

IFN- γ -enhanced allergen penetration across respiratory epithelium augments allergic inflammation

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Background: Respiratory allergen contact is the critical event in the elicitation and boosting of allergen-specific immune responses, as well as in the induction of immediate and late inflammatory reactions.

Objective: We sought to investigate the influence of various factors of allergic inflammation on the integrity and barrier function of respiratory epithelium for allergens.

Methods: We cultured the human bronchial epithelial cell line 16HBE14o– in a transwell culture system as a surrogate of intact respiratory epithelium and used purified iodine 125–labeled recombinant major birch pollen allergen (rBet v 1) to study the extent, kinetics, and factors influencing transepithelial allergen penetration.

Results: Culture supernatants from activated allergen-specific T_H1 clones decreased transepithelial resistance. A screening of various factors (histamine, IFN- γ , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-8, IL-12, and TNF- α) identified IFN- γ as a potent factor capable of reducing epithelial barrier properties and enhancing transepithelial allergen penetration. Increased submucosal allergen concentrations caused by IFN- γ -mediated reduction of epithelial barrier function provoked a more than 7-fold augmentation of histamine release from sensitized basophils.

Conclusion: These results demonstrate that the T_H1 cell-derived cytokine IFN- γ facilitates allergen penetration through the respiratory epithelium and thereby can aggravate allergic inflammation. (J Allergy Clin Immunol 2005;115:973-81.)

Key words: Allergy, allergen penetration, respiratory epithelium, IFN- γ

Allergic inflammation predominantly occurs at mucosal surfaces (eg, nose, lung, and gastrointestinal tract).¹⁻⁴ The epithelium represents a physical barrier that protects against exaggerated intrusion of antigens. However, in allergic individuals minute amounts of allergens are sufficient to activate submucosal mast cells and basophils

Abbreviations used

LDH: Lactate dehydrogenase

TER: Transepithelial resistance

to release inflammatory mediators (eg, histamine, tryptase, and leukotrienes).^{5,6} This acute allergic response is followed by a late-phase inflammatory reaction that is initiated by mast cell–derived cytokines (eg, IL-3, IL-4, IL-5, IL-8, and TNF- α) and leads to the recruitment of eosinophils, T_H2 lymphocytes, and basophils to the site of inflammation.⁷⁻⁹

Although IgE-mediated allergy is considered a primarily T_H2-mediated disease, it is well established that the chronic manifestations of atopy (eg, chronic allergic asthma and atopic dermatitis) are accompanied by the appearance of allergen-specific T_H1 cells at the sites of repeated allergen contact and chronic inflammation.¹⁰⁻¹⁵ There is evidence that these allergen-specific T_H1 cells play an important role in allergic inflammation,¹¹ but their exact role in the underlying pathomechanisms has thus far remained elusive.

In this study we have used the bronchial epithelial cell line 16HBE14o– as a surrogate of an intact respiratory epithelium to investigate the effects of various factors and cell products that are released during acute and chronic allergic inflammation on epithelial integrity and barrier function against respiratory allergens. Our experiments reveal that supernatants from allergen-specific T_H1 cell clones and, in particular, the T_H1-derived cytokine IFN- γ strongly reduced respiratory epithelial barrier function and increased transepithelial allergen penetration, which was accompanied by enhanced basophil activation. We thus demonstrate a novel mechanism of how T_H1-mediated tissue damage leads to an augmentation of allergic inflammation.

METHODS

Culture of intact human bronchial epithelial cell layers

16HBE14o– is an epithelial cell line derived from human bronchial surface epithelial cells.^{16,17} These cells form polarized monolayers when grown on transwell filters and retain properties of differentiated airway epithelial cells, including the formation of tight junctions, apical microvilli, and cilia and regulated ion transport, and are therefore considered as a surrogate for respiratory epithelium.^{16,18}

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16HBE14o– cells were grown in minimal essential medium (Gibco, Carlsbad, Calif) containing 10% FCS, 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), and 2 mM glutamine (Gibco) and were passaged when reaching approximately 80% confluence. For barrier function experiments, cells were plated at a density of 2×10^5 per 500 µL of medium to the upper chamber of 12-mm-diameter transwell inserts (polyester membranes, 0.4-µm pore size; Costar, Cambridge, Mass). The upper chamber was filled with 500 µL of medium, and the lower chamber was filled with 1500 µL of medium.

Determination of transepithelial resistance and investigation of the influence of various substances on transepithelial resistance

Transepithelial resistance (TER) served as a parameter for epithelial barrier function and was quantified with an ohmmeter (Millipore, Bedford, Mass). Baseline resistance across polyester membranes without cellular monolayers consistently ranged between 100 and 110 Ω/cm². The respective values were subtracted from TER measurements.

Experiments investigating the effects of various substances on barrier function of the epithelial monolayer were started when TER had reached at least 350 Ω/cm². 16HBE14o– monolayers were exposed to histamine (Sigma, St Louis, Mo), IL-1β, IL-2, IL-3 (Strathmann Biotech GmbH, Hannover, Germany), IL-12 (R & D Systems, Minneapolis, Minn), IFN-γ (Genzyme, Cambridge, Mass), IL-4, IL-5, IL-8, and TNF-α (Pepro Tech, Rocky Hill, NJ) at 5 different concentrations (20 pg/mL, 100 pg/mL, 1 ng/mL, 5 ng/mL, and 50 ng/mL) diluted in culture medium and to supernatants from activated basophils, mast cells, eosinophils,¹⁹ and allergen-specific T_H2 and T_H1 cell clones that had been activated by allergen exposure.²⁰

The levels of IL-4 and IFN-γ in the supernatants of the T-cell clones were measured by means of ELISA with matched antibody pairs (Endogen, Woburn, Mass), according to the manufacturer's instructions (sensitivity limits: IL-4, 4 pg/mL; IFN-γ, 15 pg/mL). Aliquots (300 µL) of supernatants and diluted substances were added to the basolateral side of cellular monolayers to mimic *in situ* conditions. Except for experiments performed with supernatants from T-cell clones, each experiment was performed in duplicate wells and compared with duplicate control wells containing only culture medium. Four independent experiments were performed with IFN-γ, 2 times in quadruplicate wells and 2 times in duplicate wells. The electrical resistance was determined every 24 hours for a period of 3 days and for a period of 5 days for one experiment with IFN-γ.

An IFN-γ-specific mouse monoclonal blocking antibody (Pepro Tech) was used to inhibit the effect of IFN-γ.

Aliquots from the apical chambers were obtained after 72 hours from wells containing increasing concentrations of IFN-γ or medium alone for measurement of lactate dehydrogenase (LDH) by using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wis). The Cytotox assays were done according to the manufacturer's instructions in duplicate wells by performing triplicate determinations, and results are shown as means ± SD.

Flow cytometry

Flow cytometric analysis of the IFN-γ receptor expression on 16HBE14o– cells was performed with a phycoerythrin-labeled anti-CD119 (IFN-γ receptor α chain) specific mAb and a phycoerythrin-labeled isotype control antibody (BD Pharmingen, San Diego, Calif) by using a FACScan (BD Biosciences). Dead cells and debris were gated out by using scatter properties of the cells and 7-amino-actinomycin D ($c = 0.025$ µg/mL) staining (Sigma, St Louis, Mo). Data were analyzed with CellQuest software (BD Biosciences).

Quantification of transepithelial allergen migration

Recombinant Bet v 1 (17 kd),²¹ the major birch pollen allergen, and recombinant Phl p 5 (28 kd), a major Timothy grass pollen allergen,^{22,23} were obtained from Biomay (Vienna, Austria). Purified dog albumin Can f 3 (69 kd), a dog dander allergen,²⁴ was obtained from Sigma. Purified allergens were iodine 125 labeled by using the chloramine T method.²⁵ The penetration of the 3 different allergens was compared by adding iodine 125–labeled allergens (500,000 cpm) to the upper chamber of intact epithelial cell layers. Two hours thereafter, aliquots of the contents of the upper and lower chamber were removed and analyzed in a gamma counter (Wallac, Turku, Finland). Counts per minute were determined for both chambers and are expressed as percentage values of permeability. Because there was no relevant difference in transepithelial penetration between these molecules, experiments were performed with Bet v 1 as a model allergen. Iodine 125–labeled Bet v 1 (250,000 cpm) was added to the upper chamber 72 hours after cytokine exposure, aliquots of the contents of the upper and lower chamber were removed, and the amount of radioactivity in both chambers was counted. Western blot analysis of allergen-containing culture medium was performed to exclude proteolytic degradation of allergens. Cells were removed from transwell filters and lysed to determine intracellular radioactivity. All experiments and measurements were performed in duplicate.

Basophil histamine release experiments

For basophil histamine release experiments, we performed transwell cultures by using the culture conditions and IFN-γ concentrations described above. After 72 hours of cytokine or medium (negative control) exposure, sets of transwell cultures were incubated with 10 ng of rBet v 1 added to the upper chamber or medium alone (negative control). After 3 hours, aliquots were removed from the lower chamber for basophil histamine release experiments. Basophils from 2 different patients with birch pollen allergy with established IgE-reactivity profiles were isolated by means of dextran sedimentation¹⁹ and exposed to equal volumes (ie, 100 µL) of culture medium from the lower chamber of transwell cultures. For control purposes, basophils were incubated with various concentrations of rBet v 1 (10^{-6} to 10^{-1} µg/mL), IFN-γ without allergen (20 pg/mL, 100 pg/mL, 1 ng/mL, 5 ng/mL, and 50 ng/mL), or medium alone. Release of histamine was measured by means of RIA (Immunotech, Marseille, France) and is expressed as a percentage of total histamine release.¹⁹ Results represent means of duplicate experiments and determinations. Experiments were performed in duplicate wells, and results represent means of triplicate determinations.

Statistical analyses

To explain the effects of different doses of IFN-γ on the relative change to the initial value, a repeated-measures ANOVA was performed. The dependent variable was the relative difference between the measurement values (x) and initial values (x_0) as follows: $(x - x_0)/x_0$. The factors dose (20 pg/mL, 100 pg/mL, 1 ng/mL, 5 ng/mL, and 50 ng/mL), time (24, 48, and 72 hours), and their interaction were included in the model. This analysis was repeated for a second experiment with fewer doses of IFN-γ (1 ng/mL, 5 ng/mL, and 50 ng/mL) but a longer observation period (24, 48, 72, 96, and 120 hours).

Concerning the histamine release experiments, a 1-way ANOVA was performed by using the factor dose to check for differences in histamine release.

We used the SAS statistical software system (version 8.2; SAS Institute Inc, Cary, NC) to carry out the calculations. *P* values of less than .05 were considered to indicate statistical significance.

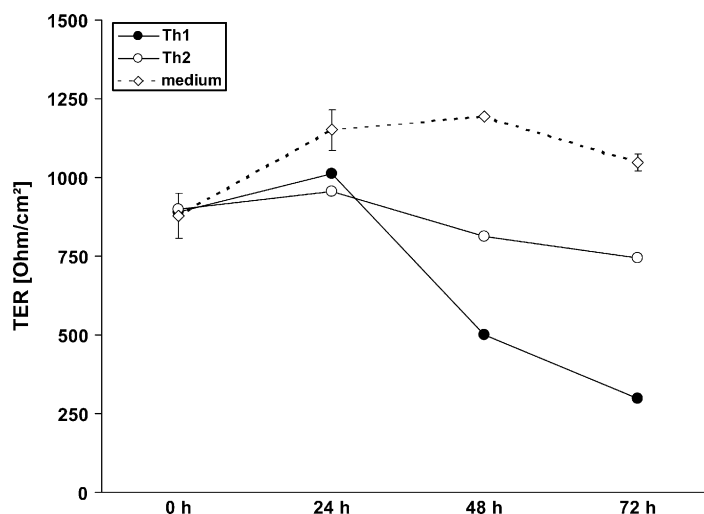


FIG 1. Supernatants from allergen-specific T_H1 cell clones reduce epithelial barrier function. TER (y-axis) was measured 24, 48, and 72 hours (x-axis) after incubation of epithelial cell monolayers with medium (duplicate wells) or supernatants from activated Bet v 1-specific T_H1 and T_H2 cell clones (single wells) from a patient with birch pollen allergy.

RESULTS

Supernatants from allergen-specific T_H1 cell clones decrease TER of human respiratory epithelial cells

We examined whether supernatants from activated T-cell clones of the T_H1 and T_H2 phenotype with specificity for the major birch pollen allergen Bet v 1 affect epithelial barrier function of human 16HBE14o– bronchial epithelial cell monolayers. After measurement of IFN- γ and IL-4 levels, supernatants were added to the basolateral side of epithelial cell layers. Results from a representative experiment are shown (Fig 1). Supernatants from T_H1 cells containing 3180 pg/mL IFN- γ induced a reduction in TER after 48 hours of culture, which peaked in a decrease to 28.3% of the baseline value (medium control) after 72 hours. Much less pronounced changes in TER (decrease to 71.1% of the medium control) were observed when a supernatant from a T_H2 clone containing 1702 pg/mL IL-4 was added (Fig 1). A second experiment was performed with supernatants from T-cell clones from another allergic donor. A strong decrease in TER to 59.6% of the baseline value (medium control) after 72 hours was noted after addition of the supernatant from a T_H1 clone containing 3028 pg/mL IFN- γ but not after adding a supernatant of T_H2 clones containing 1088 pg/mL IL-4 (100.76% of the baseline, data not shown).

IFN- γ decreases TER—no effect of IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-8, IL-12, TNF- α , and histamine

We next determined which mediator of the T_H1 response provokes the reduction in TER of 16HBE14o– epithelial cells. The effects of the 5 different T_H1 -derived cytokines IFN- γ , IL-2, IL-3, IL-12, and TNF- α on TER were investigated. Cell monolayers were exposed to

different concentrations of these cytokines (20 pg/mL, 100 pg/mL, 1 ng/mL, 5 ng/mL, and 50 ng/mL) for 72 hours, and TER was measured in 24-hour intervals. IFN- γ (1 ng/mL, 5 ng/mL, and 50 ng/mL) induced a dose-dependent decrease in TER after 48 hours and in particular after 72 hours. To verify this observation, we repeated this experiment 2 times in quadruplicate wells and once in duplicate wells with 120 hours of IFN- γ exposure. Results from 2 representative experiments are shown (Fig 2, A and B).

In total, 4 independently performed experiments revealed that the presence of 1 ng/mL IFN- γ decreased TER to a mean of $70.77\% \pm 7.73\%$ of the values of the respective medium control cultures after 72 hours. Addition of 5 ng/mL IFN- γ to 16HBE14o– cells decreased TER values to a mean of $36.85\% \pm 6.70\%$ of the respective baseline values (medium control) and treatment with 50 ng/mL IFN- γ led to a decrease in TER to a mean of $20.81\% \pm 6.57\%$ of the medium control after 72 hours. IFN- γ exposure over a period of 120 hours showed that 50 ng/mL IFN- γ decreased TER to 1.64% of the value of the medium control, which corresponds to a complete breakdown of barrier function (Fig 2, B).

Statistical analysis was done for an experiment with quadruplicate wells, with the relative difference to the initial value as the dependent variable. Performing the repeated-measures ANOVA, a significant result for dose ($P < .0001$) and time ($P < .0001$) is yielded. Furthermore, the interaction between time and dose shows statistically significant influence ($P < .0001$). This can be interpreted that for small doses, there is hardly a change over time, whereas for high doses, the values decrease with increasing time. Statistical analysis of the experiment with IFN- γ treatment for 120 hours confirmed the results of the first experiments (significant effects for dose, $P = .0004$; time, $P < .0001$; interaction dose * time $P < .0001$). Note

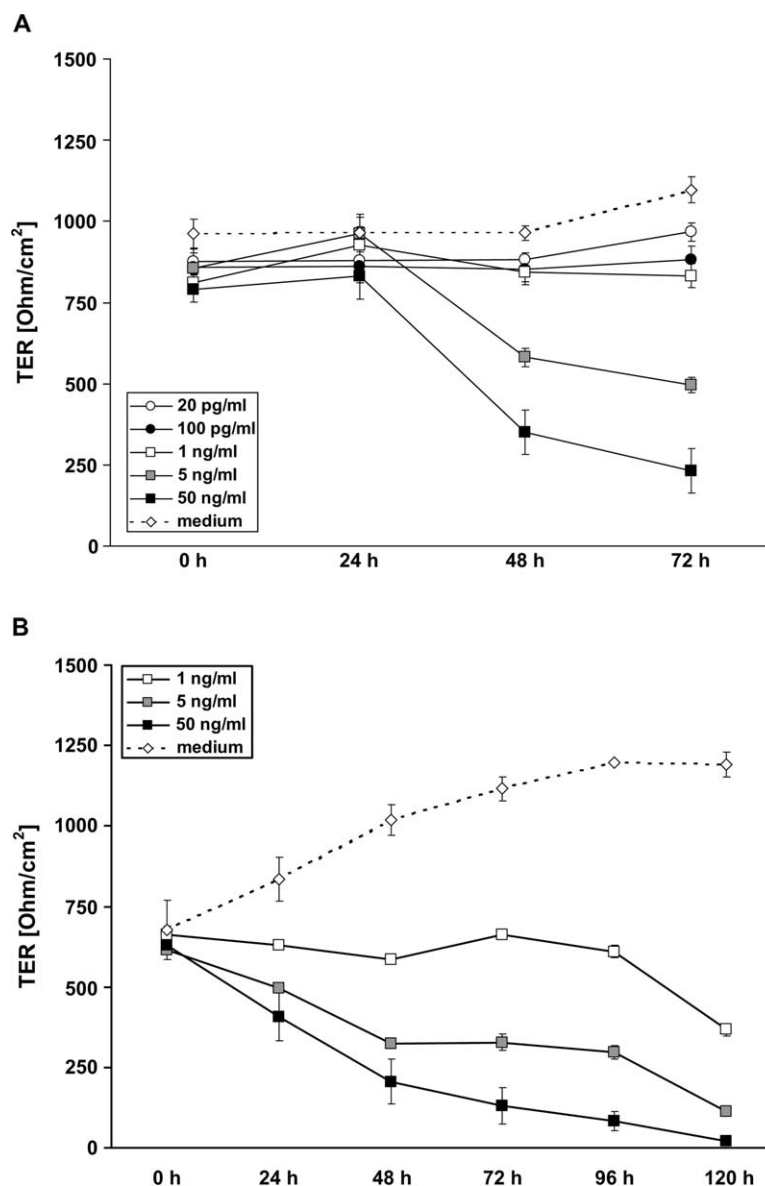


FIG 2. IFN- γ induces a specific dose- and time-dependent decrease of TER. Increasing doses of IFN- γ (*insert*) were added to monolayers of respiratory epithelial cells. The change of TER (γ -axis) is shown for different times (x -axis) for 2 independent experiments with quadruplicate (**A**) and duplicate (**B**) determinations. **C**, Effects of anti-IFN- γ antibodies on IFN- γ -mediated decrease of TER. **D**, Demonstration of IFN- γ receptors by FACS (histogram) with an anti-IFN- γ receptor α chain antibody (white) versus isotype control (black).

that in the group treated with the highest dose, the values tend towards zero after 120 hours.

The effects of IFN- γ are specific because they can be inhibited with an anti-IFN- γ antibody (Fig 2, C) and are probably mediated through the IFN- γ receptor, which could be detected by means of FACS analysis on 16HBE14o- cells (Fig 2, D).

LDH levels were determined in supernatants from IFN- γ -treated cell layers and cell layers exposed to medium alone after 72 hours. These experiments revealed no relevant differences in LDH release between cells cultured with or without IFN- γ , as indicated by OD values,

which are proportional to LDH activity (medium control, 0.654 ± 0.011 ; 1 ng/mL IFN- γ , 0.690 ± 0.037 ; 5 ng/mL IFN- γ , 0.811 ± 0.042 ; 50 ng/mL IFN- γ , 0.825 ± 0.027 ; triton-mediated necrosis, >3.00).

The other T_H1-derived cytokines, IL-2, IL-3, IL-12, and TNF- α , did not induce relevant changes in TER values, even after 72 hours of exposure. Incubation of 16HBE14o- monolayers with increasing concentrations of the T_H2-derived cytokines IL-4 and IL-5 and the epithelial cell-derived cytokines IL-1 β and IL-8 for 72 hours did not yield any relevant alterations in TER (Fig 3).

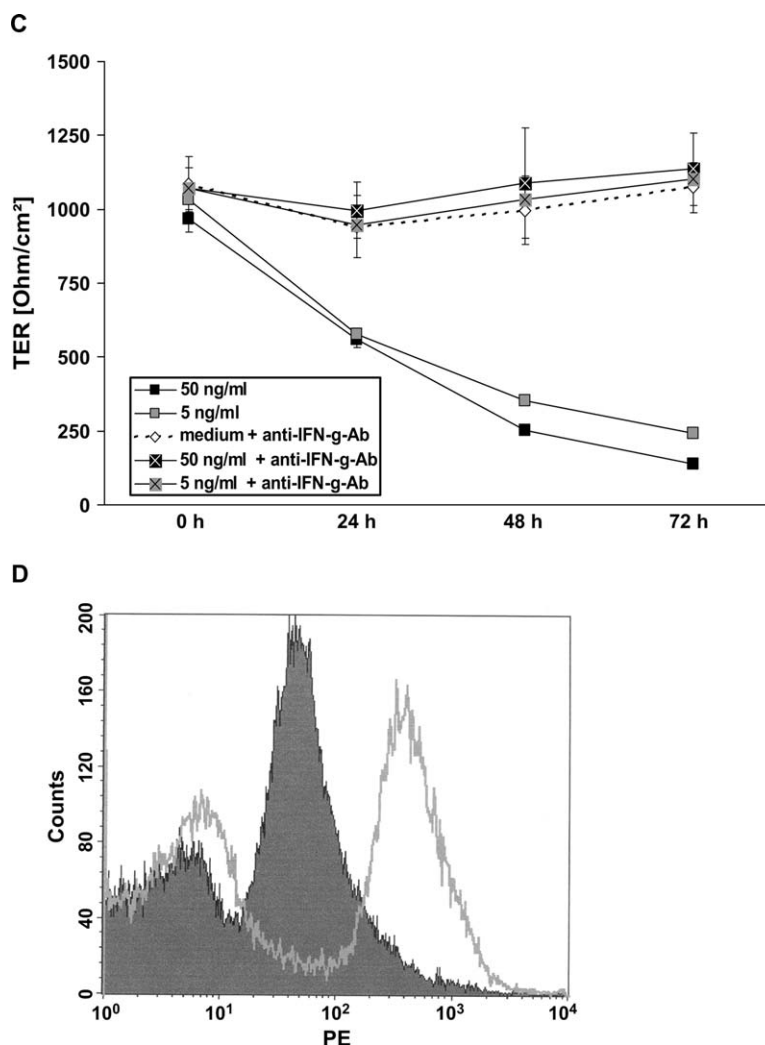


FIG 2. Continued.

The administration of different concentrations of the acute-phase mediator histamine (20 pg/mL, 100 pg/mL, 1 ng/mL, 5 ng/mL, and 50 ng/mL) did not induce any changes in TER values after 24, 48, and 72 hours compared with the baseline values (ie, medium control; Fig 3, B). Furthermore, supernatants from effector cells (activated human basophils, mast cells, and eosinophils) failed to induce decreases in TER, demonstrating that mediators of the immediate reaction other than histamine do not impair epithelial integrity (data not shown).

Decrease of TER caused by IFN- γ is associated with increased epithelial permeability for allergens

To determine whether the IFN- γ -mediated reduction of TER of respiratory epithelial cells is accompanied by enhanced transepithelial penetration of allergens, we administered iodine 125-labeled Bet v 1 to the apical side of epithelial monolayers. Alterations of allergen permeability were quantified by gamma counting (Fig 4).

The amounts of allergen passing through epithelial layers incubated with IL-4 or medium did not exceed 3.60% after 2 hours of exposure. An almost 3-fold amount of allergen (mean value, 8.68%) penetrated through epithelial cell layers treated with 50 ng/mL IFN- γ for 72 hours (Fig 4). This effect depended on the dose of IFN- γ (mean values: 1 ng/mL, 3.78%; 5 ng/mL, 6.93%; 50 ng/mL, 8.68%) and was caused by intercellular allergen transition because no radiolabeled allergens could be detected within 16HBE14o- cells (data not shown).

Increased subepithelial allergen concentrations cause augmented histamine release from sensitized basophils

Next we investigated whether increases of submucosal allergen concentrations caused by IFN- γ -mediated enhancement of transepithelial allergen passage have effects on immediate allergic inflammation. For this purpose, we

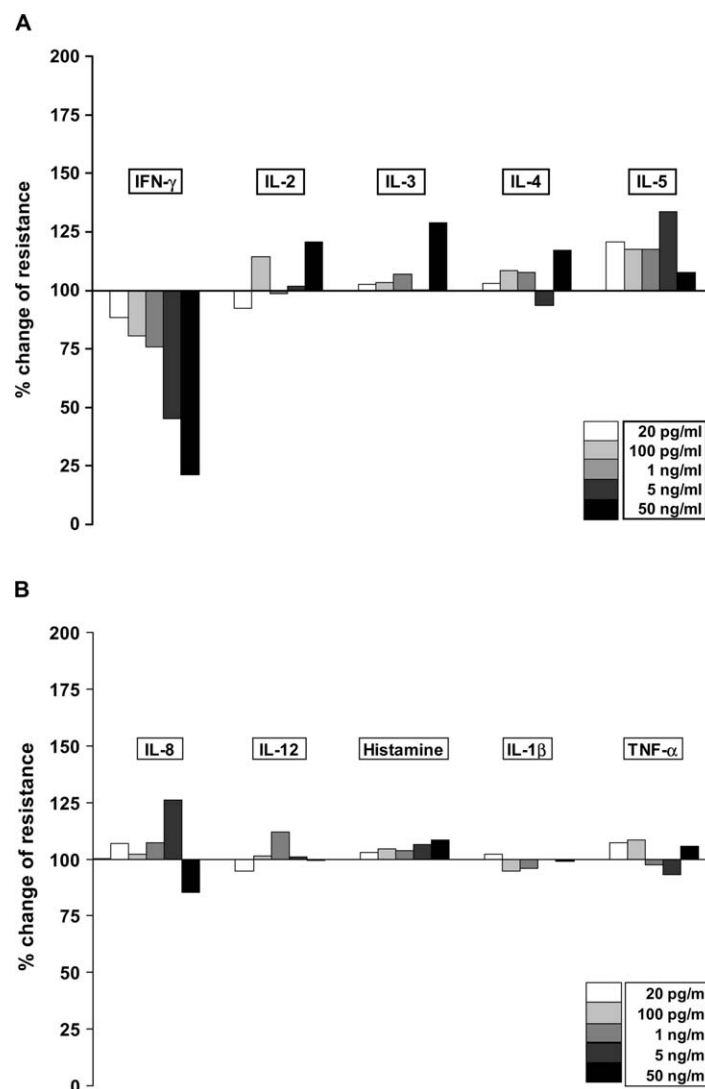


FIG 3. IFN- γ reduces TER of epithelial cells. Changes of TER after 72 hours of incubation with different mediators (**A**, IFN- γ , IL-2, IL-3, IL-4, and IL-5; **B**, IL-8, IL-12, histamine, IL-1 β , and TNF- α) are shown on the y-axis. Displayed values represent the percentage change in comparison with control wells containing only medium (100% baseline). The data for IFN- γ represent means of quadruplicate wells, and the data for the other mediators are means of duplicates.

took aliquots of culture medium from lower chambers of IFN- γ -treated monolayers that had been exposed to a constant amount (ie, 10 ng) of rBet v 1 that had been added to the upper chambers and aliquots from lower chambers of control cultures lacking either IFN- γ treatment or rBet v 1 exposure. The allergen-containing aliquots and aliquots from control cultures were then added to basophils from 2 different patients with birch pollen allergy with established IgE reactivity profiles. We found that the extent of histamine release increased strongly with increasing IFN- γ concentrations (Fig 5). When no IFN- γ was added to the cells, the maximal release of histamine was 5.46% of total histamine. The addition of 50 ng/mL IFN- γ yielded an average histamine release of 39.93%. Treatment with IFN- γ without addition of allergen did not induce relevant

histamine release (1.47%), demonstrating the allergen specificity of basophil activation (Fig 5). Statistical analysis revealed that there is a statistically significant difference between doses ($P < .0001$).

DISCUSSION

Considerable evidence has accumulated that not only T_H2 cells but also T_H1 cells and T_H1-derived cytokines play an important role in chronic manifestations of atopy.¹³⁻¹⁵ Experiments in murine models of allergy have demonstrated that transfer of allergen-specific T_H1 cells into mice with severe combined immunodeficiency syndrome caused airway inflammation,¹¹ and it has been

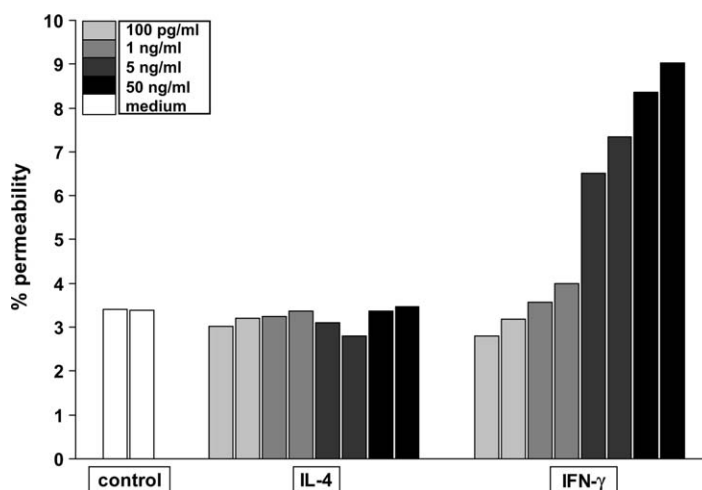


FIG 4. IFN- γ enhances allergen penetration across epithelial cell layers in a dose-dependent manner. 16HBE14o– monolayers were incubated with increasing concentrations (*insert*) of IFN- γ or IL-4 for 72 hours before iodine 125–labeled recombinant Bet v 1 was added to the upper chamber. The percentage of allergen that penetrated to the lower chamber within 2 hours is displayed on the y-axis. Bars indicate the results of duplicate wells.

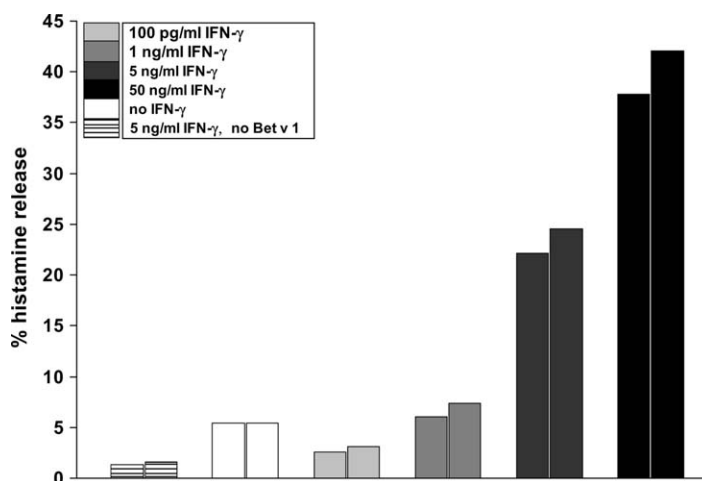


FIG 5. Increased transepithelial allergen penetration across IFN- γ -treated cell layers causes augmented histamine release from sensitized basophils. Constant amounts of rBet v 1 were added to the upper chambers of epithelial cell layers treated with different concentrations of IFN- γ (x-axis). Media obtained from the lower chambers 3 hours later were exposed to basophils. The percentage of histamine release is displayed on the y-axis. Control experiments (*white bar*, no addition of IFN- γ ; *hatched bar*, 5 ng/ml IFN- γ and no Bet v 1 added) are shown on the left side of the figure. Bars indicate the results obtained in duplicate wells with triplicate measurements.

suggested that T_H1-derived IFN- γ contributes to this process.^{10,26,27} Patients with atopic dermatitis contain allergen-specific T_H1 cells in their chronic skin lesions, which express IFN- γ .^{14,28} Similar observations have been made for chronic asthma, in which bronchoalveolar leukocytes have been shown to release increased levels of IFN- γ .^{10,26}

Our data reveal a novel mechanism by which IFN- γ produced during the chronic phase of allergic reactions aggravates acute allergic inflammation. We have identified IFN- γ as a factor that can reduce the barrier function

of respiratory epithelial cells and thus considerably enhances the penetration of allergens. This effect is dose dependent and occurs at concentrations that are secreted by allergen-specific T_H1 cells. Because we did not find increased levels of LDH in supernatants of IFN- γ -treated respiratory epithelial cells, toxic effects of IFN- γ leading to cell necrosis are unlikely. The mode of operation of IFN- γ was specific because it could be blocked with anti-IFN- γ antibodies. It might be mediated through the IFN- γ receptor either through the induction of apoptosis in respiratory cells, through an effect on the formation of

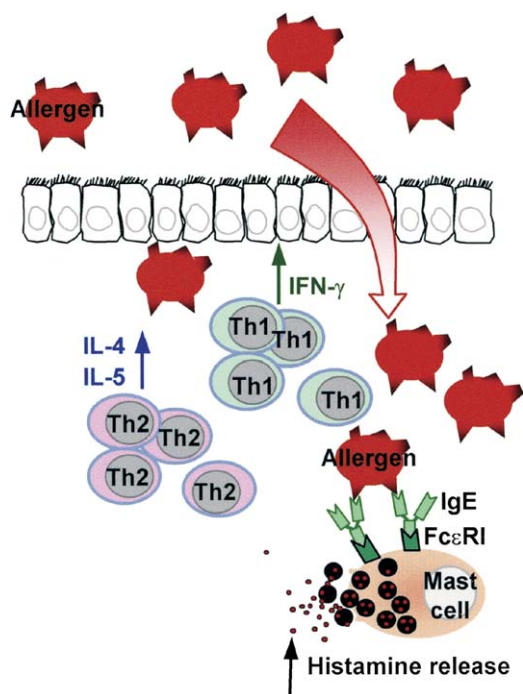


FIG 6. The T_H1 -derived cytokine IFN- γ can lead to decreased epithelial barrier function and increased allergen influx and might thus augment immediate allergic reactions. By contrast, the T_H2 cytokines IL-4 and IL-5 have no effect on epithelial integrity.

intercellular tight junctions similar to that reported for intestinal epithelial cells²⁹ and thyrocytes,³⁰ or both. The fact that no iodine 125-labeled allergens were detected within epithelial cells supports the assumption that allergen transition occurs through a paracellular mechanism and depends on the integrity of intercellular tight junctions. We found that intact epithelial cell layers allowed the penetration of less than 3.60% of allergens, whereas transition increased up to 3-fold after IFN- γ treatment. The biologic significance of increased trans-epithelial allergen migration was demonstrated by the fact that the increased allergen concentrations in the lower chambers of IFN- γ -treated epithelial cell layers caused a more than 7-fold enhanced degranulation of basophils from allergic donors. The latter finding indicates a hitherto unknown link between chronic and acute allergic reactions, which is illustrated in the schematic Fig 6: IFN- γ derived from allergen-specific T_H1 cells which occur at sites of chronic allergic inflammation can lead to increased allergen influx and thus augments immediate allergic reactions. In contrast, the T_H2 cytokines IL-4 and IL-5 and factors released from cells of immediate allergic reactions (supernatants of activated basophils, eosinophils, and mast cells) did not influence respiratory epithelial integrity and allergen penetration. Because IFN- γ has previously been shown to affect the integrity of intestinal epithelium,^{29,31,32} our observations of concomitant enhanced allergen transition and augmented submucosal histamine release might also be valid for target organs of atopy other than the respiratory tract.

In conclusion, we suggest a novel mechanism of how IFN- γ , a cytokine derived from allergen-specific T_H1 cells, can augment immediate allergic inflammation. Our data indicate an as yet unknown link between chronic and acute allergic reactions. These observations suggest that not only T_H2 but also T_H1 cells might be of importance for the maintenance and aggravation of allergic inflammation. Our finding that IFN- γ can augment allergic inflammation should be considered for the development of therapeutic antiallergic strategies that should aim to control both the acute and chronic facets of allergic inflammation.

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