (XS52), regarding its IL-1β production. Serotonin-positive platelets were increased in the inflamed skin compared with control skin. A decrease (p <0.01) in 5-HT1AR-positive mononuclear cells was evident in the eczematous skin compared with control skin, whereas 5-HT2AR- and SERT-positive cells were increased (p <0.001 for both) in the eczematous skin. Treatment of nickel-stimulated peripheral blood mononuclear cells with 5×10^{-5} mol/l of DOI inhibited (p <0.01) the proliferation of nickel-stimulated peripheral blood mononuclear cells, while no effect was found regarding IL-2 production. Citalopram at 10^{-6} mol/l tended to inhibit the production of IL-1β by the XS52 cell line. These results indicate the implication of the serotonergic system in the contact allergic reaction.
Key words: allergic contact dermatitis; serotonin; serotonin receptors; serotonin transporter protein.

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Allergic contact dermatitis (ACD) is a common inflammatory skin disorder that results from contact with a low molecular weight chemical. The antigen-presenting cells in the skin capture, process and present the antigen to sensitized T lymphocytes. Rapid activation of the T lymphocytes upon re-exposure to the relevant antigen leads to proliferation and the release of inflammatory mediators, which results in a characteristic allergic contact eczematous reaction (1).

The skin plays a significant role in the preservation of body homeostasis (2) via a neuroendocrine function. The bidirectional interplay between the nervous and immune systems has been well documented (3). In the skin, this interaction takes place through a cross-talk between the sensory nerves, autonomic nerves and immune cells via the action of neuromediators that are released by skin nerves and the immune cells *per se*, and their corresponding receptors (4, 5). These neurotransmitters and hormones can activate specific receptors on target cells and thus modulate physiological and pathophysiological effects (for review see (6)). For example, the functions of Langerhans' cells (LCs), which are key players in ACD, are affected by neuromediators (7).

Serotonin (5-hydroxytryptamine, 5-HT) is a master control neuromediator, which besides its role as a transmitter is also involved in inflammation and immunomodulation (8). Serotonin acts via up to at least 21 subtypes of receptors, of which 5-HT1AR and 5-HT2AR are the most well characterized (9, 10). The magnitude and duration of the serotonergic response is determined by the serotonin transporter protein (SERT).

The human skin expresses intrinsic serotonin biosynthetic pathways that start with the hydroxylation of L-tryptophan by tryptophan hydroxylase to hydroxytryptophan, then decarboxylation by aromatic L-aminoacid decarboxylase to form serotonin (11). Also rodent skin expresses serotonin biosynthetic pathways (e.g. see 12). In an earlier study (13), a higher concentration of serotonin was found in contact allergic compared with control skin. Also, there was a decrease in 5-HT1AR-positive dermal mononuclear cells in allergic contact eczematous compared with control skin (14).

The expression of SERT on human and murine lymphocytes (15, 16), macrophages and dendritic cells (16) has been demonstrated previously. Moreover, serotonin was suggested to mediate signalling between lymphocytes and dendritic cells (17), and the selective serotonin reuptake inhibitors (SSRIs) were shown to modulate cells of the immune system (18, 19).

In the present investigation we have further studied the contribution of the serotonergic system to the human contact allergic reaction. We used immunohistochemistry and *in vitro* methods, studying the effect of serotonergic agents on proliferation and interleukin (IL)-2 production (20) in human peripheral blood mo-

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nonuclear cells (PBMC) after stimulation by nickel sulphate, and on IL-1β production, which is important for maturation of dendritic cells (21), in a murine Langerhans' cell-like line.

MATERIALS AND METHODS

Patients

Patients with positive patch test reactions (grade 3+, according to the scoring system recommended by the International Contact Dermatitis Research Group) to 5% nickel sulphate were included in the study. Biopsies were obtained at 72 h after challenge with nickel sulphate, from the back of 13 nickel-allergic patients, from both the involved patch-tested positive reactions and the non-involved contralateral skin. Peripheral blood from 7 of these patients was drawn for *in vitro* studies.

The ethics committee at the Karolinska University Hospital, Solna, approved the study.

Processing of the biopsies

Biopsies were fixed in 10% formalin with 0.2% picric acid for 1–2 h, then rinsed in 0.1 M phosphate buffered saline (PBS) containing 10% sucrose for at least 24 h and stored at –70ºC. They were later embedded in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands) and cut (14 μm thick) using a Dittes cryostat (Leica Microsystems, Nussloch, Germany). Sections were then mounted on SuperFrost/Plus glass slides (Menzel-Glaser, Freiburg, Germany) and stored at –70ºC until immunohistochemistry. Sections were cut at approximately 100 μm intervals to include areas with different degrees of inflammation.

Chemicals

Nickel sulphate, the 5-HT2AR agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) and the SSRIs (citalopram and fluoxetine) were all purchased from Sigma-Aldrich (St Louis, USA).

Immunohistochemistry

For routine immunohistochemistry, polyclonal rabbit antibodies to serotonin (1:10,000; Diasorin, Stillwater, MN, USA) and to 5-HT1AR (S1A-170; 1:5,000), as well as monoclonal antibodies to 5-HT2AR (1:500; a generous gift from Pharmingen, San Diego, CA, USA, this antibody now being commercially available) and to SERT (dilution 1:20,000; Mab Technologies, Stone Mountain, GA, USA) were used.

Of these antibodies, the antibody to 5-HT1AR is directed against amino acids 170–186 in the second extracellular loop in rat (22). This amino acid sequence has complete homology between the human and rat. The antiserum against the extracellular portion labels a major band of 64,000 Daltons in immunoblots of solubilized protein from neonatal rat brains, and in different recombinant systems specifically labels cells transfected with constructs containing the 5-HT1AR coding sequence (23).

The mouse monoclonal antibody against the 5-HT2AR (G186-1117) is directed against amino acid residues 1–76 in the N-terminal extracellular domain of the human 5-HT2AR (24). This antibody labels human reactive, but not inactivated, astrocytes (25), Purkinje cell bodies and dendrites (26).

The mouse monoclonal anti-SERT antibody was raised against a peptide corresponding to amino acids 51–66 coupled to keyhole limpet haemocyanin (KLH) carrier protein by the addition of an N-terminal cysteine. This antibody has a high degree of specificity (27, 28).

As secondary antibodies, biotinylated goat anti-rabbit or horse anti-mouse antibodies (both diluted 1:200; Vector, Burlingame, CA, USA) were used, and to visualize the staining, the fluorochromes Cy2 (1:2,000; Amersham Biosciences, Uppsala, Sweden) or Texas Red (1:2,000; Vector) were added. Briefly, the primary antibodies were applied overnight at +4ºC and were followed by the secondary biotinylated antibodies for 40 min, after which the fluorochrome was added for 40 min and then the sections were mounted with glycerol/PBS (10:1) containing 0.1% para-phenylenediamine before being covered with glass slips.

Double staining

5-HT1A vs. tryptase-positive cells. Sections were incubated with the anti-5-HT1AR antibody, followed by a biotinylated goat anti-rabbit antibody (1:200) and Texas Red. Staining was continued with the addition of the monoclonal antibody to tryptase (dilution 1:5,000; Chemicon, Temecula, CA, USA) followed by the biotinylated horse anti-mouse antibody. Finally, Cy2 (1:2,000) was added to visualize the staining.

5-HT2A vs. CD3, CD4 and CD8. Incubation with the antibody against 5-HT2AR was followed by a biotinylated horse anti-mouse antibody, and by Texas Red, then CD4 or CD8 monoclonal antibodies (both diluted 1:80; Becton Dickinson, San Jose, CA, USA), followed by a donkey anti-mouse fluorescein isothiocyanate (FITC)-conjugated antibody (dilution 1:150; Jackson ImmunoResearch Lab., West Grove, Pennsylvania, USA). In the case of CD3, a FITC-conjugated monoclonal antibody (1:20, Becton Dickinson) was used. When these lymphocyte markers were tested separately, they exhibited the same distribution as when combined with the 5-HT2AR antibody.

SERT vs. CD1a, CD4, CD8 and CD56. Incubation with the antibody against SERT (dilution 1:20,000) was followed by the biotinylated horse anti-mouse antibody and then Texas Red. Antibodies against CD1a (1:320), CD4 or CD8 (both at 1:80), or CD56 (natural killer (NK) cell marker, dilution 1:20), all from Becton Dickinson, were then added and visualized using the donkey anti-mouse FITC-conjugated antibody (1:150).

Microscopy

Sections were examined using a Nikon Eclipse E 800 (Nikon Corp.,Yokohama, Japan) microscope equipped for epifluorescence. Cy2 fluorescent structures were visualized with a B-1E filter cube (excitation at 465–495 nm), and for Texas Red a G-1B filter cube (excitation at 540–580) was used. For quantitative analyses the slides were coded and four whole sections per slide were counted, and then the average number of positive cells was calculated. In case of serotonin-positive platelets, semi-quantification was performed, grading a low +, medium ++ or high +++ number of platelets.

Coloured images were generated using a digital video camera system (Nikon DXM 1200, Yokohama, Japan) attached to the fluorescence microscope and connected to a PC.

PBMC proliferation

PBMC were isolated from the venous blood of 7 nickel-allergic subjects using Ficoll-Paque (Amersham Biosciences). The cells were incubated at a concentration of 2×10^6 cells/ml in 96-well microplates (100 μl volume) and in macrocultures (500 μl volume) (both from Becton Dickinson). After pre-incubation for 30 min, nickel sulphate in saline (20 μl per well in the microplates and 100 μl for the macrocultures) was added, giving a final concentration of 3.8×10^{-5} mol/l. After an additional 30 min, the modulators, DOI (dissolved in deionized water and diluted in culture medium to give a final concentration of 5×10^{-5} –10^{-10}

mol/l), fluoxetine or citalopram (diluted at 10^{-5}–10^{-6} mol/l) were added in volumes of 20 µl and 100 µl, respectively.

The cells were incubated for 5 days, and 6 h before ceasing the incubation, 0.5 µCi of [methyl-^{3}H] thymidine (Amersham Biosciences) with a specific activity of 5 Ci/mmol was added in 10 µl of saline. The cells were harvested on glass fibre filters using an automatic cell harvester, then dried for 30 min and covered with melt-on scintillation sheets (all from Wallac, Upplands Väsby, Sweden). DNA incorporation was measured using a liquid scintillation and luminescence counter (Wallac) and the results were expressed as counts per minute (cpm).

IL-2 assay

For IL-2 measurements, the cells were incubated for 48 h and, following centrifugation, the supernatants were collected and used for enzyme-linked immunosorbent assay (ELISA) experiments. A DuoSet ELISA development kit (R&D systems, Abingdon, UK) that measures natural and recombinant IL-2 in cell culture supernatants was used according to the manufacturer's protocol. Briefly, 96 well-microplates were coated with capture antibody diluted in PBS 100 µl per well and incubated overnight at room temperature. Plates were blocked by adding 300 µl/well of blocking buffer (1% bovine serum albumin (BSA), 5% sucrose in PBS with 0.05% NaN_3) for 1 h. Diluted standards and samples (100 µl/well) were then added and incubated for 2 h. Then 100 µl of detection antibody was added to each well and incubated for 2 h, followed by 100 µl/well of streptavidin-horseradish-peroxidase detection reagent for 20 min. One hundred µl/well of substrate solution was added for 20 min. The plates were read at 450 nm within 30 min. Repeated washings were carried out throughout the procedure. All the incubations were performed at room temperature.

IL-1 beta assay in XS52 cell line

To study the effect of fluoxetine and citalopram on LCs, an XS52 cell line was used. This LC-like line was generated from the epidermis of newborn BALB/c mice (29) and was found to resemble LCs in their ability to present antigen, their growth factor requirement and having characteristic surface phenotypes (30). The cells were maintained in complete (c)-RPMI (Invitrogen, Paisley, UK) medium supplemented with recombinant murine granulocyte macrophage-colony stimulating factor (GM-CSF, 2 ng/ml, Sigma-Aldrich) and 10% supernatant from the NS47 cell line (see 31). Cells floating over confluent cultures were collected from the medium and used in experiments or expanded.

To quantify IL-1β production by XS52 cells in response to the doses 10^{-5}–10^{-6} mol/l of citalopram or fluoxetine, a mouse IL-1β ELISA kit (R&D systems) was used according to the manufacturer's instructions. Culture supernatants from XS52 cells were prepared by growing the cells at 5×10^4 cells/well in a 96-well flat-bottomed plate (Becton Dickinson) for 48 h in cRPMI medium without NS47 supernatant or GM-CSF. After 48 h, the cell culture medium was aspirated and 100 µl cRPMI medium containing GM-CSF (2 ng/ml) and lipopolysaccharide (LPS, 1 µg/ml, Sigma) was added, followed by the addition of 20 µl of citalopram, fluoxetine or saline (controls). After 12 h culture supernatants were collected and analysed by ELISA.

Statistics

For statistical calculation the Student *t*-test, Mann-Whitney *U* test or Wilcoxon signed-rank test was used. A *p*-value of <0.05 was regarded as being significant.

RESULTS

Serotonin

There was a higher number of serotonin-positive platelets in the eczematous (++) in contrast to the control (+) skin (data not shown).

Serotonin receptors

Immunoreactivity for 5-HT1AR was stronger in the upper part of the epidermis in the eczematous compared with control skin. 5-HT1AR expression was also detected on basal epidermal melanocyte-like cells, which were more elongated and possessed longer dendrites in the contact eczematous compared with control skin (Fig. 1A, depicted for inflamed skin). 5-HT1AR expression was also apparent on papillary dermal mononuclear cells (Fig. 1B, depicted for control skin), their number being decreased, 64±18 (mean±SD) per

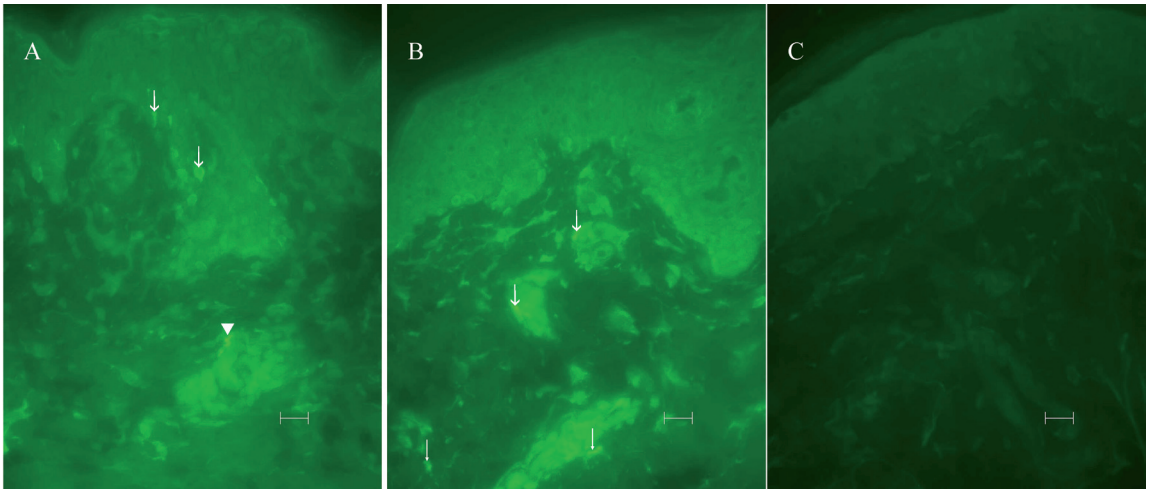

Fig. 1. Expression of the 5-hydroxytryptamin 1A receptor (5-HT1AR) on (A) epidermal melanocyte-like cells (*arrows*) and mononuclear cells in the dermis of both contact eczematous skin (*arrowhead*) and (B) control skin (*arrows*). (C) 5-HT1AR pre-absorption in control skin (note the abolishment of staining). Scale bar 20 µm in all figures.

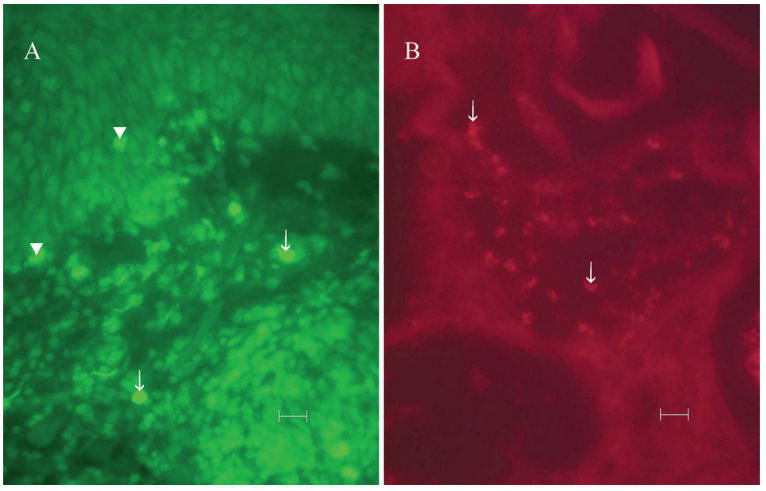

Fig. 2. Eczematous skin showing: (A) dermal 5-HT2AR-positive cells (*arrows*) infiltrating into the epidermis (*arrowheads*), and (B) serotonin transporter protein (SERT) expression on epidermal mononuclear cells in vesicles (*arrows*). Scale bars 20 μm in both figures.

section (p <0.01)) in eczematous in contrast to control skin, 97±32 (see Fig. 4A). The majority of these cells were tryptase-positive and exhibited more degranulation in the eczematous skin (not shown). 5-HT1AR immunoreactivity was abolished upon pre-absorption of the antibody with the peptide (Fig. 1C).

5-HT2AR-positive cells (Fig. 2A) were increased in number (p <0.001) from 2±2 per section in the control skin to 100±50 in the eczematous skin (see Fig. 4B). In the eczematous skin, 5-HT2AR-positive cells were seen to transmigrate vessels walls, infiltrate the epidermis (Fig. 2A) and lay within vesicles. 5-HT2AR-positive cells were also positive for CD3 (not shown), CD4 (Fig. 3A and B) or CD8 (not shown).

SERT

SERT expression was evident on dermal mononuclear cells, which were also demonstrated to infiltrate the epidermis and lay within vesicles in the contact allergic skin (Fig. 2B). The number of these cells was higher (p <0.001), 96±19 cells/section, in the eczematous compared with the control skin, 2±2 (Fig. 4C). Double staining revealed the co-localization of SERT with

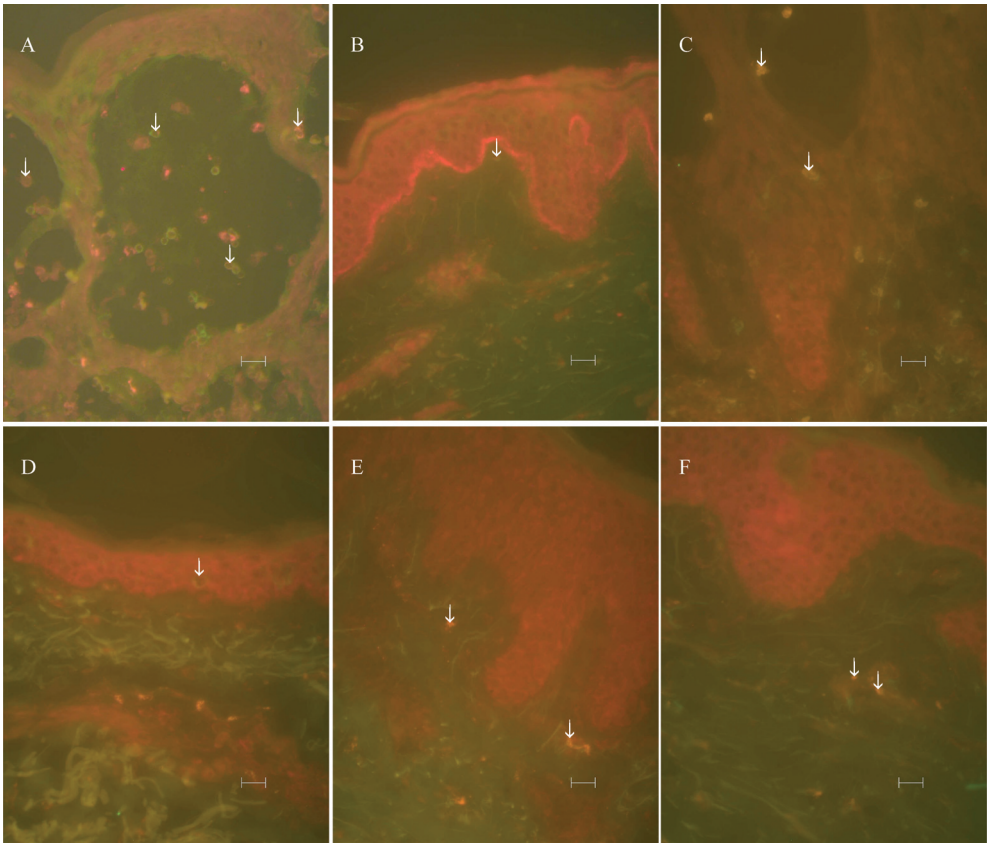

Fig. 3. Overlay photomicrographs showing co-localization of 5-HT2AR and CD4 on mononuclear cells (*arrows*) in (A) inflamed skin and (B) control skin. Also co-localization of serotonin transporter protein (SERT) with CD1a is seen in (C) inflamed skin (*arrows*) and (D) control skin (*arrow*). (E) Inflamed skin and (F) control skin, show SERT co-localization with CD56 (*arrows*). Scale bar 20 μm in all figures.

CD1a- (Fig. 3C and D), CD4-, CD8- (both not shown) or CD56- (Fig. 3E and F) positive cells.

PBMC proliferation and IL-2 production

PBMC were stimulated with nickel sulphate, which gave a 31-fold stimulation of the proliferation compared with non-stimulated cells, 649±857 cpm, and 192±268 pg/ml for IL-2 production, compared with the control. IL-2 for saline-treated cultures could not be detected in most of the experiments, but was 0.7 pg/ml in one experiment.

Adding DOI at 5×10^{-5} mol/l, inhibited (p <0.05) the proliferation of the nickel-stimulated cells (Fig. 5) (median 2104 cpm, quartile deviation 2952, compared to the control, median 16861 cpm, quartile deviation 15385), while no significant effect on their IL-2 production was obtained. Adding ketanserin (5-HT2AR antagonist) at 5×10^{-7} mol/l moderately (72%) blocked the inhibitory effect of DOI on the nickel-stimulated cells (data not shown). For the SSRIs, fluoxetine at 10^{-5} mol/l gave a 47% decrease (p=0.17) of the proliferation (not shown).

XS52 and IL-1β

The basic values, in the control cultures, for XS52 cell IL-1β production, being median 94 pg/ml, quartile deviation 109, showed a tendency (p=0.07) to decrease in response to 10^{-6} mol/l of citalopram, median 57, quartile deviation 46, while fluoxetine had no effect (data not shown).

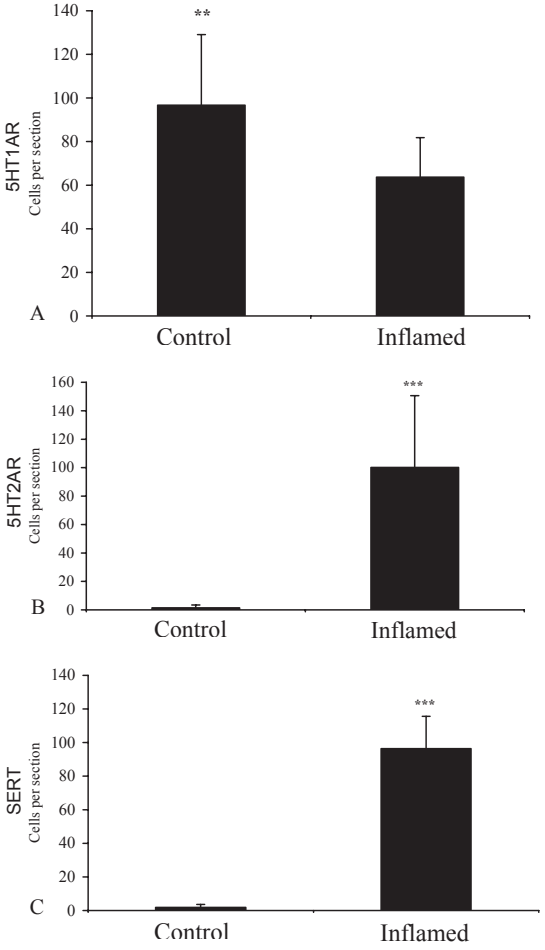

Fig. 4. (A) A decrease in 5-HT1AR-positive cells in the eczematous (inflamed) compared with control skin (**p <0.01). (B) An increase in the number of 5-HT2AR-positive cells in inflamed compared with control skin (***p <0.001). (C) The number of serotonin transporter protein (SERT) immunoreactive cells, being higher in the inflamed compared with control skin (***p <0.001). Skin biopsies (n=26).

DISCUSSION

In this study, the involvement of the serotonergic system in allergic contact dermatitis is indicated by the findings with a decrease in the number of 5-HT1A receptor-positive cells, an increase in 5-HT2A receptor-positive and SERT-positive cells in the contact eczematous, compared with the control skin, and the increased number of serotonin-positive platelets in the former skin. In earlier investigations an agonist to the 5-HT1A receptor, buspirone (32), and an antagonist to the 5-HT2A receptor, ketanserin (33), were found to cause a decreased contact allergic reaction in mice. 5-HT and ketanserin, both at 10^{-4} mol/l, gave a decreased nickel-induced proliferation of PBMC from nickel-allergic patients (34); however, these effects were not blocked.

In the present study, 5-HT1A receptors were localized on mast cells and melanocyte-like cells, 5-HT2A receptors only on lymphocytes, and SERT on lymphocytes, NK cells and LCs. In an earlier publication, Slominski et al. (35) reported an expression of the seroto-

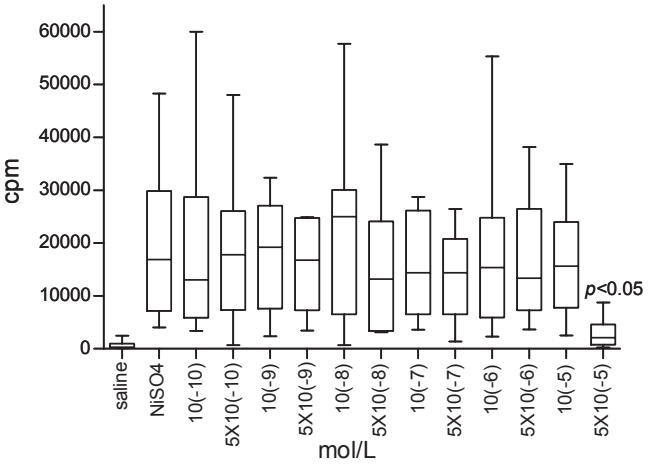

Fig. 5. Effect of the 5-HT2AR agonist 2,5-dimethoxy-4-iodoamphetamine (DOI) on nickel-stimulated peripheral blood mononuclear cells proliferation. Note the decreased proliferation (* p <0.05) using 5×10^{-5} mol/l of the agonist (boxplot represents median and, upper and lower quartiles, number of experiments =7).

nergic receptors (e.g. 5-HT1A and 5-HT2A) on human keratinocytes, melanocytes and dermal fibroblasts, being actively functional when tested in cell cultures. Also 5-HT receptors have earlier been reported in lymphocytes, dendritic cells and macrophages (see 36). In addition, SERT has been reported to be expressed by lymphocytes and dendritic cells (36), also dependent on their maturation (17). In addition, NK cells have been reported to be dependent on a serotonergic response for their function (37).

Treatment of nickel-stimulated PBMC with the 5-HT2AR agonist DOI, at the highest concentration, decreased the cellular proliferation; this effect was moderately blocked by ketanserin, whereas treatment with fluoxetine gave a tendency to a decrease of the proliferation. Microscopically, 5-HT2AR and SERT immunoreactive cells in adjacent sections showed similar distribution in the inflamed skin. Both 5-HT2AR and SERT were positive on CD3 immunoreactive cells, which indicate a co-expression on lymphocytes. A connection between the SERT inhibitor (fluoxetine) and 5-HT2AR has been earlier described (38), where a fluoxetine-induced decrease in lymphocyte proliferation was reported and suggested to be mediated by an activation of central 5-HT2AR. In the present study there was a tendency to an inhibition of the XS52 cell line production of IL-1β in response to the addition of SSRIs. This might support a general inhibitory effect of SSRIs on immune cells (38, 39).

The contribution of serotonin to skin physiology and pathology depends on its numerous vasoactive and immunomodulatory properties, which lead to involvement in the development of allergic reactions (for review see 40). The effects of serotonin *in vitro* may vary depending on the concentration and condition of the cell culture medium leading to the stimulation or inhibition of cells (35, 41, 42).

In conclusion, our results indicate that the serotonergic system contributes to the human contact allergic reaction and that the serotonin receptors 5-HT1AR, 5-HT2AR and the SERT protein might be targets for therapy in allergic contact eczema.

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