Histamine 4 receptor plays an important role in auto-antibody-induced arthritis

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Abstract

Rheumatoid arthritis is a widespread autoimmune disease. In the murine K/B×N arthritis model, anti-GPI (anti-glucose 6-phosphate isomerase) antibodies lead to the formation of immune complexes. In the course of pathogenesis, these complexes activate the immune system and induce degranulation of mast cells, which are essential in this model of rheumatoid arthritis. A major mediator in mast cell granules is histamine, which is proven to be indispensable for joint inflammation in K/B×N mice. Histamine is known to bind to four different receptors (HR1–4), which have different expression profiles and exert a variety of different functions, including activation of the immune system. To analyze the contribution of the different histamine receptors, we employed histamine receptor antagonists (cetirizine, ranitidine, thioperamide and clozapine) blocking the receptors in C57BL/6 mice. Arthritis was induced via K/B×N serum injection. The results demonstrated that mice treated with all four histamine receptor antagonists simultaneously showed no arthritic symptoms, while positive control mice injected with K/B×N serum and vehicle suffered from severe symptoms. When antagonists specific for HR1–4 were applied individually, only the HR4 antagonist clozapine could protect mice from arthritis, reflecting its expression and functionality in the immune system.

Keywords: autoimmune disease, histamine receptor antagonist, K/B×N model

Introduction

Rheumatoid arthritis (RA) is prominent autoimmune disease. Auto-antibodies form immune complexes on the cartilage surface and induce an inflammation (1). The inflammation of the synovial tissue is mediated by macrophages and fibroblasts, which form the thin layers of the synovium and, upon inflammation and influx of inflammatory cells from the circulation, proliferate to form the pannus tissue.

Diseased K/B×N mice suffer from thickened ankles and pannus tissue similar to human RA patients. Other similarities are leukocyte invasion, synovocyte proliferation, synovitis and cartilage/bone erosion (2–4). Auto-antibodies are directed against glucose 6-phosphate isomerase (GPI), a natural enzyme involved in glycolysis but also a ‘moonlighting protein’ with diverse chemotactic properties (5–7). Although a transgenic TCR is the triggering factor for developing arthritis, the auto-antibodies can be used to transfer the disease into naive mice.

Mast cells are present in human synovial tissue (8) and have been shown to be pivotal in the K/B×N model of arthritis (9, 10). Furthermore, preventing degranulation of mast cells blocks the development of arthritis (10). Previously, we showed that mice deficient in histidine decarboxylase, who consequently cannot produce histamine, developed a milder form of auto-antibody-induced arthritis (11).

The actions of histamine are mediated via four known G-protein coupled histamine receptors designated HR1, HR2, HR3 and HR4. All four differ in their expression profiles, signal transduction capabilities and functions (12–14).

HR1 is expressed on many cells such as airway and vascular smooth muscle cells, hepatocytes, chondrocytes, nerve cells, endothelial cells, dendritic cells, monocytes, neutrophils, T cells and B cells (15). Its activation leads to airway and vascular smooth muscle contraction. Furthermore, allergic rhinitis, atopic dermatitis, conjunctivitis, urticaria, asthma and anaphylaxes are mediated through HR1 (16). HR1-deficient mice, reported symptoms are impairment of memory, learning, locomotion, and nociception and aggressive behavior (17). HR2 is coupled to the adenylyl cyclase and the phosphoinositot second messenger system (18). The activated receptor can inhibit many functions in the immune system such as antibody production, T-cell proliferation, cell-mediated cytolyis and cytokine production.
Moreover, HR2 negatively regulates histamine release in basophils and mast cells. HR2 is co-expressed with HR1, but it is more involved in antagonizing functions of HR1 (15, 19). Both HR1 and HR2 were shown to play no function in the K/BxN model of arthritis by using the respective gene-deficient mice (11). HR3 is predominantly expressed in the central and peripheral nervous systems as a pre-synaptic receptor, controlling the release of histamine and neurotransmitters such as dopamine, serotonin, noradrenaline, gamma amino butyric acid and acetylcholine (20). Besides, HR3 is an autocrine receptor, regulating the release of histamine and the negative feedback mechanism for reducing central histaminergic activity. There seems to be a mast-cell–neuron feedback loop, which is controlled by the HR3. The third histamine receptor is further involved in cognition, sleep–wake status, energy homeostatic regulation and inflammation (19). HR4 is about 37–43% homologous with HR3 and is expressed in bone marrow, the spleen, and on hematopoietic cells, neutrophils, eosinophils and T cells; low expression is found in the thymus, lung, small intestine, colon and heart. Additionally, basophils and mast cells possess the HR4 (21). HR4 appears to be involved in inflammatory processes, recruiting and activating inflammatory cells like eosinophils, mast cells, neutrophils, T cells and dendritic cells (22–24). The receptor is also involved in immune regulatory functions such as chemotaxis or cytokine secretion (25, 26) and induces intracellular Ca²⁺ mobilization (24). Surprisingly, it does not induce vasodilation, which is an important function of histamine in inflammatory responses. Nevertheless, the HR4 plays an important role in inflammatory cascades such as histamine release by mast cells.

Here we analyzed the importance of the histamine receptors in auto-antibody-induced arthritis and show that HR4 is most important for the development of arthritis.

Methods
Experimental animals
C57BL/6 (B6) mice were obtained from our animal facility and were maintained under pathogen-free conditions. KRN-T cell-receptor transgenic (KRN) mice and Non-obese diabetic (NOD/Lt) mice were obtained from C. Benoist and D. Mathis (Strasbourg, France). Additionally, 8-week-old C57BL/6 mice were obtained from Charles River laboratories, Germany, KRN and NOD/Lt mice were crossed, and their offspring (K/BxN) were screened for arthritis.

Statistical analysis
Data were expressed as mean ± standard error (SEM). Statistical analysis was performed with GraphPad Prism, which was used to calculate statistical significance with an unpaired, two-tailed t-test or ANOVA. A post hoc analysis with Dunnett’s Multiple Comparison Test was performed after the ANOVA. Statistical significance was set at P ≤ 0.05.

Antagonist study
Histamine receptor antagonists were applied orally to the mice for analyzing their effects on auto-antibody-induced arthritis.

The antagonists were dissolved in sterile water or in ethanol, dependent on their solubility and mixed with methylcellulose. Cetirizine hydrochloride (Sigma–Aldrich, Deisenhofen, Germany) was used at 10 mg ml⁻¹ in H₂O; ranitidine hydrochloride (Sigma–Aldrich) was used at 1.8 mg ml⁻¹ in H₂O; thioperaide maleate salt (Sigma–Aldrich) was used at 10 mg ml⁻¹ in H₂O; and clozapine (Sigma–Aldrich) was used at 1 mg ml⁻¹ in ethanol.

Induction of arthritis by K/BxN serum transfer and arthritis assessment
Arthritis was induced in the recipient mice by an intraperitoneal injection of 200 µl of the K/BxN serum at day 0. Increase in ankle thickening was measured using a micrometer (Hann and Kolb, model no. 33185). Every morning (9–11 a.m.) and evening (4–6 p.m.) for a period of 14 days, all mice received 300 µl of the respective antagonist orally. Differences of ankle thickness compared with day 0 of the four limbs per individual were determined and expressed as the mean. The Clinical Index score, independent of the number of ankles affected, was measured on a scale ranging from values of 0 (no symptoms); 0.25; 0.5; 0.75 to 1 (severe symptoms). A dose of 4 mg kg⁻¹ for cetirizine and clozapine, 10 mg kg⁻¹ for ranitidine and 3 mg kg⁻¹ for thioperaide was used. The vehicle mixture corresponds to the volumes of ethanol and water in the antagonist mixtures.

Blood cell count
Whole blood (10 µl) was taken from the tail vein of four clozapine-treated and four control mice and anticoagulated with EDTA. Red blood cells were lysed with a hypotonic buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Paraformaldehyde-fixed cells were analyzed on a FACS Calibur using CellQuest software. A gate was set for lymphocytes and granulocytes and the cell numbers statistically evaluated with Prism 6 (GraphPad Software, La Jolla, CA, USA).

Histology
Joints were fixed in 4% phosphate-buffered formaldehyde (Sigma–Aldrich) for 24–48 h. Tissue was then decalcified by rocking for 2 weeks in 14% EDTA solution (Merck, Darmstadt, Germany) that was daily renewed. The samples were washed in PBS and dehydrated with different concentrations of ethanol (50, 70, 96 and 100%). After dehydration, the tissue was embedded in paraffin, and 4 µm thick sections were cut and stained with H&E. H&E-stained ankle sections were scored for acute and chronic inflammation defined by synovial hyperplasia and degree of infiltration of the synovial tissue with polymorphonuclear and mononuclear leukocytes as follows: 0 = normal structures, 1 = mild hyperplasia and infiltration, 2 = moderate changes and 3 = severe changes. The extent of damage of cartilage surface and bone structures was evaluated on a scale of 0–3, where 0 = no damage, 1 = mild destruction, 2 = moderate destruction and 3 = severe destruction of cartilage and bone (extensive area of cartilage destruction and deep invasive bone erosions).
Results

Simultaneous application of HR1–4 antagonists blocks arthritis

To study the effect of histamine receptor antagonists on arthritis, C75BL/6 mice were first treated with a mixture of HR1–4 antagonists or vehicle dose and then injected with K/BxN serum to induce arthritis. Mice treated with the HR1–4 antagonists simultaneously showed a near complete protection from arthritis upon serum injection (Fig. 1a, circles). This is in contrast to K/BxN serum-treated mice given the vehicle dose only, which developed severe arthritis (Fig. 1a, triangles). Analyzing the average Clinical Index of C57BL/6 mice treated with K/BxN serum and vehicle dose showed a high Clinical Index score (Fig. 1b), reflecting typical arthritis pathogenesis for the K/BxN serum transfer model in contrast to HR1–4 antagonist-treated mice.

HR4 is the most important HR in the arthritis pathogenesis in the K/BxN serum transfer model

To delineate the contribution of the individual HRs in the development of arthritis, we applied all four antagonists individually. The average difference of measured ankle thickness was taken and SEM was calculated. An ANOVA test was performed comparing the different treatments for statistical significant differences. Additionally, an unpaired, two-tailed t-test was used to analyze significance for each day. Treatment of mice with antagonists against HR1, HR2 and HR3 (cetirizine, ranitidine and thioperamide, respectively) resulted in arthritic scores almost as high as in mice treated with K/BxN serum and vehicle dose and did not reveal any statistically significant differences. In contrast to this, application of the HR4 antagonist clozapine protected mice from arthritic symptoms. In these mice, ankle thickness was significantly different compared with C57BL/6 mice treated with K/BxN serum and vehicle dose (Fig. 2). Significance was highest on days 8 and 10 at P ≤ 0.001.

Histological analysis

Anteke joints were taken from mice sacrificed on day 8 post serum injection and analyzed histologically using H&E staining. Fig. 3 contains sections of mice given all four antagonists or a vehicle dose. Histological sections presented in Fig. 3b and e display strong arthritic symptoms with inflammation of the whole joint, pannus formation and partial destruction of cartilage and bone. Periarticular bone destruction and tenosynovitis are also evident in K/BxN serum-treated mice. Experimental mice given all four antagonists are protected against this inflammation (Fig. 3c and f). Their ankles possess intact bone tissue and cartilage layers. Joints of these mice are comparable with completely healthy mice (Fig. 3a and d).

Mice given cetirizine, ranitidine or thioperamide individually were not protected from arthritic symptoms and displayed strong inflammation and joint destruction (Fig. 4a–c and e–g). Only mice given the HR4 antagonist clozapine revealed no obvious inflammation of the joint. Cartilage and bone are intact and no pannus tissue is detectable (Fig. 4d and h). There are no signs of periarticular inflammatory processes along the shaft.

Comparing the scorings for inflammation and destruction of tissue presented in Figs 3 and 4 revealed that only mice given K/BxN serum and a vehicle dose show high values. These are significantly different to healthy mice and mice treated with all four antagonists or with clozapine alone (Fig. 5). Clozapine may lead in about 1% of human patients to leukopenia (27). Studies in rats, rabbits and guinea pigs (28, 29) did not show leukopenia. To exclude that leukopenia would account for the effects observed in clozapine-treated
mice, we measured white blood cell counts on days 3 and 8 in clozapine-treated and untreated C57BL/6 mice injected with K/B×N sera. No significant difference comparing both experimental groups were found and therefore clozapine did not result in leukopenia in the C57BL/6 mice (Fig. 6).

Discussion

Here we show that HR4 plays an important role in antibody-induced arthritis, employing the K/B×N model. K/B×N serum injection in cetirizine (HR1)-treated and ranitidine (HR2)-treated mice revealed that ankle thickness and the Clinical Index scores were not different from those of mice given K/B×N serum + vehicle dose. These results confirm former studies, showing that HR1- and HR2-deficient mice were not protected from auto-antibody-induced arthritis (11). Although thioperamide (HR3)-treated mice showed no statistically significant difference of symptoms compared with mice given K/B×N serum and vehicle dose only, their pathogenesis was slightly lower in the beginning until day 8. It is possible that HR3-antagonist thioperamide also partially blocked HR4, as HR4 is about 40% homologous to HR3 (21) by sequence and only about 20% compared with HR1 or HR2 (30). This may explain why mice treated with all four antagonists (Fig. 1) displayed a more pronounced attenuation of arthritis compared with clozapine-treated mice (Fig. 2). Further there might be a basal level of signaling via the HR1–3, which got blocked by the combined antagonist treatment and thereby reduced arthritic activities above those blocked by HR4 antagonist only. Because HR3 is mostly expressed in the nervous system regulating release of neurotransmitters and controlling histaminergic activity in the CNS there might be an indirect involvement of HR3 in arthritis (19).

Because of its expression mainly on immune cells and its chemotactic properties, HR4 is defined as the immune system histamine receptor (24) and induces mast cell attraction towards histamine without affecting mast cell degranulation (23). This effect leads to an accumulation of mast cells in inflammatory regions. Its expression on macrophages and fibroblasts of the synovium (26) might be directly linked to the influx of macrophages and other immune cells into the inflamed joint. Expression of HR4 mRNA in synovial cells of RA patients is directly correlated with severity or arthritis (31). Moreover, signaling via HR4 leads to increased IL-6 expression and TLR signaling (32) and thus contributes to the inflammatory microenvironment of the arthritic joint.

Fig. 2. Average (with SEM) difference in ankle thickness of mice treated with all four antagonists individually. Ankle thickness was measured for 14 days after injection of K/B×N. Antagonist or vehicle dose were given twice a day. Ankle thickness (in mm) was measured with a caliper every second day. Five different types of experimental and control mice were measured: C57BL/6 with K/B×N serum and vehicle dose, C57BL/6 mice with cetirizine (HR1 antagonist), ranitidine (HR2 antagonist), thioperamide (HR3 antagonist) or clozapine (HR4 antagonist) dose and K/B×N serum were used as test mice. Treatment with cetirizine, ranitidine or thioperamide had no effect on ankle thickness, whereas clozapine notably decreases the thickness. Statistical significance is expressed using asterisks, where * P ≤ 0.05, ** P ≤ 0.01 and *** P ≤ 0.001.
Fig. 3. Histological sections of ankle joints from (a) mice given C57BL/6 serum and all four antagonists (40x) and (d) (100x), (b) mice given K/BxN serum and a vehicle dose (40x) and (e) (100x), (c) mice given K/BxN serum and all four antagonists (40x) and (f) (100x). Healthy mice presented in (a) and (d) display no inflammation with a thin synovial membrane (thick arrow), intact cartilage and bone tissue (thin arrows). Joints of arthritic mice in (b) and (e) exhibit a strong intra-articular inflammation, including hyperplasia of the synovial lining layer, infiltration of inflammatory cells into the synovial tissue (asterisk) and pannus formation. Cartilage and bone are partly destroyed (thin arrow), and periar-ticular inflammation (tenosynovitis) is observed (thick arrow). Joints of mice given the antagonists display no symptoms in (c) and (f) and are comparable with healthy joints of (a) and (d).

Fig. 4. Histological sections of ankle joints from (a) mice given K/BxN serum and cetirizine (HR1; 40x) and (e) (100x), (b) mice given K/BxN serum and ranitidine (HR2; 40x) and (f) (100x), (c) mice given K/BxN serum and thioperamide (HR3; 40x) and (g) (100x) and (d) mice given K/BxN serum and clozapine (HR4; 40x) and (h) (100x). Mice given cetirizine (a), ranitidine (b) or thioperamide (c) display strong symptoms of intra- and peri-articular inflammation, including hyperplasia, infiltration of inflammatory cells (asterisks), pannus formation, cartilage and bone destruction (thin arrows) and tenosynovitis (thick arrow). Mice given clozapine (d) reveal no inflammation of the joint with intact bone and carti-lage (thin arrows), and thin synovial membrane (thick arrow).
In conclusion, our experiments proved that symptoms of arthritis could be reduced with the application of HR4 antagonist clozapine. Our results support the hypothesis that histamine receptors, in particular HR4, are involved in the pathogenesis of arthritis.

References

Fig. 5. Inflammation score of histological analysis. (a) Mice were given C57BL/6 serum or K/BxN serum with all four antagonists or K/BxN serum with vehicle dose. Bars of mice given C57BL/6 or K/BxN serum with antagonists are surrogates as all values were zero. (b) Mice were given K/BxN serum and antagonist individually (cetirizine, ranitidine, thioperamide and clozapine). Bars of clozapine-treated mice are surrogates as all values are zero. Scores range from 0 (no symptoms) to 4 (severe symptoms). Acute Infl. = acute inflammation; Chron. Infl. = chronic inflammation; Destr. Cart. = destruction cartilage; Destr. Bone = destruction of bone. Statistical significance is expressed using asterisks, where * P ≤ 0.05 and ** P ≤ 0.01.

Fig. 6. Relative white blood cell (WBC) count of clozapine-treated mice. WBC counts of control mice were set as 100% and the corresponding values of clozapine-treated mice on day 3 and 8 are shown. The blood cell counts between control and treated mice do not differ significantly on both days and between day 3 and day 8 in clozapine-treated mice. Error bars indicate the SEM, n = 4.


