



Modulation of expression and function of Toll-like receptor 3 in A549 and H292 cells by histamine

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Abstract

It was reported recently that histamine induced Toll-like receptor (TLR)2 and TLR4 expression in endothelial cells and enhanced their sensitivity to Gram-positive and Gram-negative bacteria; and that TLRs were expressed in airway epithelial cells and that several inflammatory mediators modulated their expression. However, little is known of potential influence of histamine on TLRs in pulmonary epithelial cells. In the present study, effects of histamine on expression of TLRs in both human A549 and NCI-H292 cell lines were examined by using real-time quantitative RT-PCR analysis, flow cytometry and immunofluorescent staining. The results revealed that both cell types constitutively expressed mRNAs for TLR1–TLR10. Histamine up-regulated the expression of TLR3 mRNA by 12.3- and 11.6-fold, respectively in both cell types. The time course showed that histamine induced TLR3 mRNA expression was initiated at 30 min, nearly reached peak levels after 2 h and was sustained at least until 12 h. Histamine also induced TLR3 protein expression in A549 and NCI-H292 cells. Histamine and poly (I:C), a specific TLR3 ligand stimulated interleukin (IL)-8 secretion from both cell types. Moreover, histamine enhanced poly (I:C)-induced IL-8 secretion and phosphorylation of NF-kappaB in the two cell types, and histamine H₁ receptor antagonists inhibited the action of histamine. In conclusion, histamine selectively up-regulated expression of TLR3, and stimulated IL-8 secretion from the cells. Histamine also enhanced poly (I:C) induced IL-8 secretion and phosphorylation of NF-kappaB. These observations suggest that histamine might play an important role in enhancing the innate immune responses of airway to viral infection.

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1. Introduction

Histamine is a well-known mediator of allergic inflammation. In addition to its well-recognized function as a vasoactive substance, this mediator can also modulate the body defense reactions against infection through its regulatory effects on the induction of inflammatory cell accumulation (He et al., 1997) and release of inflammatory cytokines from monocytes/macrophages (Dileepan et al., 1993), eosinophils (Raible et al., 1992), T cells (Ogden and Hill, 1980), neutrophils (Beer et al., 1984), endothelium (Delneste et al., 1994), and epithelium

(Takizawa et al., 1995; Rugolo et al., 1996; Li et al., 1995). The receptors for histamine discovered so far include H₁, H₂, H₃ and H₄ (Hough, 2001; Morse et al., 2001), which are a group of G-protein-coupled receptors. A number of studies have shown that some histamine-induced inflammatory responses of human airway epithelial cells are mediated through H₁ receptor (Takizawa et al., 1995; Rugolo et al., 1996; Li et al., 1995).

The respiratory epithelium is an important interface between the body and the environment and is constantly exposed to invading pathogens. In addition to providing a barrier against the entry of foreign pathogens, epithelial cells have been shown to be able to produce a variety of biologically active mediators, including cytokines and chemokines (Diamond et al., 2000) in response to microorganisms. Recent studies have shown that airway epithelial cells express a family of pattern recognition receptors called Toll-like receptors (TLRs) and that the expression of TLRs can be regulated by cytokines, corticosteroids or microbes (Sha et

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al., 2004; Armstrong et al., 2004; Homma et al., 2004; Monick et al., 2003).

TLRs play a central role in detecting microbes by initiating innate immune responses and linking innate and adaptive immunity (Akira et al., 2001). Since Medzhitov and Janeway (Medzhitov and Janeway, 1997) discovered the first mammalian TLR, 11 family members have been identified in mammalian systems (Akira, 2003; Zhang et al., 2004). These type I transmembrane proteins share significant similarities with the IL-1 receptor and all contain the highly homologous toll/IL-1 receptor cytoplasmic domain. Many functions of TLRs are carried out through a group of signaling molecules, including MyD88, IL-1 receptor-associated kinase, TNF receptor associated factor-6, mitogen-activated protein kinases and NF- κ B (Shizuo, 2003). The homology present in the toll/IL-1R is contrasted with significant variation in the leucine-rich repeats of the extracellular domains, which permits discrimination of a variety of pathogen-associated molecular patterns. TLR2 and TLR4 recognize endotoxin, lipoproteins and some extracellular matrix molecules; TLR3 recognizes double-stranded RNA; TLR5 recognizes bacterial flagellin; The synthetic imidazoquinolines activate immune cells via TLR7; TLR9 recognizes CpG-containing DNA. Briefly, TLRs are capable of sensing organisms ranging from protozoa to bacteria, fungi and viruses (Beutler, 2004).

Respiratory infections are thought to be a leading cause of exacerbation of asthma (Johnston et al., 1995; Nicholson et al., 1993). In asthma, airway epithelial damage is commonly observed and thus more sufficiently exposed to inflammatory environment. Mast cells, frequently located in close proximity to airway epithelium, not only provide a stable pool of histamine, but also spontaneously release this mediator in response to stimulation by several cytokines (Oh and Nakano, 1988). A recent study by Talreja et al. has shown that histamine can induce TLR2 and TLR4 expression in endothelial cells and enhance their sensitivity to Gram-positive and Gram-negative bacteria (Talreja et al., 2004). Another study by Corne et al. has shown that people with atopic asthma suffer from more frequent lower respiratory tract infections and have more severe and longer-lasting lower respiratory tract symptoms (Corne et al., 2002).

Based on the information above, we attempted to determine whether histamine could regulate the expression of TLRs and IL-8 secretion from two pulmonary tumour cell lines in the present study.

2. Materials and methods

2.1. Reagents and antibodies

DMEM, antibiotics, glutamine and EDTA, TRIzol reagent, dNTP were from Invitrogen (Carlsbad, CA). FCS was purchased from Hyclone (Logan, UT). Polyinosinic-polycytidylic acid [poly (I:C)], histamine and diphenhydramine hydrochloride, terfenadine were obtained from Sigma Chemical Corp (Saint Louis, MO). FITC-conjugated mouse monoclonal antibody against human TLR3 (Clone: 40C1285) and isotype control (FITC-conjugated mouse IgG₁, Clone: Ci4) were from

Immugenex Corp (Los Angeles, CA) and Serologicals Corp (Chemicom, Australia), respectively. Rabbit monoclonal antibodies against phospho-NF- κ B p65 (Ser 536) and polyclonal antibodies against NF- κ B p65 were from Cell Signaling Technology (Beverly, MA). HRP-conjugated goat anti-rabbit secondary antibody was from Pierce (Rockford, IL). RNasin and moloney murine leukemia virus reverse transcriptase were from Promega (Madison, WI). SYBR Green I Stain was from Cambrex (San Diego, CA). RNase-free DNase I, ProofStart DNA Polymerase and QuantiTetTM SYBR Green PCR Kit were from Qiagen (Valencia, CA). Uracil-N-Glycosylase was from MBI Fermentas (Hanover, MD). Most of other reagents such as salt and buffer components were analytical grade and obtained from Sigma (St. Louis, MO).

2.2. Cell culture

The human lung carcinoma cell line A549 and the human mucoepidermoid carcinoma cell line NCI-H292 were obtained from the American Type Culture Collection (Manassas, VA). A549 and NCI-H292 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C with 5% CO₂ in humidified air. For challenge experiments, cells were transferred into 12-well plastic culture plates, cultured until the adherent cells reached ~80% confluence. The cells were then cultured with the serum-free basal medium for an additional 8 h before challenge.

2.3. Cell challenge

Cells were challenged with various concentrations of histamine, diphenhydramine hydrochloride, terfenadine or poly (I:C) for different periods. After centrifugation, culture supernatants were collected and stored at –80 °C until use, and cells were harvested for extracting total RNA. The cell priming experiments were also performed for investigation of effect of histamine on poly (I:C)-induced IL-8 release. Briefly, cells were preincubated with 100 μ M of histamine (or for some experiments, 10 μ M of diphenhydramine or terfenadine were added to cells for 1 h before addition of histamine) for 12 h, and then washed twice with the fresh medium before incubation with 25 μ g/ml of poly (I:C) or medium alone for 12 h.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from confluent A549 cells and NCI-H292 cells, using TRIzol reagent and treated with RNase-free DNase I to remove contaminated DNA according to the manufacturer's protocol. After the first-strand cDNA was obtained by incubating RNA with oligo (dT)₁₈ primers, 1 μ l of each cDNA sample (corresponding to 100 ng total RNA) was used for standard PCR and real-time PCR. The primer sequences and sizes of amplicons for TLR1–TLR10 and β -actin are listed in Table 1. Standard PCR was performed in a Peltier thermal cycler apparatus (MJ Research, Watertown, MA) using the ProofStart DNA Polymerase. The thermocycling protocol was as follows: 95 °C

Table 1
Primers for RT-PCR and/or real-time RT-PCR

Target gene	Locus	Forward sequence (5'–3')	Reverse sequence (5'–3')	Amplicon size (bp)
TLR1	NM.003263	gcccaaggaaaagagcaaac	aaagcagcaatatcaacaggag	135
TLR2	NM.003264	tctccatttcgctctttt	ggcttggtgttcattatcttc	125
TLR3	NM.003265	taaaactgaacctgcactct	tatgacgaaaggcacctatc	101
TLR4	NM.003266	ccgcttcctggtcttatcat	tctgctgcaactcatttc	141
TLR5	NM.003268	ttgtcaaacacctggacac	ctgctcacaagacaaacgat	148
TLR6	NM.006068	cccaaggagaaaagcaaac	ttcaccatcatccaagtaaat	156
TLR7	NM.016562	agcattttactgtcccatc	gccactcaaggacagaact	140
TLR8	NM.016610	caaccaaagcaagaaaacag	tgcaagccaagtaaaaaag	87
TLR9	NM.017442	ggcaaaagtgggcgagatgag	agtggtgggtgtccctggtc	483
TLR10	NM.030956	ctccaacttgtccagaat	tgggtgggaatgcaatagaat	132
β -actin	BC002409	gttgcggttacacctttctt	accttcaccgttccagttt	148

Primers were designed according to cDNA sequences from the NCBI database.

for 5 min, 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 30 s. Amplification products containing SYBR Green I Stain were resolved in a 1.5% agarose gel. Gels were recorded with an Ultra-Lum system (Ultra-Lum, Claremont, CA).

2.5. Real-time PCR

Real-time PCR was performed in an ABI PRISM 7700 Sequence Detection System Thermal Cycler (PE Applied Biosystems, Foster City, CA) to quantify TLR1–TLR10 and β -actin mRNAs with QuantiTetTM SYBR Green PCR Kit. In a 0.2 ml PCR tube (Bio-Rad, Hercules, CA), 1.0 μ l of cDNA was diluted 1 in 9 in water and 9 μ l of it were added to a PCR reaction mixture containing 12.5 μ l of 2 $\times$ QuantiTetTM SYBR Green PCR Master Mix, 0.3 μ M of each sense and antisense primers, and 0.5 U of Uracil-*N*-Glycosylase, which gave a total volume of 25 μ l. Cycle parameters were 50 °C for 2 min to activate UNG, 95 °C for 15 min to activate HotStarTaq DNA Polymerase, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 30 s. Specificities of the amplification products were controlled by melting curve analysis. No amplification of non-specific products was observed for any of the primer sets used. The gene specific threshold cycle (C_t) for each sample (TLR1–TLR10) was corrected by subtracting the C_t for the housekeeping gene β -actin (ΔC_t). Untreated controls were chosen as the reference samples, and the ΔC_t for all experimental samples were subtracted by the ΔC_t for the control samples ($\Delta\Delta C_t$). The magnitude change of test gene mRNA was expressed as $2^{-\Delta\Delta C_t}$ (Sha et al., 2004).

2.6. Flow cytometry

A549 cells grown in six-well culture plates were incubated in the presence or absence of 100 μ M histamine for 2, 12 and 24 h. For some experiments, cells were preincubated with 10 μ M diphenhydramine hydrochloride, a histamine H₁ receptor antagonist for 1 h before addition of 100 μ M histamine for 12 h. Cells were then harvested for immunocytochemical analysis. For intracellular staining, cells were fixed and permeabilized by

incubating them with a solution containing 2% paraformaldehyde and 0.2% Tween 20 in PBS on ice for 60 min. For each analysis, cells were washed twice with PBS containing 0.2% BSA and incubated with 50 μ l of FITC-conjugated anti-TLR3 antibody (40C1285, 100 μ g/ml) or FITC-conjugated isotype control (mouse IgG1, 5 μ g/ml) in the dark for 30 min at 4 °C. Fluorescence analysis was performed using a FACScan Flow Cytometer (BD Biosciences, San Diego, CA). Results were expressed as the mean fluorescence intensity (MFI), comparing the stimulated cells with the unstimulated cells.

2.7. Immunofluorescent staining

A549 and NCI-H292 cells in Lab-Tek[®] II chamber slides were incubated with 100 μ M histamine for 2, 12 and 24 h. For some experiments, cells were preincubated with 10 μ M diphenhydramine hydrochloride for 1 h before addition of 100 μ M histamine for 12 h. Cells were fixed in 2% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 60 min, then stained with FITC-conjugated anti-TLR3 antibody (40C1285, 100 μ g/ml). In control experiments, cells were incubated with FITC-conjugated mouse IgG₁ (5 μ g/ml) as isotype control. The cells were washed and viewed under a confocal microscope.

2.8. Determination of IL-8 levels in culture supernatants

IL-8 concentrations were determined using a human IL-8 ELISA kit (Pierce, Rockford, IL).

2.9. Western blot analysis

After confluent, A549 and NCI-H292 cells were plated in 75 mm culture dishes and incubated for 12 h with 100 μ M histamine or medium alone. For some experiments, cells were preincubated with 10 μ M diphenhydramine for 1 h before addition of 100 μ M histamine for 12 h. The cells were then stimulated with 25 μ g/ml poly (I:C) (or medium alone) for 30 min before being lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 25 mM β -glycerolphosphate, 2 mM sodium

pyrophosphate, 1% protease inhibitor cocktail and 0.5 mM dithiothreitol. Cell debris was removed by centrifugation of the lysate at $13,000 \times g$ for 10 min. The supernatants were mixed with equal volumes of $2\times$ sodium dodecyl sulphate sample buffer and heated to 100°C for 5 min. An equal volume of sample was fractionated by SDS-PAGE on a 10% acrylamide gel and transferred onto a polyvinylidene difluoride membrane with a Bio-Rad semidry transfer system, according to the manufacturer's instructions. After blocking non-specific binding sites with 5% non-fat milk in TBS/Tween for 1 h, membranes were probed with anti-NF- κB or anti-phospho-NF- κB antibodies overnight followed by incubated with HRP-conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. Densitometry analysis of immunoblots was carried out using a ScionImage software. The relative levels of phospho-NF- κB were expressed as the ratio to NF- κB .

2.10. Statistical analysis

Data are expressed as mean $\pm$ S.E.M. Statistical significance of differences was determined with a one-way ANOVA and a post hoc test using the SPSS version 11. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Expression of TLR1–TLR10 mRNAs in airway epithelial cells

TLR1–TLR10 mRNAs were constitutively expressed in both A549 and NCI-H292 epithelial cells (Fig. 1). The sizes of amplicons for TLR1–TLR10 were between 101 and 156 bp, except for TLR8 and TLR9, which had sizes of 87 and 483 bp, respectively

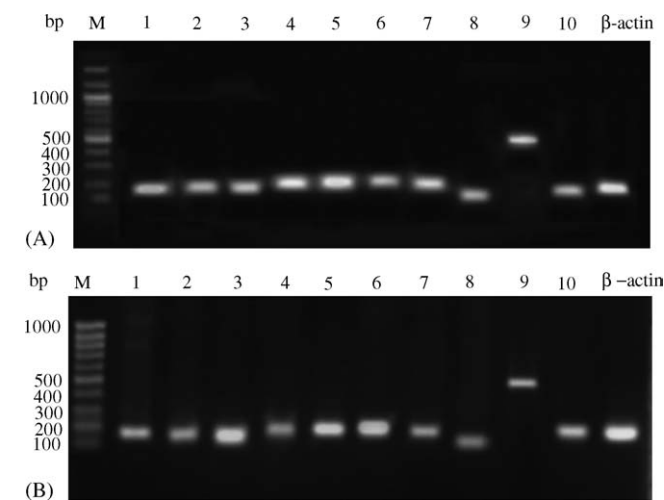


Fig. 1. RT-PCR analysis of the expression of mRNAs for TLR1–TLR10 in (A) A549 cells and (B) NCI-H292 cells. Total cellular RNA was extracted and subjected to RT-PCR using specific primers to human TLR1–TLR10 and β -actin. Amplification products were resolved on a 1.5% agarose gel containing SYBR Green I Stain (image shown was a representative of three experiments). Lanes 1–10 represented TLR1–TLR10, respectively. M = DNA marker; bp = base pair.

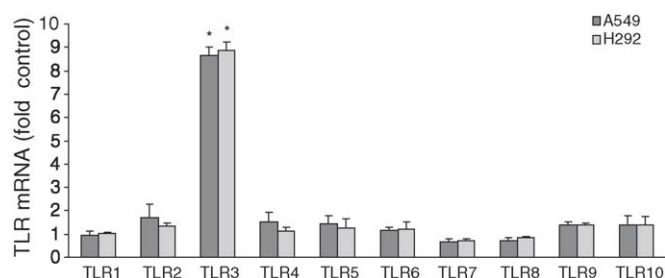


Fig. 2. Analysis of histamine induced expression of TLR mRNAs in A549 and NCI-H292 cells using real-time PCR. Total cellular RNA was isolated, reverse transcribed to cDNA, and the cDNA was used for real-time PCR. The ratio of histamine induced TLR1–TLR10 mRNA expression to the basal expression of TLR1–TLR10 mRNAs in A549 and H292 cells was shown. Cells were treated with $100 \mu\text{M}$ of histamine at 37°C for 2 h. The data were normalized to the housekeeping gene (β -actin gene) and were expressed as mean $\pm$ S.E.M. fold of control for four separate experiments performed in duplicate. * $p < 0.05$ compared with the baseline control.

(Table 1). These sizes of the amplicons were matched well with their corresponding predicted sizes. RT-PCR analysis of β -actin expression confirmed the quality of all mRNA preparations used for RT-PCR.

3.2. Effect of histamine on expression of TLRs mRNA in airway epithelial cells

The ratio of histamine induced TLR1–TLR10 mRNA expression to the basal expression of TLR1–TLR10 mRNAs in A549 and H292 cells is shown in Fig. 2. Histamine at $100 \mu\text{M}$ induced up-regulation of expression of TLR3 mRNA by 8.7- and 8.9-fold, respectively, but had no effect on expression of the other TLR mRNAs in A549 and NCI-H292 cells.

3.3. Up-regulation of expression of TLR3 mRNA by histamine in airway epithelial cells

Histamine at concentrations of 1, 10 and $100 \mu\text{M}$ stimulated a dose-dependent increase in expression of TLR3 mRNA in both A549 and NCI-H292 cells following 2 h incubation period. As little as $1 \mu\text{M}$ ($0.11 \mu\text{g/ml}$) of histamine was able to induce approximately 7.8- and 5.8-fold increase in expression of TLR3 mRNA in NCI-H292 and A549 cells, respectively. The maximum increased expression of TLR3 mRNA was 12.3- and 11.6-fold over basal expression in A549 and NCI-H292 cells, respectively induced by $100 \mu\text{M}$ of histamine (Fig. 3A). The time courses for both A549 and NCI-H292 cells showed that $100 \mu\text{M}$ histamine induced TLR3 mRNA expression was initiated at 30 min, peaked around 2 h and was sustained until at least 12 h before declining at 24 h (Fig. 3B).

3.4. Inhibition of histamine-induced expression of TLR3 mRNA by anti-histamines

Histamine H_1 receptor antagonist diphenhydramine at concentration of $10 \mu\text{M}$ inhibited approximately 85% and 83% histamine induced up-regulation of TLR3 mRNA expression in A549 and NCI-H292 cells, respectively after a 2 h

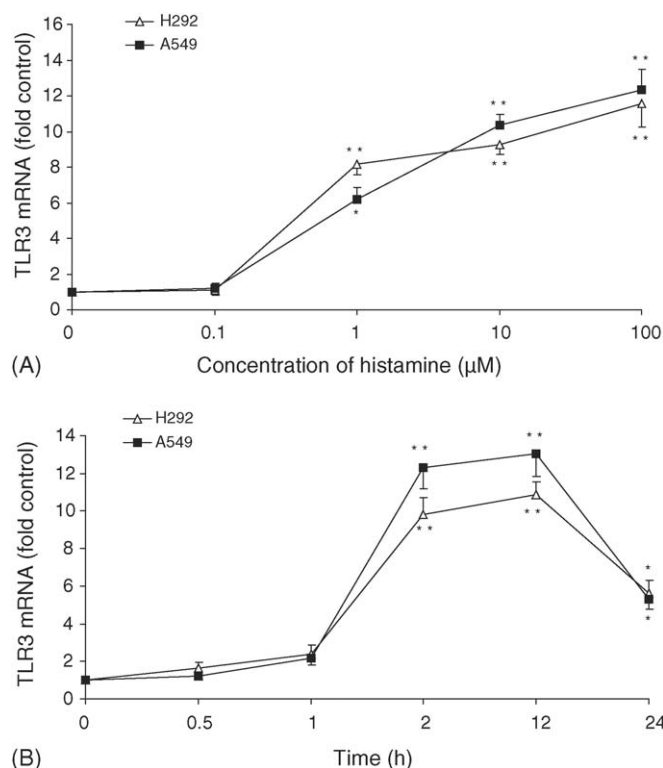


Fig. 3. Influence of histamine on expression of TLR3 mRNA in A549 and NCI-H292 cells. (A) Dose dependent curves of histamine induced up-regulation of expression of TLR3 mRNA in A549 and NCI-H292 cells. Cells were incubated with various concentrations of histamine at 37°C for 2 h. The data were calculated as described in materials and methods. * $p < 0.05$, ** $p < 0.01$ compared with the gene expression induced by histamine at the concentration of $0.1 \mu\text{M}$ ($n = 4$). (B) Time course study of histamine induced up-regulation of expression of TLR3 mRNA in A549 and NCI-H292 cells. Cells were incubated with $100 \mu\text{M}$ of histamine at 37°C for different periods. The data were calculated as described in materials and methods. * $p < 0.05$, ** $p < 0.01$ compared with the gene expression induced by histamine for 0.5 h ($n = 4$).

incubation period. Similarly, another histamine H_1 receptor antagonist terfenadine at a concentration of $10 \mu\text{M}$ reduced histamine stimulated up-regulation of TLR3 mRNA expression by 89% and 88% in A549 and NCI-H292 cells, respectively (Fig. 4).

3.5. Up-regulation of expression of TLR3 by histamine in airway epithelial cells

Immunofluorescent staining revealed that TLR3 protein was constitutively expressed in A549 and NCI-H292 cells. Histamine at a concentration of $100 \mu\text{M}$ was able to markedly up-regulate the expression of TLR3 in A549 and NCI-H292 cells, which was particularly evident following 12 h incubation period. Diphenhydramine at $10 \mu\text{M}$ clearly diminished histamine-induced expression of TLR3 in A549 and NCI-H292 cells (Fig. 5A and B). Flow cytometry analysis showed that histamine at $100 \mu\text{M}$ significantly enhanced the expression of TLR3 in A549 cells (Fig. 6A) with the mean fluorescence intensity being increased by approximately 3.5-fold (Fig. 6B). Histamine induced up-regulation of expression of

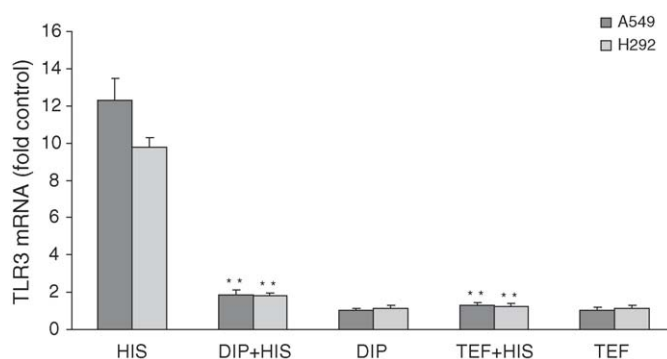


Fig. 4. Effect of antihistamines on histamine induced up-regulation of expression of TLR3 mRNA in A549 and NCI-H292 cells. Histamine H_1 receptor antagonists diphenhydramine and terfenadine were added to cells for 1 h before addition of $100 \mu\text{M}$ histamine for 2 h. The data were calculated as described in materials and methods. ** $p < 0.01$ compared with the uninhibited gene expression induced by histamine ($n = 4$). HIS = histamine; DIP = diphenhydramine; TEF = terfenadine.

TLR3 in A549 was blocked by diphenhydramine at $10 \mu\text{M}$ (Fig. 6A and B).

3.6. Effect of histamine and poly (I:C) on IL-8 secretion from airway epithelial cells

Histamine at a concentration of $100 \mu\text{M}$ stimulated a 2.3- and 2.6-fold increase in IL-8 secretion from A549 and NCI-H292 cells, respectively following a 12 h incubation period. Similarly, poly (I:C), a specific TLR3 ligand at $25 \mu\text{g/ml}$ provoked a 2.2- and 2.4-fold increase in IL-8 secretion from A549 and NCI-H292 cells, respectively. Diphenhydramine at $10 \mu\text{M}$ by itself had no influence on IL-8 secretion, but it inhibited approximately 91% and 94% histamine induced IL-8 secretion from A549 and NCI-H292 cells, respectively (Fig. 7A).

In order to determine if histamine had a priming effect on A549 and NCI-H292 cells, cells were preincubated with histamine ($100 \mu\text{M}$) for 12 h, and washed twice before adding $25 \mu\text{g/ml}$ of poly (I:C) for 12 h. Histamine was able to enhance the response of A549 and NCI-H292 cells to poly (I:C) by approximately 2.3- and 2.5-fold, respectively. The priming action of histamine on cells was completely abolished when diphenhydramine or terfenadine was preincubated with cells before addition of histamine (Fig. 7B).

3.7. Effect of histamine and poly (I:C) on phosphorylation of NF- κB in A549 and NCI-H292 cells

While histamine at a concentration of $100 \mu\text{M}$ had little effect on the phosphorylation of NF- κB after 12 h, poly (I:C) at concentration of $25 \mu\text{g/ml}$ induced 3.4- and 2.3-fold increase in phospho-NF- κB in A549 and NCI-H292 cells respectively after a 30 min incubation period. Preincubation of cells with histamine for 12 h was able to enhance the poly (I:C) induced phosphorylation of NF- κB in both cell types. Once again, pre-treating the cells with $10 \mu\text{M}$ of diphenhydramine blocked the action of histamine on cells (Fig. 8).

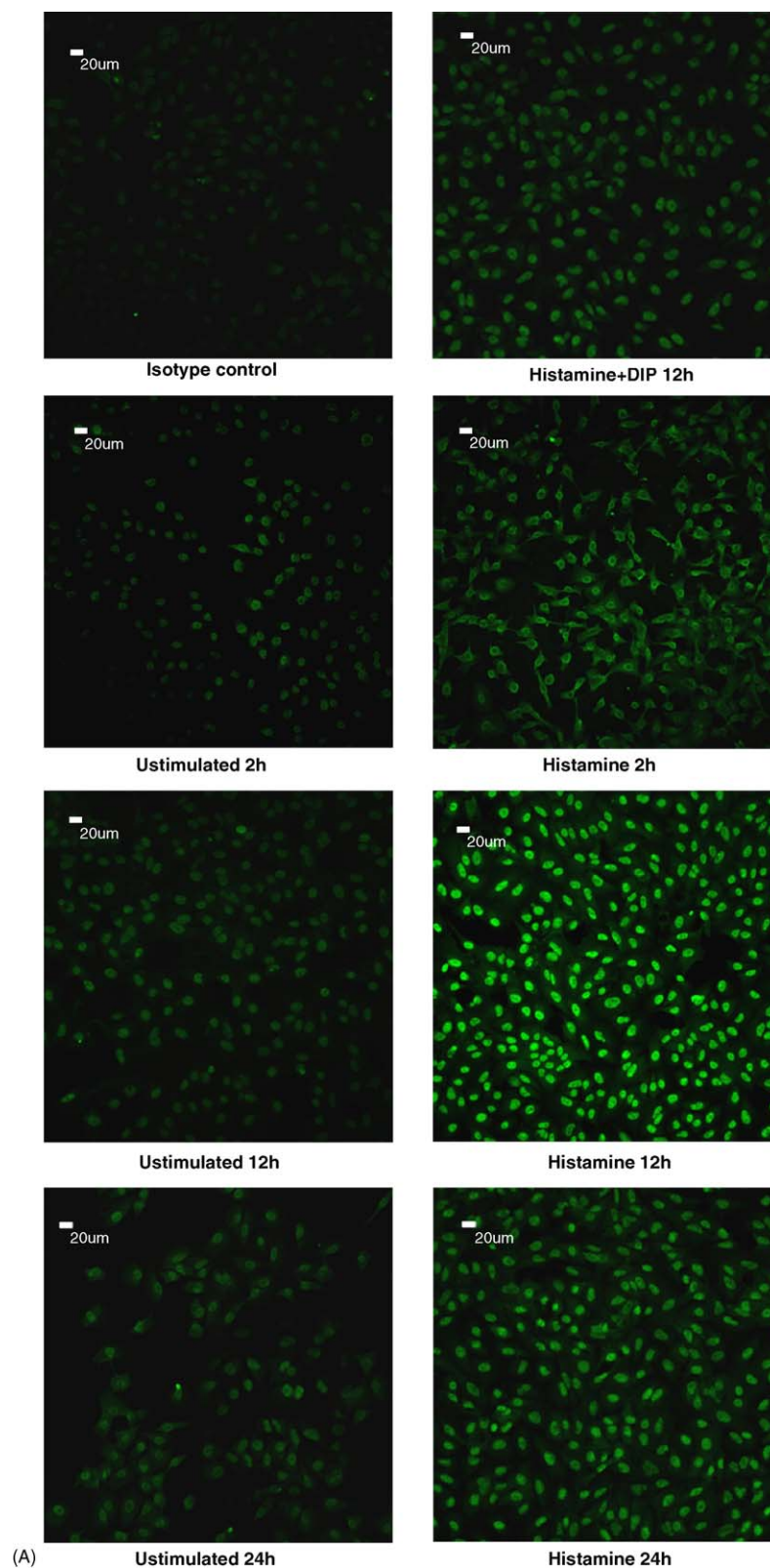


Fig. 5. Immunofluorescent staining of intracellular TLR3 protein in (A) A549 and (B) NCI-H292 cells. Cells were incubated with medium alone (constitutive expression) or histamine alone (100 μ M) at 37 $^{\circ}$ C for 2, 12, and 24 h before being stained with FITC-conjugated anti-TLR3 antibody. Cells were also incubated with histamine (100 μ M) and diphenhydramine (10 μ M) at 37 $^{\circ}$ C for 12 h. FITC-conjugated mouse IgG₁ was used as isotype control. The stained cells were viewed under a confocal microscope.

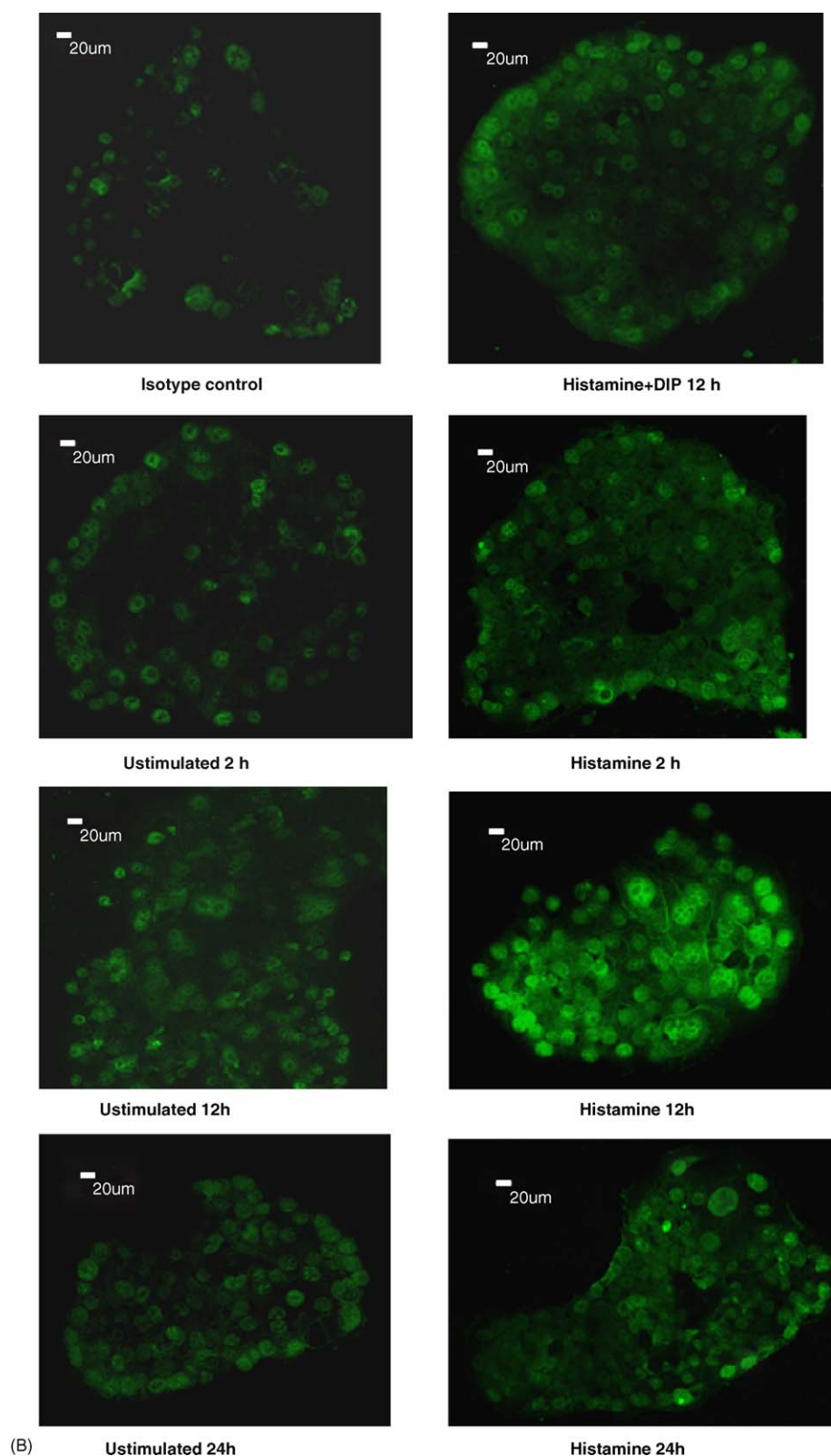


Fig. 5. (Continued).

4. Discussion

We have demonstrated for the first time that histamine, a key mediator of allergic inflammation, is able to induce up-regulated

expression of TLR3 in airway epithelial cells. This observation suggests a novel connection between the major mast cell mediator and innate immune responses. We have also shown that histamine is a potent stimulator of IL-8 release from A549 and

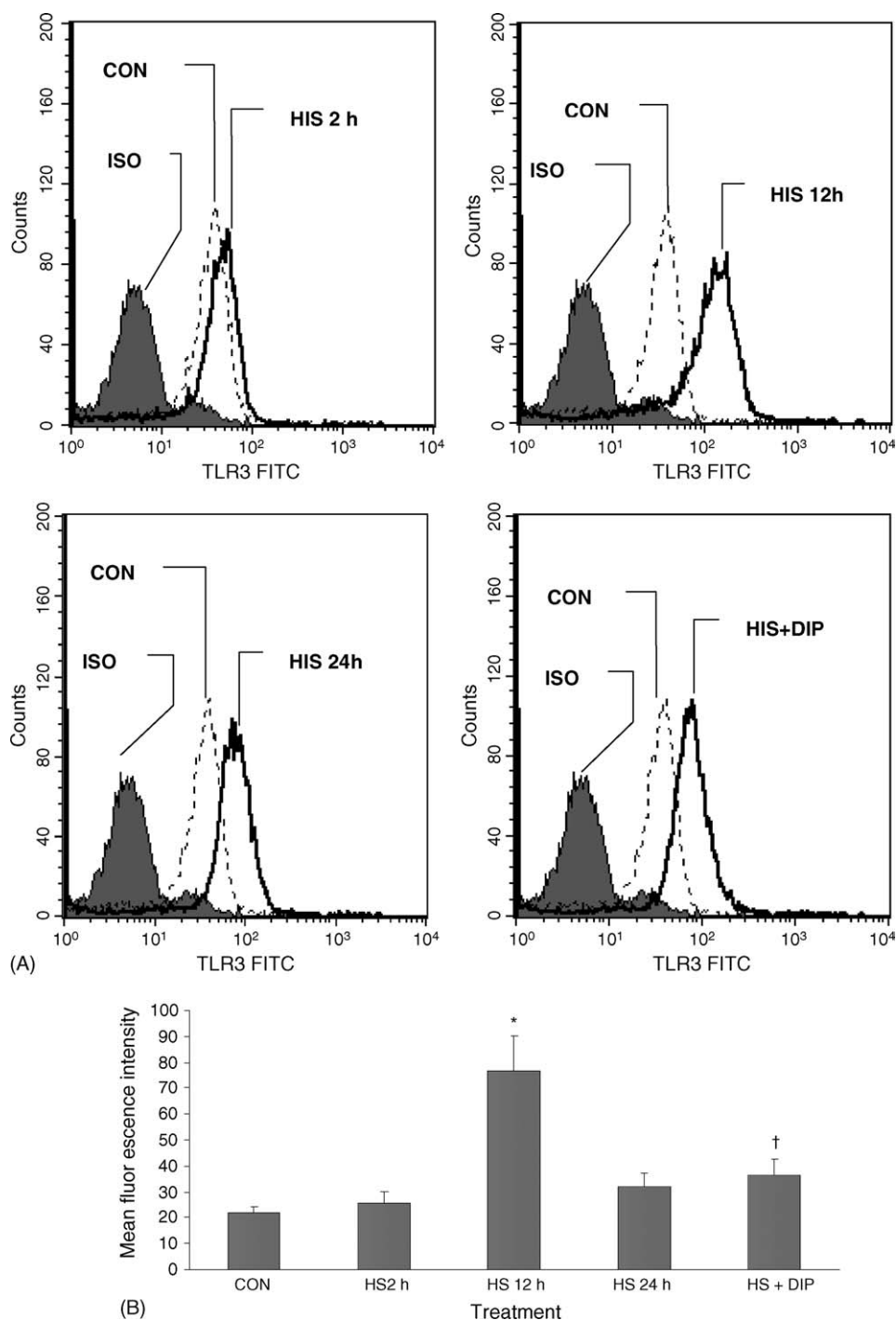


Fig. 6. (A) Flow cytometry analysis of intracellular TLR3 protein in A549 cells. ISO=isotype control; CON=medium alone control; HIS=histamine; DIP=diphenhydramine. (B) Mean fluorescence intensity data ($n=4$) from FACS analysis were shown. Cells were incubated with medium alone (constitutive expression) or histamine alone (100 μ M) at 37 °C for 2, 12, and 24 h before being stained with FITC-conjugated anti-TLR3 antibody. Cells were also incubated with histamine (100 μ M) and diphenhydramine (10 μ M) at 37 °C for 12 h. FITC-conjugated mouse IgG₁ was used as isotype control. * $p < 0.05$ compared with the constitutive baseline expression. † $p < 0.05$ compared with the uninhibited expression induced by histamine.

NCI-H292 cells, enhancing the cellular response to poly (I:C), a specific TLR3 ligand.

As little as 1 μ M (0.11 μ g/ml) histamine was able to induce up-regulated expression of the TLR3 gene in A549 and NCI-H292 cells by approximately 6.0- and 8.1-fold, which proved that it was a potent stimulator of TLR3 gene expression. Under similar conditions, poly (I:C) at a concentration of 25 μ g/ml was

only able to stimulate some 13.5-fold increase in TLR3 gene expression in BEAS-2B cells (Sha et al., 2004). Up-regulated expression of TLR3 protein was also observed in the present study when histamine was incubated with A549 and NCI-H292 cells. While the influence of histamine on TLR3 expression does not appear to have been studied previously, the report which found histamine up-regulated TLR2 and TLR4 expression in

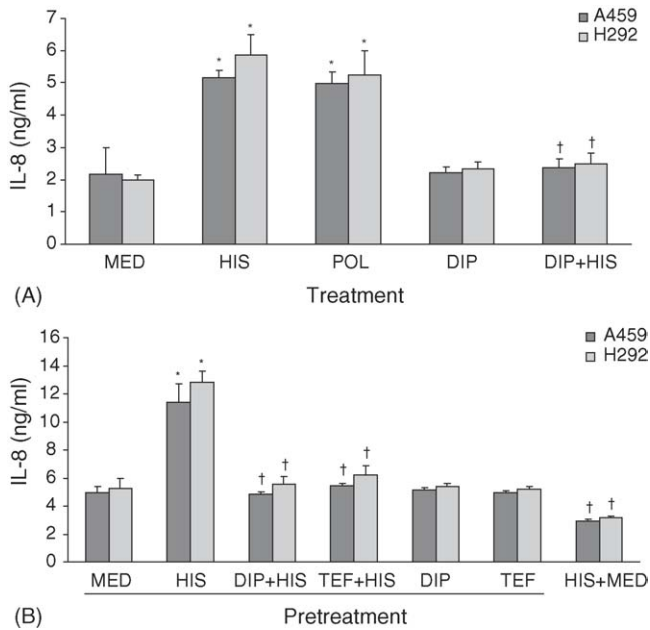


Fig. 7. Effect of histamine and poly (I:C) on IL-8 secretion from A549 cells and NCI-H292 cells. (A) Cells were incubated with 100 μ M of histamine (with or without 10 μ M of diphenhydramine), 25 μ g/ml of poly (I:C) or 10 μ M of diphenhydramine alone at 37 $^{\circ}$ C for 12 h. (B) Cells were preincubated with 100 μ M of histamine (or for some experiments, 10 μ M of diphenhydramine or terfenadine were added to cells for 1 h before addition of histamine), 10 μ M of diphenhydramine or 10 μ M of terfenadine for 12 h, and then washed twice with the fresh medium before incubation with 25 μ g/ml of poly (I:C) or medium alone for 12 h. Data were presented as the mean $\pm$ S.E.M. of four separate experiments. * p < 0.05 compared with control. † p < 0.05 compared with the response to histamine alone. MED = medium alone; HIS = histamine; DIP = diphenhydramine; TEF = terfenadine; POL = poly (I:C).

human umbilical vein endothelial cells (Talreja et al., 2004) may support our current findings.

Although it is difficult to rule out the possible differences in TLR function, cell signaling and secretion properties between normal bronchial epithelial cells and tumour-derived epithelial cell lines, we found that, like primary bronchial epithelial cells (Sha et al., 2004), both A549 and NCI-H292 cells constitutively express TLR1–TLR10 genes, providing us with a solid foundation to study TLR expression and function with these cells.

Up-regulated expression of TLR3 mRNA induced by histamine in both A549 and NCI-H292 cells was initiated at 30 min, nearly reached peak values after 2 h and was sustained at least until 12 h before decline at 24 h. To our knowledge, this is the first time course study on TLR3 gene expression in vitro in human epithelial cells (including cell lines), which indicates that histamine induced TLR3 gene expression is a relatively fast event and lasts for a quite long time. However, the maximum expression of TLR3 protein in both A549 and NCI-H292 cells induced by histamine occurs at 12 h. This 10-h time difference between the maximum TLR3 gene and protein expression may represent the time that is required for generation of TLR3 protein from TLR3 mRNA.

Moreover, histamine was found to be a potent secretagogue of IL-8 release from A549 and NCI-H292 cells. It also enhanced

poly (I:C) induced IL-8 secretion. These observations suggest that histamine may play an important role in stimulation of body defense mechanisms against viral infection. While lack of direct evidence on histamine stimulating IL-8 secretion by itself and enhancing poly (I:C) induced IL-8 secretion from airway epithelial cells, it is known that poly (I:C) can induce IL-8 secretion from BEAS-2B cells (Sha et al., 2004). Moreover, the reports that INF- α enhances poly (I:C) induced IL-28 and IL-29 gene expression in A549 cells (Tissari et al., 2005) and that histamine enhances LPS induced IL-6 release (Talreja et al., 2004) from human umbilical vein endothelial cells may support our present findings.

In the present study, the histamine H₁ receptor antagonists diphenhydramine and terfenadine at least partially inhibited histamine induced up-regulation of TLR3 gene and protein expression, histamine-stimulated IL-8 secretion and histamine-enhanced poly (I:C)-induced IL-8 secretion, indicating strongly that the actions of histamine on A549 and NCI-H292 cells are via H₁ receptor. The similar observation that diphenhydramine inhibited histamine-induced up-regulation of TLR2 and TLR4 gene expression in endothelial cells (Talreja et al., 2004) is consistent with our current findings.

While poly (I:C) at concentration of 25 μ g/ml induced 3.4- and 2.3-fold increase in phospho-NF- κ B in A549 and NCI-H292 cells, respectively after a 30 min incubation period, histamine at a concentration of 100 μ M had little effect on phosphorylation of NF- κ B at 12 h following incubation. This finding seems consistent with a very recent report on the relationship between poly (I:C) and NF- κ B, showing that poly (I:C) up-regulated expression of TLR3 was primarily regulated by NF- κ B, and subsequently caused up-regulation of IL-8 gene expression in HT29 human intestinal epithelial cell line (Schneeman et al., 2005). Although histamine by itself failed to induce phosphorylation of NF- κ B in both cell types after 12 h incubation it did enhance the poly (I:C) induced phosphorylation of NF- κ B. Hence, histamine primed poly (I:C)-induced IL-8 secretion was also likely through phosphorylation of NF- κ B. The observation that histamine had little effect on phosphorylation of NF- κ B at 12 h following incubation may be because this time point is not the best for investigation of the phosphorylation events (Schneeman et al., 2005).

A very recent report revealed that increased TLR2, 3 and 4 protein expression was found in nasal mucosa of patients with allergic rhinitis following allergen challenge, but only increase in TLR3 mRNA expression was observed during pollen season, suggesting the importance of TLR3 in allergic airway inflammation (Fransson et al., 2005). The other evidence for the importance of TLR3 in allergic airway inflammation is that TLR3 is a major receptor of viral infection (Mogensen and Paludan, 2005), rhinovirus replication increased the expression of TLR3 on BEAS-2B cells (Hewson et al., 2005) and respiratory viral infections are strongly associated with asthma exacerbation (Pattemore, 2003). Our findings that histamine induces up-regulation of TLR3 gene and protein expression and enhances poly (I:C)-induced IL-8 secretion imply further that this mast cell mediator play a crucial role in allergic airway inflammation.

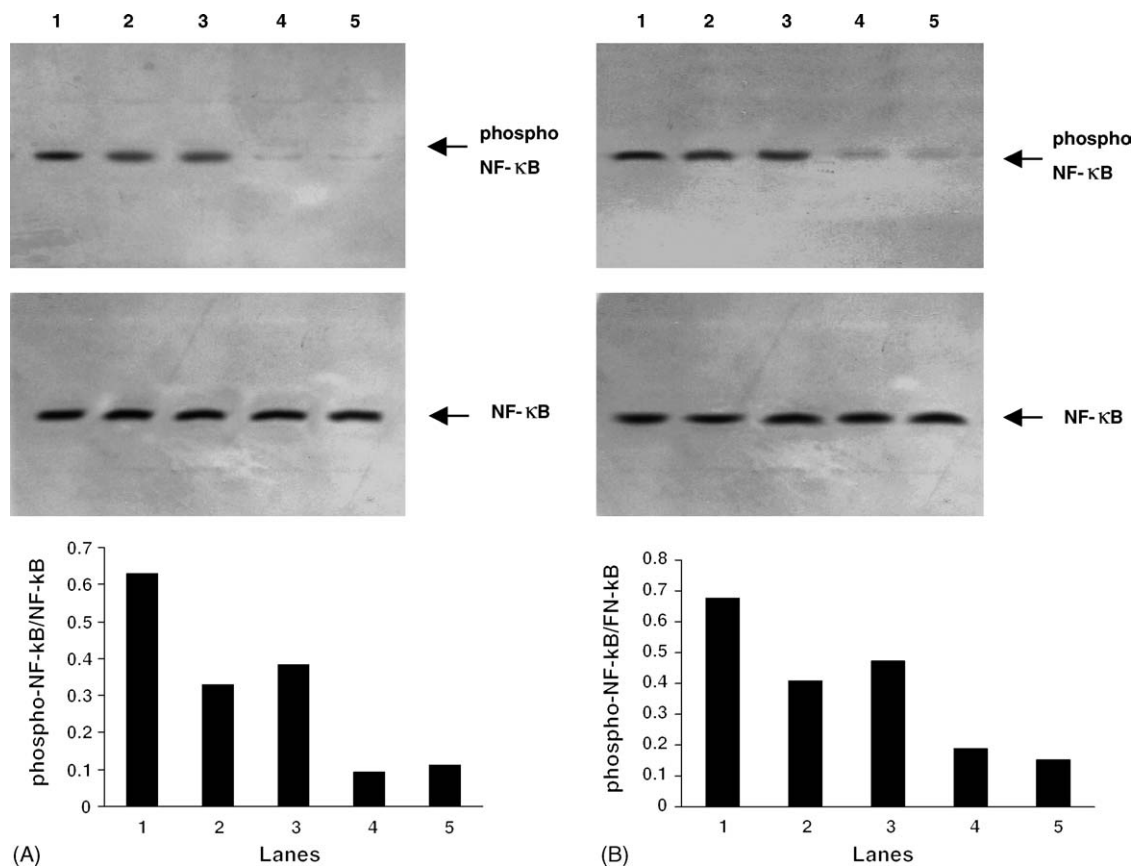


Fig. 8. Effect of histamine and poly (I:C) on phosphorylation of NF- κ B in A549 cells and NCI-H292 cells. Cells were incubated for 12 h with histamine (100 μ M), or for some experiments, cells were preincubated with diphenhydramine (10 μ M) for 1 h before addition of histamine for 12 h. The cells were then stimulated with 25 μ g/ml of poly (I:C) for 30 min before being lysed. Levels of NF- κ B and phospho-NF- κ B in (A) A549 and (B) NCI-H292 cells were determined by Western blot with specific antibodies. Lane 1: histamine 12 h + poly (I:C) 30 min; lane 2: medium 12 h + poly (I:C) 30 min; lane 3: diphenhydramine 1 h + histamine 12 h + poly (I:C) 30 min; lane 4: medium 12 h and 30 min; lane 5: histamine 12 h and medium 30 min. Densitometry analysis of immunoblots was carried out using a ScionImage software. The relative levels of phospho-NF- κ B were expressed as the ratio to NF- κ B.

Histamine H_1 receptor antagonists have been reported to be able to reduce total symptom score and virus concentrations in nasal secretions of the patients with the common cold (Gwaltney et al., 2002). They can also inhibit rhinovirus-induced ICAM-1 up-regulation and NF- κ B activation in respiratory epithelial cells (Papi et al., 2001). Since histamine H_1 receptor antagonists inhibit virus-induced TLR3 and ICAM-1 up-regulation, they may also reduce the influence of virus on epithelial cells, and therefore reduce the symptoms of the common cold. On the other hand, histamine H_1 receptor antagonists diminish the histamine induced up-regulation of TLR3 gene and protein expression and IL-8 secretion and histamine enhanced poly (I:C)-induced IL-8 secretion, therefore may affect the development of the protective immune response in airway mucosa.

In conclusion, histamine not only by itself stimulates IL-8 release, but also through selective up-regulation of TLR3 expression enhances poly (I:C)-induced IL-8 secretion from both A549 and NCI-H292 cells. The potentiation of poly (I:C)-induced IL-8 secretion by histamine appeared to associate with NF- κ B activation in these cells. Since histamine H_1 receptor antagonists diminish the ability of histamine to stimulate a protective immune response in A549 and NCI-H292 cells, these findings

may have implications for our understanding of the role of respiratory viral infections in the development of exacerbations in the patients with asthma, and the efficacy of treatment with anti-histamines.

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