

H1 histamine receptor mediates inflammatory responses in human keratinocytes

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Background: Keratinocytes participate in initiation and amplification of T-cell-mediated skin diseases. During these disorders, histamine can be released from both residents skin cells and immigrating leukocytes, and can affect the functions of dendritic cells, monocytes, and lymphocytes. Little information is available on the effects of histamine on keratinocytes.

Objective: To examine the presence of functional H1 receptors on human keratinocytes and the capacity of histamine to modulate the expression of inflammatory molecules in these cells. **Methods:** Primary cultures of resting and cytokine-activated keratinocytes from healthy subjects were analyzed for histamine H1 receptor expression and the production of inflammatory mediators after exposure to histamine receptor agonists and antagonists.

Results: Cultured keratinocytes constitutively expressed the H1 receptor mRNA and protein, which was not influenced by IFN- γ , TNF- α , or IL-4. H1 but not H2 agonists induced calcium fluxes in keratinocytes. Treatment of keratinocytes with histamine (10^{-7} to 10^{-4} mol/L) or β -histine increased the IFN- γ -induced expression of membrane intercellular adhesion molecule 1 and MHC class I but not MHC class II molecules. Moreover, H1 stimulation promoted basal CC chemokine ligand (CCL)-5/RANTES and GM-CSF secretion and augmented IFN- γ -induced release of the chemokines CCL2/monocyte chemoattractant protein 1, CCL5/RANTES, CCL20/macrophage inflammatory protein 3 α , and CXC chemokine ligand 10/IFN-induced protein of 10 kd, as well as GM-CSF. Administration of the H1 antihistamine levocetirizine, but not of the H2 antihistamine cimetidine, abolished histamine-dependent expression of all of the investigated proinflammatory molecules in a dose-dependent manner (0.01-10 μ mol/L). Levocetirizine at higher doses also reduced intercellular adhesion molecule 1, CCL5/RANTES, and GM-CSF release induced solely by IFN- γ . **Conclusion:** Histamine exerts proinflammatory effects on keratinocytes via the H1 receptor, and these effects are efficiently inhibited by levocetirizine. (J Allergy Clin Immunol 2004;114:1176-82.)

Key words: Keratinocytes, histamine receptors, chemokines, GM-CSF, adhesion molecules

Abbreviations used

CCL: CC chemokine ligand
CXCL: CXC chemokine ligand
EGTA: Ethyleneglycol-bis-(β -aminoethylether)-N,N,N',N'-tetraacetic acid
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
ICAM-1: Intercellular adhesion molecule 1
IP-10: IFN-induced protein of 10 kd
MCP-1: Monocyte chemoattractant protein 1
MIP-3 α : Macrophage inflammatory protein 3 α

Numerous studies have definitively demonstrated that keratinocytes participate relevantly in cutaneous immune responses by expressing membrane and soluble mediators, which directly and indirectly modulate the recruitment and local activation of T cells and other leukocytes.¹⁻⁴ These mediators include adhesion molecules, MHC molecules, growth factors, cytokines, and chemokines. The most efficient signals driving keratinocyte activation are lymphokines secreted by T cells, monocytes, and dendritic cells. In particular, IFN- γ , TNF- α , and IL-17 are among the most potent activators of keratinocytes.^{5,6}

Histamine is stored in mast cells and basophils and is an essential effector molecule of IgE-mediated allergic responses. However, histamine may also play an important role in cell-mediated immune responses. It is produced by different leukocyte populations such as monocytes, T cells, and neutrophils in response to cytokines or bacterial products,^{7,8} as well as by keratinocytes on exposure to ultraviolet radiation.⁹ On the other hand, histamine receptors are widely expressed by cells of the innate and adaptive immunity. In these cells, histamine modulates the synthesis of adhesion and costimulatory molecules and cytokines with important functional consequences.^{7,8,10} At least 4 types of histamine receptors have been characterized.¹¹ The H1 receptor, which is coupled to G_{i/o/q} proteins and induces Ca²⁺ release from intracellular stores, mediates most of the proinflammatory effects of histamine.

Few studies have addressed the role of histamine on the inflammatory functions of keratinocytes.¹²⁻¹⁵ We examined the presence of H1 receptor on normal human keratinocytes and the capacity of histamine to modulate inflammatory molecule expression. The results indicate that the H1 histamine receptor mediates inflammatory activity on keratinocytes and strongly augments IFN- γ -induced keratinocyte activation.

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Supported by the Ministero della Sanità and UCB Pharma SpA.

Received for publication June 11, 2004; revised July 21, 2004; accepted for publication July 21, 2004.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2004.07.054

METHODS

Chemicals

Histamine, β -histine, dimaprit, and cimetidine were purchased from ICN Biomedicals (Aurora, Ohio). Levocetirizine dihydrochloride was provided by UCB Farchim SA (Bulle, Switzerland).

Keratinocyte cultures

Keratinocyte cultures were prepared from skin biopsies taken from healthy subjects ($n = 3$; 2 female subjects and 1 male subject; age, 30–40 y). Biopsies were disaggregated to single-cell suspensions by using 0.25% trypsin (Biochrom, Berlin, Germany). Primary cultures were prepared by seeding cell suspensions on a feeder layer of irradiated 3T3/J2 mouse fibroblasts and cultured according to an optimized Rheinwald and Green culture technique.⁴ Second or third passage keratinocytes were used in all experiments, with cells cultured in 6-well plates in serum-free medium (Keratinocyte Growth Medium; Clonetics, San Diego, Calif) for at least 3 to 5 days before performing experiments. Keratinocytes were stimulated with 200 U/mL IFN- γ , TNF- α , or IL-4 (R&D Systems, Abingdon, Oxon, United Kingdom) for 24 hours. Treatment with histamine, selective histamine receptor agonists, or antagonists and/or cytokines was performed in medium devoid of hydrocortisone and bovine pituitary extract, but supplemented with 0.1% BSA (Sigma-Aldrich, Milan, Italy). PBMCs were separated by centrifugation over Ficoll-Hypaque (Lymphoprep; Nycomed-Pharmacia, Oslo, Norway) according to standard methods.

RT-PCR analysis

Total RNA was extracted from cultured keratinocytes by using the Trizol reagent (Invitrogen Italia, Milan, Italy). RNA (1 μ g) was reverse-transcribed to cDNA by using oligo(dT) primers and then amplified with the GeneAmp RNA PCR kit (Perkin-Elmer Applied Biosystems, Norwalk, Conn) according to the manufacturer's protocol. The following synthetic oligonucleotides were used: for H1 receptor, 5'-CAT TCT GGG GGC CTG GTT TCT CT-3' and 5'-CTT GGG GGT TTG GGA TGG TGA CT-3' (402-bp amplification product); and for H2 receptor, 5'-CCC GGC TCC GCA ACC TGA-3' and 5'-CTG ATC CCG GGC GAC CTT GA-3' (495-bp amplification product). As control, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used with primers 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' (983-bp amplification product). Thirty-five PCR cycles were run at 94°C (45 seconds), 60°C (45 seconds), and 72°C (45 seconds). PCR products were subjected to electrophoresis in a 1% agarose gel and visualized by means of ethidium bromide staining. For semi-quantitative analysis, RNA concentrations, primers, and PCR cycles were titrated to obtain standard curves to verify linearity and to permit analysis of signal strength. The levels of mRNA expression were quantified by densitometric analysis, with the values normalized against GAPDH.

Western blot analysis

Keratinocyte protein extracts were prepared by solubilizing cells in radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate in PBS) containing a mixture of protease and phosphatase inhibitors. Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Membranes were then blocked and probed with the rabbit anti-H1 and anti-H2 receptor antibodies (Alpha Diagnostics International, San Antonio, Tex) diluted in PBS containing 5% nonfat dried milk. To verify band specificity, anti-H1 and anti-H2 receptor antibodies were also preincubated with a 100 $\times$ molar excess of blocking

peptides. Filters were developed with a horseradish peroxidase-conjugated antirabbit immunoglobulin by using the ECL-plus detection system (Amersham-Pharmacia-Biotech, Milan, Italy), followed by autoradiography.

Intracellular Ca^{2+} changes

Ca^{2+} transients were measured in normal keratinocytes loaded with the Ca^{2+} indicator Fura-2/AM (Calbiochem, La Jolla, Calif) by using the digital fluorescence microscope unit Attofluor (Zeiss, Oberkochen, Germany), as described previously. Traces were followed fluorospectrometrically, and Ca^{2+} transients were determined by multiple cell acquisitions with the 340/380 wavelength excitation ratio at an emission wavelength of 505 nm. Curves shown are representatives of the whole cell population.

Flow cytometry analysis

Keratinocyte expression of membrane intercellular adhesion molecule 1 (ICAM-1), HLA-DR, and HLA-A,B,C was evaluated by using fluorescein isothiocyanate-conjugated anti-ICAM-1 (clone 84H10; Immunotech, Marseille, France), anti-HLA-DR (clone L243; BD PharMingen, San Diego, Calif), and anti-HLA-A,B,C (clone G46-2.6, BD PharMingen) mAbs. In control samples, staining was performed by using isotype-matched control antibodies. Cells were analyzed with a FACScalibur equipped with Cell Quest software (Becton Dickinson, Mountain View, Calif). Results are expressed as net mean fluorescence intensity, which represents the mean fluorescence intensity subtracted from the fluorescence of isotype-matched control antibodies.

ELISA

Cell-free supernatants from resting or stimulated keratinocyte cultures were tested for CC chemokine ligand (CCL)-5/RANTES content by using the antibody pair, rabbit polyclonal 20581D for coating and 20582D for detection (BD PharMingen). CXC chemokine ligand (CXCL)-10/IFN-induced protein of 10 kd (IP-10) was assayed by using the purified 4D5/A7/C5 and the biotinylated 6D4/D6/G2 antihuman CXCL10/IP-10 mAbs, and CCL20/macrophage inflammatory protein 3 α (MIP-3 α) release was measured by using MAB-360 and BAF-360 mAbs (BD PharMingen). GM-CSF and CCL2/monocyte chemoattractant protein 1 (MCP-1) were measured with OptEIA kits (BD PharMingen) as per the manufacturer's protocol. The plates were analyzed in an ELISA reader model 3550 UV Bio-Rad (Hercules, Calif). Keratinocyte cultures were performed in triplicate for each condition. Results are given as mean ng/10⁶ cells $\pm$ SD.

Statistical analysis

The Wilcoxon signed-rank test was applied to compare differences in membrane and secreted proteins. Significance was assumed at a P value of .05 or less.

RESULTS

Human keratinocytes express functional H1 histamine receptor

In the first set of experiments, we determined whether normal human keratinocytes from healthy individuals express histamine receptors. Unstimulated keratinocyte cultures as well as freshly procured epidermal cell suspensions deprived of CD1a⁺ and CD3⁺ cells expressed both H1 and H2 receptor mRNAs (Fig 1, A, and data not shown). Treatment of cultured keratinocytes with IFN- γ and/or TNF- α for 24 hours did not alter H1 receptor mRNA accumulation, but upregulated by about 3-fold

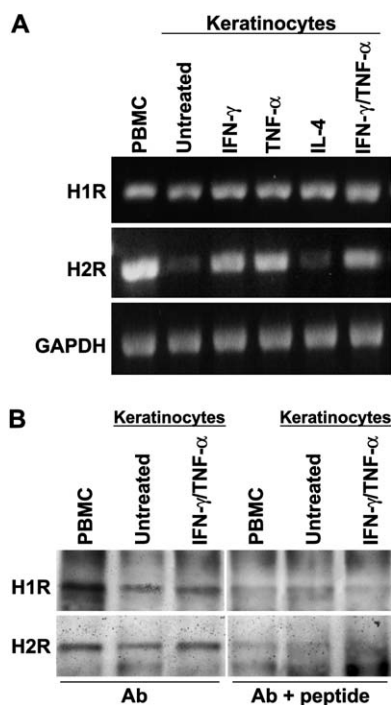


FIG 1. Human keratinocytes express the H1 and H2 histamine receptors at both the mRNA and protein levels. Primary cultures of keratinocytes were prepared from the skin of healthy individuals ($n = 3$). **A**, RT-PCR analysis was performed on mRNA from resting keratinocytes or cells incubated for 24 hours with cytokines. **B**, Western blot analysis was performed by using rabbit polyclonal anti-H1 and anti-H2 receptor antibodies. In control experiments, anti-H1 and anti-H2 antibodies were preincubated with specific blocking peptides. PBMC served as control.

H2 receptor mRNA. In contrast, IL-4 had no effect of histamine receptor expression. Western blot analysis confirmed the presence of H1 and H2 receptor proteins in resting keratinocytes, with TNF- α /IFN- γ -stimulated cells expressing more H2 receptor than resting cells (Fig 1, B). To assess receptor functionality, we measured Ca^{2+} contents in keratinocytes treated with histamine or a H1 specific agonist. Stimulation of keratinocytes with histamine induced a rapid and dose-dependent Ca^{2+} increase with maximal responses at 10^{-4} mol/L concentration (Fig 2, A). A similar response was obtained with the H1 agonist β -histine, but not with the H2 agonist dimaprit (Fig 2, B and C). Stimulation of keratinocytes with histamine or β -histine in a Ca^{2+} -free ethyleneglycol-bis-(β -aminoethylether)-N,N',N',N'-tetraacetic acid (EGTA)-containing saline solution (4 mmol/L EGTA) did not abrogate histamine-induced Ca^{2+} increase, suggesting that Ca^{2+} rise was a result of mobilization from intracellular stores (not shown).

Histamine and β -histine upregulate the expression of ICAM-1, chemokines, and GM-CSF in keratinocytes

Resting cultured keratinocytes express on their cell surface MHC class I molecule, but only limited amounts

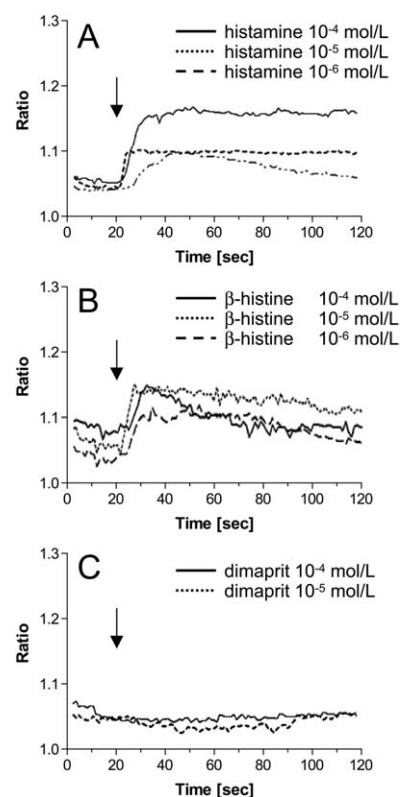


FIG 2. Histamine induces Ca^{2+} transients in resting keratinocytes. Keratinocytes were loaded with the Ca^{2+} indicator Fura-2/AM and stimulated with different concentrations of histaminergic agonists: **(A)** histamine, **(B)** β -histine (H1 receptor agonist), **(C)** dimaprit (H2 agonist). One representative experiment of 3 similar experiments is shown.

of ICAM-1 and no MHC class II molecules. MHC molecules are essential for antigen presentation, whereas ICAM-1 provides a major adhesion pathway for the retention of T lymphocytes in the epidermis. Moreover, ICAM-1 serves as an important costimulatory molecule for the cytotoxic activity of CD4^{+} and CD8^{+} T lymphocytes against keratinocytes.³ Therefore, we next examined whether histamine could affect ICAM-1 and MHC molecule expression on keratinocytes. Histamine, β -histine, or dimaprit alone did not influence baseline expression of ICAM-1 and MHC molecules. Keratinocytes exposed to IFN- γ showed high ICAM-1 expression, which was markedly upregulated when histamine was used together with IFN- γ (Figs 3 and 4). The effects of histamine was dose-dependent, with the more effective concentration in the 10^{-4} mol/L range (Fig 4). Histamine also increased IFN- γ -induced expression of MHC class I, but not of HLA-DR. Similar results were reproduced by the H1 agonist β -histine, whereas the H2 agonist dimaprit had no effect.

Keratinocytes can synthesize and release several chemokines and cytokines active on a variety of leukocytes. The release of these mediators is important for the attraction and activation of leukocytes in the skin environment. Resting keratinocytes exposed to histamine or

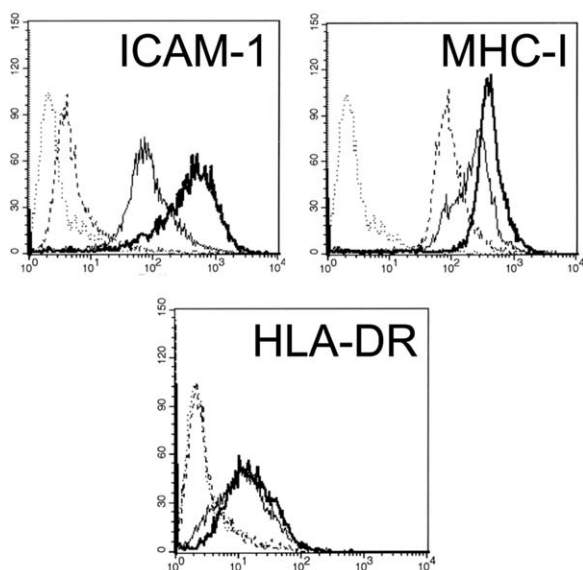


FIG 3. Histamine and β -histine increase IFN- γ -induced ICAM-1 and MHC class I on keratinocytes. Representative histograms showing the fluorescence intensity of unstimulated keratinocytes (dashed lines), IFN- γ -activated cells (continuous thin line), and keratinocytes cotreated with IFN- γ and 10^{-4} mol/L histamine (continuous bold line). Dotted lines are cells stained with isotype control immunoglobulin.

β -histine released higher amounts of the cytokine/growth factor GM-CSF and of the chemokine CCL5/RANTES (Fig 5). In contrast, histamine or β -histine did not affect the secretion of CCL2/MCP-1, CCL20/MIP-3 α , and CXCL10/IP-10 in resting keratinocytes. Histamine (10^{-4} to 10^{-6} mol/L) and β -histine were also able to enhance significantly the release of all of the aforementioned mediators from keratinocytes concomitantly activated with IFN- γ (Fig 5). In contrast, dimaprit did not affect either resting or IFN- γ -activated keratinocytes.

H1 reverse agonist levocetirizine blocks histamine-dependent upregulation of inflammatory molecules in activated keratinocytes

Antihistamine drugs act by binding and stabilizing the inactive form of the receptor and are thus better defined as inverse agonists.¹¹ We thus evaluated whether levocetirizine, a potent and selective inverse agonist of H1 histamine receptor, could counteract the effects of histamine on keratinocytes. Fig 6 shows that levocetirizine inhibited in a dose-dependent manner ICAM-1 and MHC class I expression induced by costimulation with IFN- γ and histamine. Interestingly, high doses (10 μ mol/L) of levocetirizine also reduced keratinocyte ICAM-1 and class I induced by IFN- γ alone, confirming previous findings using cetirizine.¹⁶ HLA-DR expression was not affected by levocetirizine. Levocetirizine also blocked in a dose-dependent fashion histamine-dependent release of GM-CSF and chemokines (Fig 7), and, at high doses (10 μ mol/L), inhibited secretion of CCL5/RANTES and

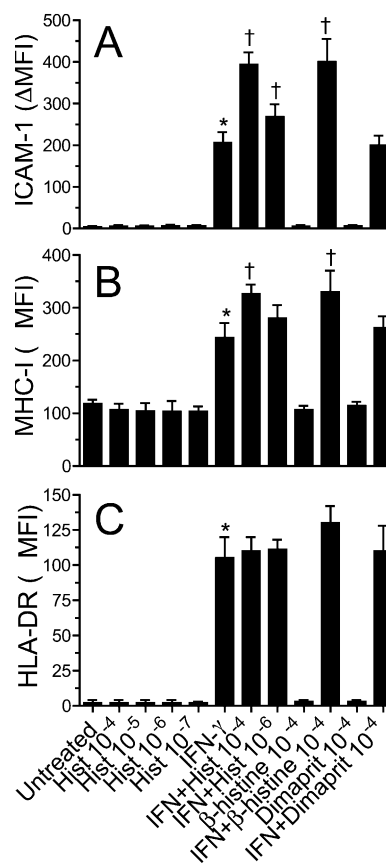


FIG 4. Histamine and β -histine augment IFN- γ -induced ICAM-1 and MHC class I on keratinocytes. Keratinocytes were treated as indicated for 24 hours and then analyzed by flow cytometry for surface expression of (A) ICAM-1, (B) MHC class I, and (C) HLA-DR. Results are expressed as the mean ($\pm$ SD) net fluorescence intensity (Δ MFI) from 3 independent experiments. * $P < .05$ versus untreated cells; † $P < .05$ versus cells treated with IFN- γ alone.

GM-CSF induced solely by IFN- γ . Addition of the H2 antihistamine cimetidine to keratinocyte cultures stimulated with either IFN- γ alone or IFN- γ plus histamine did not change the expression of membrane or secreted inflammatory mediators. The combined use of cimetidine and levocetirizine did not further inhibit histamine effects on keratinocytes compared with levocetirizine alone (data not shown).

DISCUSSION

The primary function of keratinocytes is to provide the structural and physical integrity of the skin, thereby protecting the body against external injury. Moreover, keratinocytes produce an array of antimicrobial peptides, which empower the skin against microbial insults.¹⁷ As part of their commitment in host defense, keratinocytes are actively engaged in skin inflammatory responses by attracting leukocytes and modulating their functions. These activities are also exerted during chronic inflammatory

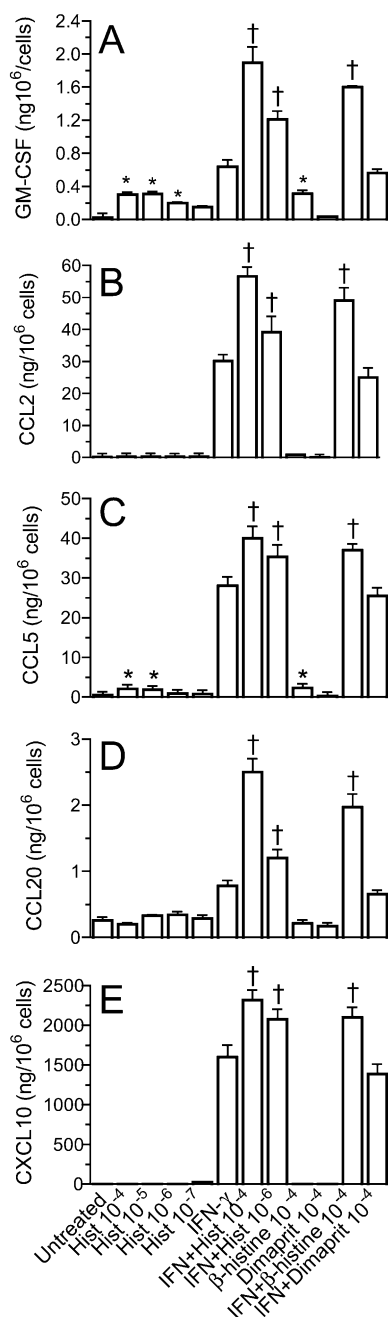


FIG 5. Histamine and β -histidine enhance the release of GM-CSF and chemokines from keratinocytes. Keratinocytes were treated as indicated for 24 hours, and the supernatants were then analyzed by ELISA for the amount of (A) GM-CSF, (B) CCL2/MCP-1, (C) CCL5/RANTES, (D) CCL20/MIP-3 α , and (E) CXCL10/IP-10. Results are expressed as mean ng/10⁶ cells. * P < .05 versus untreated cells; † P < .04 versus cells treated with IFN- γ alone.

skin disorders such as psoriasis and atopic dermatitis. In these conditions, the proinflammatory performances of keratinocytes may be genetically altered and may become detrimental.¹ Keratinocytes are particularly sensitive to leukocyte-derived cytokines and are thus easily inserted

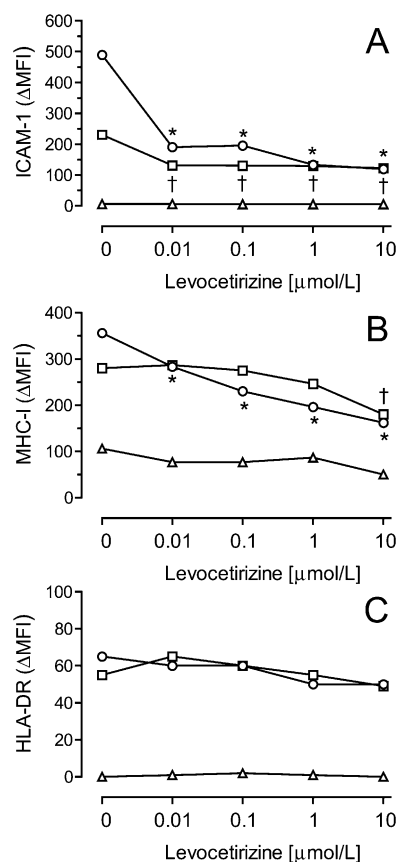


FIG 6. Levocetirizine inhibits ICAM-1 and MHC class I expression on keratinocytes. Keratinocytes were left untreated (triangles) or were stimulated with IFN- γ (squares) or IFN- γ plus 10⁻⁴ mol/L histamine (circles). Levocetirizine was added at the indicated concentrations. Data are the mean of 3 independent experiments. * P < .05 versus cells treated with IFN- γ and histamine; † P < .05 versus cells treated with IFN- γ alone.

into vicious circles that amplify skin inflammation. Other than protein mediators, a variety of other substances can participate in skin inflammation. Histamine is stored in mast cells and basophils, but it is also produced by other cell types, including neutrophils, monocytes, and T cells, in which histidine decarboxylase is rapidly induced on activation by cytokines or bacterial products.⁷ Moreover, keratinocytes themselves can represent an additional source of histamine.⁹ Indeed, histamine is present at higher levels in inflamed skin of patients with atopic dermatitis or psoriasis.^{18,19} Moreover, mast cell degranulation is a prominent feature of human allergic contact dermatitis reactions.²⁰

In this study, we show that human keratinocytes express functional H1 receptor as indicated by the capacity of selective H1 agonists to induce calcium fluxes. Histamine and β -histidine were sufficient to induce modest but significant release of CCL5/RANTES and GM-CSF in resting keratinocytes, adding further evidence about the capacity of this amine to induce cytokine release from keratinocytes.¹³⁻¹⁵ More important, histamine and the

β -histine were able to increase markedly the expression of membrane molecules (ICAM-1 and MHC class I) and the release of inflammatory soluble factors induced by IFN- γ . Histamine and IFN- γ use distinct signal transduction pathways, and this can lead to a stronger activation of inflammatory genes. In particular, histamine increases both activator protein 1 and nuclear factor κ B transcriptional activity, whereas IFN- γ uses Stat1 and Stat3 transcription factors.^{15,21} The H2 agonist dimaprit did not induce any of the inflammatory molecules studied, even in combination with IFN- γ , although the H2 receptor was present in resting keratinocytes and upregulated in response to IFN- γ and TNF- α . A synergistic activity of histamine with TNF- α in inducing ICAM-1 expression in keratinocytes has been already described by Mitra et al.¹² Consistent with this study, we found no effect of histamine on MHC class II expression. Histamine also enhanced expression of MHC class I, which is essential in CD8⁺ T-cell-mediated cytotoxicity of keratinocytes.³ Together with the increased ICAM-1, this mechanism may thus render more efficient immune-mediated epidermal damage. H1 receptor engagement led to the upregulation of several chemokines abundantly expressed in inflamed skin, including CCL2/MCP-1, CCL5/RANTES, and CXCL10/IP-10, active predominantly on monocytes and T lymphocytes.^{1,22} CCL20/MIP-3 α , which recruits immature Langerhans cells, dendritic cells, and cutaneous lymphocyte-associated antigen-positive T cells,^{23,24} was also significantly increased. These proinflammatory activities of histamine were not unexpected, because histamine has been shown to modulate the synthesis of adhesion and costimulatory molecule and cytokines in other cell types, including monocytes, dendritic cells, T cells, and endothelial cells, mostly via the H1 receptor.^{7,8,10} CCL5/RANTES and CCL2/MCP-1 induce mast cell accumulation and histamine release when injected into rat skin.²⁵ CCL5/RANTES, and less effectively CCL2/MCP-1, also upregulate histidine decarboxylase transcripts,²⁵ indicating the existence of a complex histamine-chemokine cross-talk in the skin. In contrast with normal cells, neoplastic keratinocytes may show a different response to histamine. In particular, it has been shown that histamine inhibits the production of CXCL10/IP-10 from human squamous cell carcinoma and melanoma cell lines stimulated with IFN- γ . This effect was mediated primarily by the H2 receptor and suggests that histamine may suppress antitumor immune responses.²⁶

We have also shown that levocetirizine very efficiently blocks all of the proinflammatory actions of histamine on keratinocytes. In contrast, the H2 antagonist cimetidine did not inhibit these inflammatory actions of histamine, as previously observed.¹⁵ The activity of levocetirizine was dose-dependent, with as little as 0.01 μ mol/L (4.6 ng/mL) showing significant effects in many instances. Levocetirizine is the R-enantiomer of racemate cetirizine. Compared with cetirizine, it possesses higher affinity and a slower dissociation kinetics for H1 receptors, and is very poorly metabolized.^{27,28} Interestingly enough, levocetirizine inhibited ICAM-1 expression as well as CCL5/

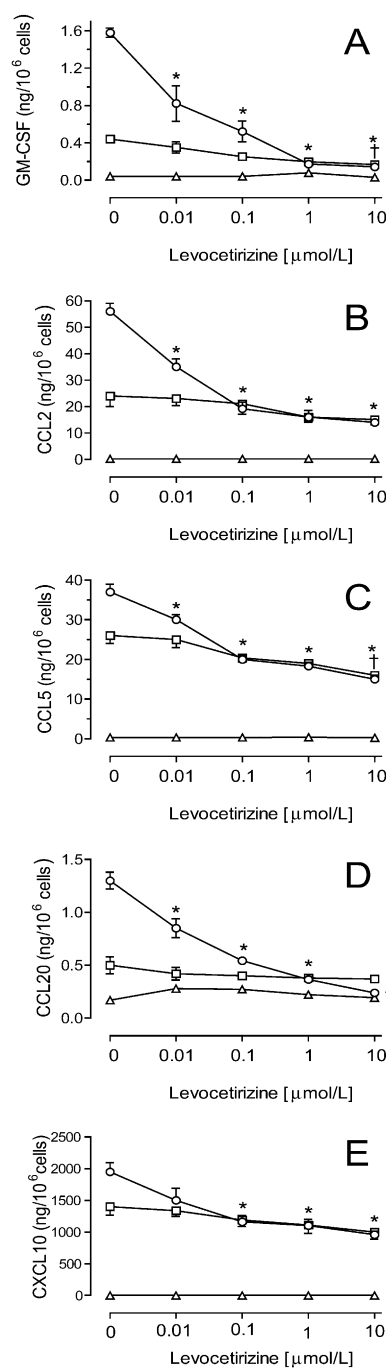


FIG 7. Levocetirizine reduces chemokine and GM-CSF release from keratinocytes. Keratinocytes were left untreated (triangles) or were stimulated with IFN- γ (squares) or IFN- γ plus 10^{-4} mol/L histamine (circles). Levocetirizine was added at the indicated concentrations. * $P < .05$ versus cells treated with IFN- γ and histamine; † $P < .05$ versus cells treated with IFN- γ alone.

RANTES and GM-CSF release induced solely by IFN- γ , indicating an anti-inflammatory activity of the drug independent of added histamine. Similar activity has been demonstrated for cetirizine¹⁶ and may be the consequence of the inhibition of endogenous histamine produced by

keratinocytes in response to IFN- γ . Experiments are currently underway to test this hypothesis. At this time, it is difficult to extrapolate the *in vivo* relevance of the anti-inflammatory activity shown by levocetirizine *in vitro*. The skin levels of levocetirizine after oral intake are not known. However, skin concentrations of cetirizine are approximately 50% of the corresponding plasma values 3 hours after a single oral dose, and are in the order of 150 to 300 ng/mL.^{29,30} Levocetirizine is not further metabolized, and it is thus possible that skin levels of the drug at least similar to those of cetirizine are reached in the skin.

In conclusion, our study indicates that histamine exerts relevant proinflammatory activity on keratinocytes *in vitro*, mostly by augmenting the IFN- γ -induced activation of keratinocytes. These effects are mediated via the H1 receptor, and they can be efficiently inhibited by levocetirizine.

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