

ORIGINAL PAPER

Medication with antihistamines impairs allergen-specific immunotherapy in mice

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Clinical and Experimental Allergy

Summary

Background Histamine released from activated mast cells and basophils is an important mediator in allergy. Therefore, antihistamines are efficiently and widely used to suppress allergic symptoms.

Objective This study evaluated the role of antihistamines in sensitization against allergens and in the efficiency of allergen-specific immunotherapy.

Methods CBA mice were sensitized and de-sensitized with bee venom allergen extracts and the major allergen phospholipase A2. Clemastine was used to test the effect of a histamine-1 receptor antagonist on the immune responses to phospholipase A2.

Results The results demonstrated that sensitization against bee venom was strongly enhanced during treatment with antihistamines. Clemastine increased IgE production while decreasing IgG2a production against bee venom. This T-helper type 2 shift of the humoral response appeared to be caused by reduced IFN- γ and enhanced IL-4 secretion from allergen-specific T cells. We also found reduced TNF- α , IL-6 and major histocompatibility complex class-II expression by macrophages. In sensitized mice, the efficiency of allergen-specific immunotherapy was reduced by clemastine treatment.

Conclusion Antihistamines may enhance allergic sensitization and reduce the efficiency of allergen-specific immunotherapy. Future studies will need to demonstrate to what extent pre-medication with antihistamine also affects allergen-specific immunotherapy in humans.

Keywords allergy, antihistamine, immunotherapy, vaccination

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Introduction

Allergy to *Hymenoptera* stings is mediated by IgE and strongly allergic patients are typically treated by venom-specific immunotherapy (SIT). After SIT over 5 years, the clinical protection rate is high [1, 2]. However, systemic allergic side-effects due to the venom injection are frequent and occur in 20–40% of the patients [1]. The strategies proposed to reduce allergic side-effects include the use of recombinant allergens [3–5], DNA vaccines [6] and synthetic peptides with the most important T cell epitopes [7]. The most frequently used strategy, however, is pre-medication with antihistamines, which has been shown to reduce local and systemic side-effects [8–12]. Concomitant medication with antihistamines has also been reported to improve the efficacy of SIT [8].

Allergen-triggered activation of basophils and tissue mast cells induces the release of histamine, as well as

prostaglandins, leukotrienes and numerous other mediators. Especially, histamine exerts a variety of rapid inflammatory effects including bronchoconstriction, vasodilation, increased vascular permeability and stimulation of afferent sensory nerve terminals [13]. These effects are mostly mediated by interaction with histamine-1 (H_1) receptors and they are responsible for the majority of acute symptoms in bronchial asthma, allergic rhinitis and urticaria [14]. However, histamine may also influence immune responses by modulating the activity of T cells, monocytes and macrophages [15]. Histamine receptors are present on all these leucocytes [16–22].

In this study, we therefore investigated to what extent medication with antihistamine affected the immunogenicity of bee venom allergens in mice. Surprisingly, the results showed that medication with the H_1 -receptor antagonist clemastine induced a T-helper type 2 (Th2) shift of the humoral immune response against bee venom.

Also, our data suggest that pre-treatment with H₁-receptor antagonists might be counterproductive when administered during SIT.

Methods

Animals and materials

Six-week-old female CBA mice were purchased from Harlan (Horst, the Netherlands) and typically left to adapt 1–2 weeks in our germ-free animal facilities before any experimental procedures were performed. Bee venom allergen extract (BV) was purchased from ALK-Abelló (Trimed Ltd, Bruttisellen, Switzerland), while the bee venom major allergen phospholipase A2 (PLA2) was purchased in a purified form from Sigma-Aldrich (Buchs, Switzerland). Before use, the allergens were mixed with a 2% aqueous adjuvant suspension of aluminium hydroxide (Alu-Gel-S[®]) from Serva (Heidelberg, Germany). The H₁-receptor antagonists clemastine (Tavegil[®]) and dimetindene (Fenistil[®]) were both purchased from Novartis (Basel, Switzerland).

Immunization

Mice anaesthetized with ketamine and xylazine by intraperitoneal (i.p.) injections were immunized three times at 2-week intervals by intralymphatic (i.ln.) injections of 0.3 µg BV or 0.1 µg PLA2 as described [23, 24]; typically, groups of four mice were used, and the experiments were repeated once for testing of reproducibility. Either allergen was contained in 10 µL saline including 3 µL of the adjuvant aluminium hydroxide (60 µg Al³⁺). Serum was prepared from clotted blood and kept frozen at –20 °C until analysed by ELISA. When indicated, mice were given 100 µg clemastine i.p. on days –1, 0, 1, 2 and 3 with reference to the time-point of immunization. Clemastine treatment caused a weak transient sedation of the animals.

In a therapeutic approach, mice were first sensitized by six-weekly i.p. injections of 0.1 µg BV in 50 µL saline also containing 15 µL aluminium hydroxide (300 µg Al³⁺). After a dormant period of 3 weeks, the mice underwent a de-sensitization protocol, during which they received three i.ln. injections of 0.3 µg PLA2 on aluminium hydroxide as described above. In parallel to the de-sensitization, some mice were also treated with daily i.p. injections of 100 µg of clemastine as described above.

For induction of anaphylactic responses, sensitized mice were challenged intraperitoneally with 10 µg of BV in saline. Rectal temperature was measured with a calibrated digital thermometer before and 30 min after the challenge. All animal experiments were performed according to guidelines formulated by the federal Swiss Veterinary Office.

Antibody determination

For detection of anti-PLA2 antibodies, a previously described method was utilized [24, 25]. Briefly, the plates were coated overnight with 5 µg/mL PLA2 in buffered carbonate (pH 9.4). Subsequent to incubation with the individual mice sera, the plates were incubated with 1 µg/mL biotinylated goat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, CA, USA) and developed with streptavidin(SA)-conjugated horseradish peroxidase (HRP) (BD Pharmingen) and the enzyme substrate 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) di-ammonium salt (Sigma-Aldrich). The absorption was read at 405 nm on a microplate reader.

For detection of PLA2-specific IgE, plates were coated with 5 µg/mL of a mouse anti-IgE antibody (6HD5) kindly provided to us by Rolf M. Zinkernagel. As a secondary reagent to bound mouse serum, biotinylated PLA2 (in-house preparation) was used at 3 µg/mL. The plates were developed in SA-HRP and ABTS as above.

Cytokine secretion from splenocytes

To evaluate cytokine secretion from lymphocytes in immunized CBA mice, the spleens were isolated and homogenized. After lysis of erythrocytes and washing, 5×10^5 splenocytes were cultured with 5 µg/mL heat-denatured PLA2 protein in 200 µL complete medium [Iscove's modified Dulbecco's medium (IMDM) supplemented with L-glutamine, FCS, mercaptoethanol, streptomycin and ampicillin]. After 24 h, 50 µL rIL-2 (Roche, Rotkreuz, Switzerland) was added to yield 20 U/mL. Supernatants were collected after a 96-h incubation, and IL-4 and IFN-γ concentrations were determined by ELISA according to the manufacturer's protocol (R&D Systems, Abingdon, UK).

Cytokine secretion and surface expression of major histocompatibility complex class II on macrophages

The effect of clemastine on the phenotype of antigen-presenting cells (APCs) was studied in immature macrophages isolated from the peritoneum of naïve C57BL/6 mice. The peritoneal cells were washed once, re-suspended in IMDM medium supplemented with 5% FCS, glutamine, pyruvate, mercaptoethanol, penicillin and streptomycin and plated in Costar 48-well culture plates (Sigma-Aldrich) at 37 °C and 5% CO₂ for 90 min for removal of non-adherent cells by gentle washing. To the adherent macrophages, fresh medium was added, as well as 10 µg/mL heat-denatured PLA2 and 1.0 µg/mL lipopolysaccharide (LPS) derived from *Escherichia coli* clone 026:B6 (Sigma-Aldrich). To selected samples, 10 µg/mL clemastine was also added. After 60 min of incubation, the supernatant was collected for analysis of secreted TNF-α

and IL-6 by ELISA according to the manufacturer's protocol (R&D Systems).

Alternatively, mature macrophages were induced in C57BL/6 mice by an i.p. injection of 100 µg concanavaline A (Sigma-Aldrich). After 4 days, the peritoneal cells were harvested and adherent cells were selected on six-well culture plates as described above. The cells were then incubated with 100 ng/mL LPS and 5 µg/mL heat-denatured PLA2. To some samples, clemastine or histamine (ALK-Abelló) was additionally added at 10 µg/mL. After 16–20 h of incubation, the adherent cells were removed with 5 mM EDTA in phosphate buffer saline (PBS) for 30 min and washed once in PBS/FCS 2% for subsequent fluorescence-activated cell sorting (FACS) analysis. After blocking of Fc receptors with anti-CD16/CD32 for 5 min, the cells were stained with labelled anti-I-A^b (clone AF6-120.1) and anti-CD11b monoclonal antibodies (BD Pharmingen) in PBS/FCS 2% and at 4 °C for 20 min. Finally, the cells were fixed in 4% paraformaldehyde for 10 min and acquired on a FACS Calibur flow cytometer and analysed using CellQuest software from BD Biosciences (San Jose, CA, USA).

Statistics

Differences between independent groups were tested by non-parametric statistical analysis. Data were presented as means $\pm$ standard deviation and compared using a two-sided independent Mann-Whitney *U*-test or by one-way ANOVA and Bonferroni's multiple comparison post-test. The significance level was set at 5%.

Results

We first analysed the effect of antihistamine treatment on the immunogenicity and allergenicity of bee venom allergens. After three injections of BV, the IgG response was lower in mice treated with clemastine than in untreated mice (Fig. 1, left panel). Especially, the production of the Th1-dependent immunoglobulin subclass IgG2a was strongly impaired ($P=0.007$ at the serum dilution 1/640), but also the less Th1-dependent subclass IgG1 was significantly lower than that in untreated controls ($P=0.037$ at the serum dilution 1/640). In contrast, clemastine treatment enhanced the bee venom-specific IgE response when compared with untreated controls ($P=0.003$ at the serum dilution 1/40). The ratio of IgG2a to IgG1, a parameter for the relative contribution of the underlying Th1 and Th2 polarization, decreased as a function of clemastine treatment.

In order to exclude the possibility that the observed effects of clemastine were due to direct neutralization of residual histamine in the BV extracts, we repeated the above experiments with a purified preparation of the bee venom major allergen PLA2 (Fig. 1, right panel). Again,

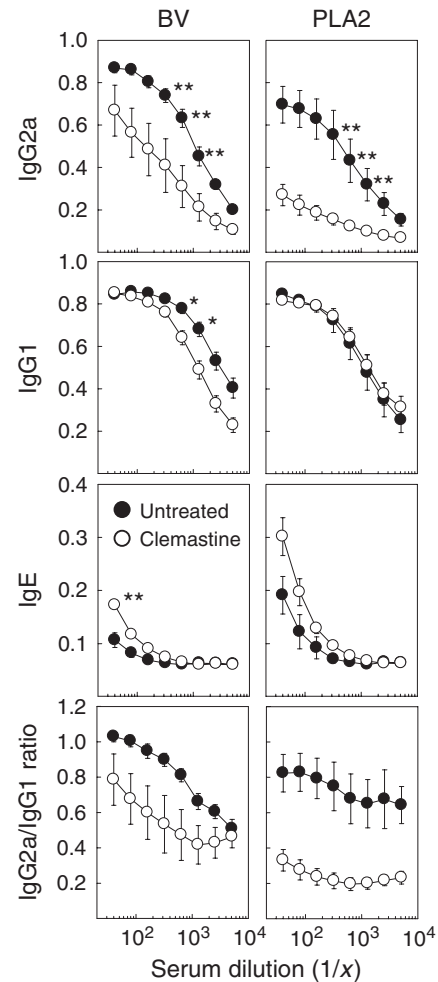


Fig. 1. The effect of antihistamine on the induction of antibody responses in mice. Groups of four CBA mice were immunized thrice at 2-week intervals by an intralymphatic injection of 0.3 µg BV crude extract (left panel) or 0.1 µg purified phospholipase A2 (PLA2) (right panel) both adsorbed on 60 µg Al³⁺ from the adjuvant aluminium hydroxide. Some mice received clemastine by four daily intraperitoneal injections of each 100 µg, starting 1 day before the immunization. Individual sera prepared 5 weeks after the last immunization were tested on ELISA for PLA2-specific IgG2a, IgG1 and IgE. The results are illustrated as PLA2-specific antibody levels in optical density as a function of the serum dilution. The ratios of IgG2a-to-IgG1 are calculated as the product of the two isotypes at 1 : 640 serum dilutions. Similar data were obtained for sera prepared 3 and 7 weeks after the last immunization. *P*-values are indicated for significant differences between clemastine-treated and untreated groups of mice as tested by the Mann-Whitney *U*-test: * $P<0.05$; ** $P<0.01$. The illustrated data are representative of two independent experiments with each four mice. BV, bee venom allergen extract.

the production of Th1-associated IgG2a was highly compromised in mice treated with clemastine ($P<0.001$ at the serum dilution 1/640), while the production of IgG1 was similar in clemastine treated and untreated groups. IgE secretion increased in clemastine-treated mice, and as for BV-immunized mice, the IgG2a to IgG1 ratio was reduced in mice treated with clemastine.

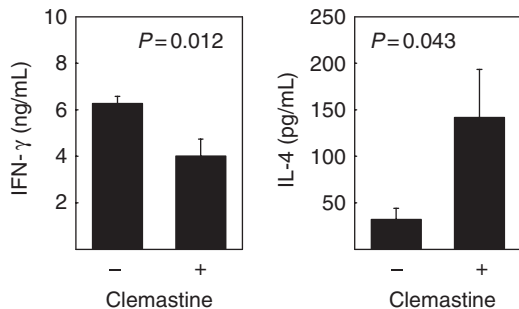


Fig. 2. Clemastine treatment resulted in T-helper type 2-skewed cytokine secretion. Groups of mice were immunized with phospholipase A2 (PLA2) as described in Fig. 1, and were given a boost injection of PLA2 7 weeks after the last injection. Two weeks thereafter, splenocytes were isolated and stimulated with heat-denatured PLA2 (5 µg/mL) and IL-2 (20 U/mL). After 96 h, the supernatants were harvested and tested for IFN-γ and IL-4 contents by ELISA. *P*-values, as calculated by the Mann-Whitney *U*-test, are indicated for the effect of clemastine treatment on IFN-γ and IL-4. The data illustrated are representative of two independent experiments, each with four mice. BV, bee venom allergen extract.

Seven weeks after the last of three i.ln. PLA2 injections, mice received another boosting injection of PLA2 with or without clemastine. Two weeks thereafter, splenocytes were isolated and stimulated *in vitro* with heat-denatured PLA2 in the presence of IL-2 for another 96 h. In line with the antibody data above, clemastine reduced IFN-γ secretion and enhanced IL-4 production, suggesting a Th2 polarization (Fig. 2). Similar effects on the immunogenicity of PLA2 were obtained on replacing clemastine with dimetindene, another H₁-specific antihistamine (not shown).

To test whether antihistamine treatment affected venom immunotherapy, BV-sensitized mice were de-sensitized by three i.ln. injections of PLA2. Such direct targeting of the allergen to secondary lymphatic organs such as the lymph node corresponds to repeated high-dose subcutaneous venom administration used during specific allergen immunotherapy in humans (manuscript in preparation). Before SIT, all mice showed high levels of IgE and IgG1 while having no detectable levels of IgG2a (Fig. 3a, left panel). SIT caused a strong increase in IgG2a and a slight increase in IgG1 and IgE (Fig. 3a, right panel). Clemastine treatment significantly reduced the increase of IgG2a ($P < 0.05$) and led to a stronger increase of IgE ($P < 0.05$) when compared with control mice receiving SIT without antihistamine. The effects of antihistamine treatment were also illustrated in IgG2a to IgG1 antibody titre ratios (Fig. 3b). Medication with clemastine significantly reduced the ratio. Hence, clemastine prevented the restoration of the Th1–Th2 balance intended by SIT. When mice were challenged with a sub-lethal dose of BV, anaphylaxis was observed in sensitized mice that had not received SIT. A few minutes after the BV challenge, these mice displayed a

reluctance to move, a hunched appearance, had piloerection and a significant 3 °C decline in body temperature (Fig. 4). In contrast, mice receiving SIT were protected against the BV challenge and showed little or no symptoms of stress or sickness. Their body temperature declined by merely 0.8 °C. Importantly, concomitant antihistamine medication and de-sensitization with clemastine reduced this protection. These mice showed clinical signs of anaphylaxis and the body temperature declined by approximately 2.0 °C, which was significantly more than the SIT control group ($P = 0.003$). Hence, clemastine treatment impaired the efficacy of SIT.

Macrophages play a crucial role in antigen presentation and are known to produce histamine upon stimulation with bacterial products [26, 27]. To test whether antihistamine treatment affected APCs, macrophages were cultured with heat-denatured PLA2 and LPS in the presence or in the absence of clemastine. The secretion of the pro-inflammatory cytokines TNF-α and IL-6 was abolished in clemastine-treated macrophages (Fig. 5a). Although not significantly ($P = 0.07$), the expression major histocompatibility complex (MHC) class II was reduced as compared with cells not treated with antihistamine (Fig. 5b). In contrast, histamine caused increased MHC class-II expression. The viability, as tested by trypan-blue incorporation, was not affected by this treatment.

Discussion

The objective of this study was to determine the role of antihistamine in prophylactic and therapeutic vaccination. We could demonstrate that clemastine treatment strongly compromised the immunogenicity of bee venom allergens. The effect was particularly strong for the Th1 arm of immunity with inferior IgG2a and IFN-γ production from lymphocytes and TNF-α and IL-6 production from macrophages. Most importantly, pre-medication with antihistamine made sensitized mice more susceptible to anaphylaxis after systemic administration of a high dose of bee venom allergen.

The conclusion of our study is in contrast to reports by Müller et al. [8] and Ohashi et al. [12], who suggested that antihistamines not only reduced local and systemic allergic side-effects, especially in the early dose-increasing period of the immunotherapy, but could also improve the efficacy of SIT. Treatment with terfenadine or fexofenadine, both H₁-receptor antagonists, lowered systemic allergic reactions to bee venom re-exposure, and the authors suggested that antihistamines during the initial dose-increase phase enhanced the long-term efficacy of bee venom SIT [8, 11] or allergic rhinitis [12]. Consequently, pre-medication with antihistamines has gained popularity in venom immunotherapy.

The potential immunosuppressive effects of antihistamine on immunogenicity and the Th1–Th2 balance

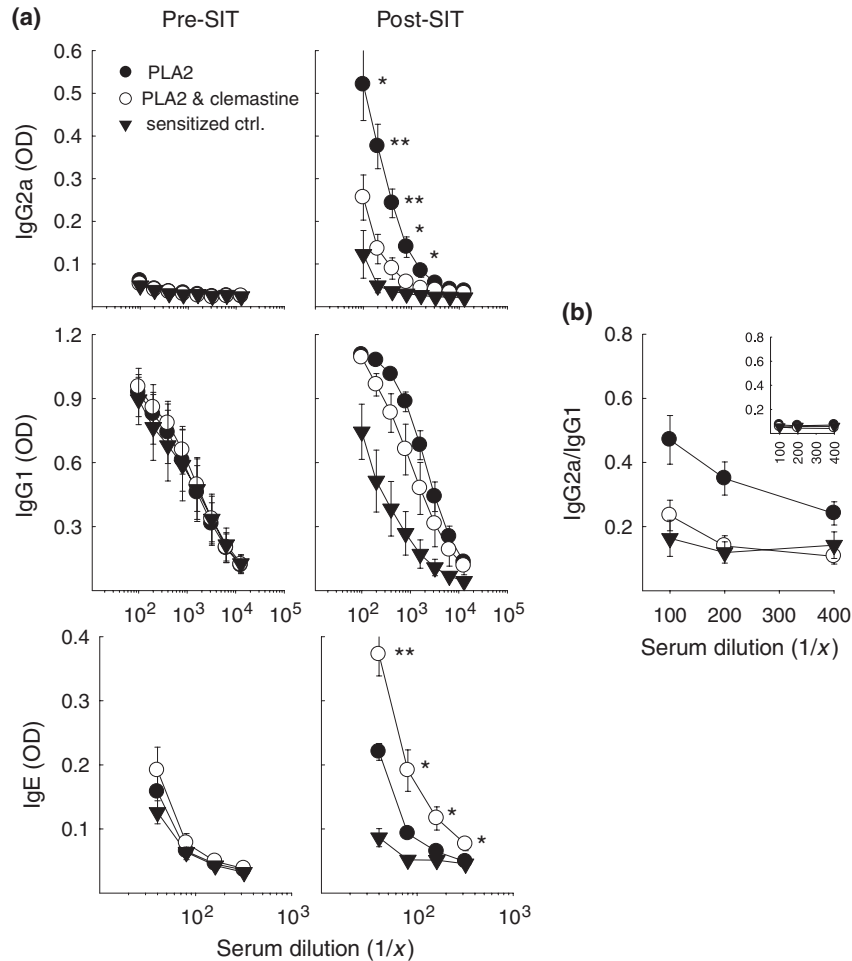


Fig. 3. Clemastine affected antibody production after venom-specific immunotherapy (SIT). Groups of four mice were sensitized by six-weekly intraperitoneal (i.p.) injections of 0.1 µg BV in aluminium hydroxide (300 µg Al³⁺). Three, 5 and 7 weeks after the last sensitization, one group of mice was de-sensitized by three intralymphatic injections of 0.3 µg phospholipase A2 (PLA2) (●) as described in Fig. 1. In one group of mice, the SIT was accompanied by four daily injections of 100 µg clemastine (i.p.) starting 1 day before PLA2 SIT (○). One group was left untreated as sensitization control (▼). (a) Before SIT (Pre SIT) and 5 weeks after the last injection (Post SIT), blood was collected for analysis of IgG2a, IgG1 and IgE in serum. The results are illustrated as PLA2-specific antibody levels in optical density as a function of the serum dilution. (b) The ratio of IgG2a-to-IgG1 before (inset) and after SIT is illustrated as the OD ratio for the serum dilutions 1 : 100, 1 : 200 and 1 : 400. **P* < 0.05; ***P* < 0.01 (Mann-Whitney *U*-test comparing SIT/clemastine-treated mice with SIT-treated mice). BV, bee venom allergen extract.

demonstrated in our study are supported in a row of published observations. Bäuerle et al. [28] showed that H₁-receptor stimulation activated the prototypic pro-inflammatory transcription factor nuclear factor (NF)-κB, which has multiple secondary effects on the immune system, including the stimulation of pro-inflammatory cytokines. Indeed, H₁-receptor antagonism can suppress TNF-α secretion, which further prevents TNF-α-mediated activation of epithelial [29] and endothelial cells [30] as well as the recruitment of inflammatory cells [31]. TNF-α also forms synergism with IFN-γ in the activation of macrophages. Activated macrophages secrete IL-12 [32, 33], which acts as a chemo-attractant for other macrophages, promotes the migration of APCs, is associated with several pathogenic inflammatory responses

and directs the differentiation of activated naïve CD4 T cells into Th1 effector cells.

Upon stimulation with bacteria or bacterial products such as LPS, macrophages produce histamine, which acts as an autocrine signal exocytosis and for IL-6 production [26, 27, 34]. When, in our hands, antihistamines impaired this IL-6 production, it suggested that the immunomodulating effects observed may in part be an effect on the innate immunity. The adjuvant and the allergen stimulate histamine secretion in macrophages but on blocking the histamine receptor, activation is impeded and migration is arrested; hence, the antigen presentation loses efficiency.

Furthermore, in the differentiation process of lymphoid or myeloid dendritic cells from monocytes, H₁ and H₃ receptors act as positive stimulants that increase antigen-

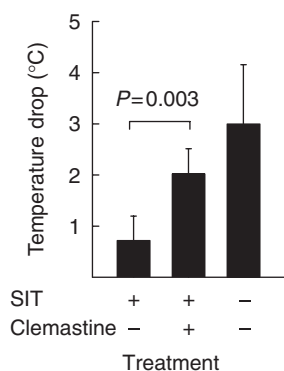


Fig. 4. Clemastine impaired the efficacy of venom immunotherapy. Groups of four mice were sensitized and de-sensitized as described in Fig. 3. Subsequently, the mice were challenged with 10 µg BV in phosphate buffer saline by an intraperitoneal injection, and anaphylaxis was measured by means of the change in rectal temperature after 30 min. BV, bee venom allergen extract.

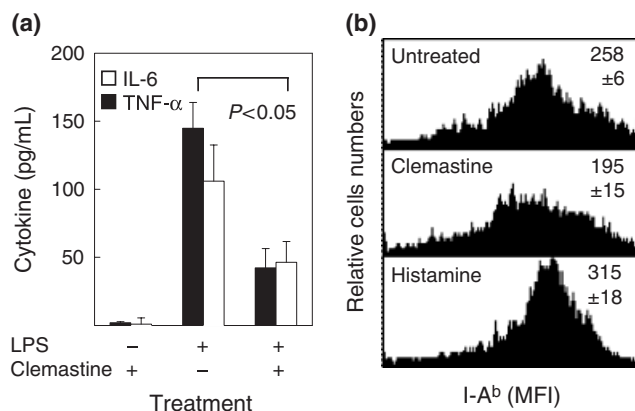


Fig. 5. Clemastine reduces the secretion of TNF-α and IL-6 from immature and major histocompatibility complex (MHC) class-II expression on mature peritoneal mouse macrophages. (a) Immature peritoneal macrophages were collected from naïve C57BL/6 mice and incubated for 90 min with 5 µg/mL phospholipase (PLA2) with and without 1 µg/mL lipopolysaccharide (LPS) and 10 µg/mL clemastine ($n=4$). IL-6 and TNF-α in the supernatants were measured by ELISA. (b) MHC class-II expression (I-A^b) was measured by fluorescence-activated cell sorting analysis on ConA-generated mature peritoneal macrophages after overnight incubation with 5 µg/mL PLA2 and 0.1 µg/mL LPS and with 10 µg/mL clemastine or histamine ($n=3$). Numbers within the histogram plots represent the mean fluorescence intensity (MFI ± SEM) of I-A^b gated on CD11b-positive cells. The experiments were repeated twice with comparable results. There was a significant difference in I-A^b expression between the clemastine- and the histamine-treated group as analysed by one-way ANOVA and Bonferroni's multiple comparison test ($P<0.01$).

presentation capacity, pro-inflammatory cytokine production and Th1 lymphocyte-priming activity [19]. Immature dendritic cells exposed for 24 h to histamine-stimulated memory T cells more efficiently than untreated dendritic cells. In addition, H₁-receptor stimulation transiently up-regulated the expression of co-stimulatory and accessory molecules, such as CD80, CD86, CD40, CD49d, CD54 and MHC class II. It further induced the potent production of

IL-6, IL-8, MCP-1 and MIP-1α by immature dendritic cells and up-regulated IL-1β, RANTES and MIP-1β but not TNF-α and IL-12 mRNA expression. Finally, and as demonstrated in the present investigation, antihistamines are likely to skew the immune response towards Th2 through its effect on NF-κB and pro-inflammatory cytokines. H₁-receptor blocking has also been shown to have implications for animals' resistance to bacterial infections. The intracellular growth of both *Mycobacterium bovis* BCG was improved in mice genetically depleted at the level of the H₁ receptor [35], and we have observed that mice treated with clemastine succumb to listeriosis at sub-lethal doses (unpublished observations). Both these observations further suggest a clear immunosuppressive effect of antihistamine.

Besides its effect on the innate immune system, histamine has been shown to have a direct effect on the function of leucocytes. Gelfand et al. studied the effect of antihistamines on the elicitation phase of the allergic response in ovalbumin (OVA)-asthmatic mice. Lung eosinophilia as well as inflammation by lymphocytes were strongly reduced in mice that received fexofenadine along with an aerosolic OVA challenge [36]. Moreover, the proliferation of T cells could be enhanced by exogenous and endogenous histamine through H₁-receptor-mediated IL-2 production [37], suggesting that histamine may act as an autocrine regulator of T cell proliferation. Likewise, Akdis and colleagues reported that H₁-receptor knock-out mice were characterized by dominant secretion of Th2 cytokines IL-4 and IL-13 as well as IgE in addition to suppressed IFN-γ production [18]. The same study also demonstrated that H₂-receptor knock-out mice secreted more Th1 cytokine than their wild-type littermates, suggesting diametrically opposite immunological consequences of H₁- and H₂-receptor stimulation. However, clemastine and dimetindene are selective H₁-receptor inverse agonists and therefore do not mediate competing H₂-specific immune responses.

From a patho-physiological point of view, it also makes sense that the immune system will try to counteract an allergic reaction in the best possible way. Hence, for IgE-mediated immediate-type allergies, i.e. Th2-type reactions, the most appropriate defence mechanisms would be (i) to impede the effects produced by released histamine and other chemical mediators of allergy; (ii) to prevent further degranulation of mast cells and basophils by IgG-mediated neutralization or opsonization of allergens before their cross-linking of cell-bound IgE; and (iii) to generate Th1-like immune responses, which, through cytokines and allergen-specific T cell-mediated suppression, create environmental conditions unfavourable for sustained allergic reactions. Consequently, antihistamine treatment may compromise the immune system's capability to act with a histamine-mediated self-repair response to allergic reactions.

Taken together, this study showed that concurrent antihistamine therapy and bee venom immunotherapy was accompanied by immune regulatory responses that impaired the efficacy of SIT. This raises important questions concerning the rationale of applying such a combination. While the benefits of antihistamine premedication with regard to local and systemic allergic side-effects of SIT, especially in rush-immunotherapy, are indisputable, the further immunological effects on the SIT are ambiguous and should be addressed in future clinical studies.

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