

Leukocyte responses to experimental infection with human rhinovirus

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The respiratory tract infection (RTI) is one of the world's most common and expensive ailments. It has been increasingly recognized that viral infections can exacerbate asthmatic symptoms¹⁻⁵ and cause airway hypersensitivity that persists weeks after the virus itself has been eradicated.⁶ The major viruses responsible for RTI include rhinoviruses, parainfluenza viruses, and adenoviruses. In particular, rhinovirus infection accounts for approximately 50% of clinical colds in adults.⁷

Infection by rhinovirus is thought normally to be restricted to the upper part of the respiratory tract. This is supported by the virus' optimal replication temperature of 33° C, which could prevent deeper body penetration under normal conditions. Evidence for any lower respiratory tract infection is thus far inconclusive,^{8, 9} and there is no evidence for the virus spreading any farther than this. However, the infection can often compromise lower airway function⁸ and have systemic actions. Therefore it is likely that indirect methods such as leukocyte activation, mediator release, and neurogenic modulation contribute to the systemic effects of RTI.¹⁰

We are interested in the changes in leukocyte function under the influence of a rhinoviral infection and the possible role of mediators in orchestrating the indirect actions of the virus. Many aspects of virus-induced colds resemble events in inflammation; thus, cellular mechanisms are likely to be common to both. Studies of various leukocytes have revealed alterations in number, site of accumulation, and activation state after viral in-

Abbreviations used

F-met peptide:	Formyl methionyl-leucyl-phenylalanine
HRV-16:	Human rhinovirus-16
PAGCM:	Pipes with albumin, glucose, calcium, and magnesium
PIPES:	Piperazine- <i>N,N'</i> -bis(2-ethanesulfonic acid)
RTI:	Respiratory tract infection
TCID ₅₀ :	Tissue culture infective dose
TRIS:	Tris(hydroxymethyl)amino-methane

fection. There is a decrease in the total number of peripheral lymphocytes in rhinovirus infection¹¹ and in particular a drop in the ratio of CD4⁺ helper to CD8⁺ suppressor cells. This, together with increased levels of virus-specific IgE antibodies, has been correlated with severity of wheezing in patients infected with respiratory syncytial virus or parainfluenza virus.¹²

Studies have found elevated levels of neutrophils in both nasal biopsy specimens and lavages during RTI.^{13, 14} In conjunction with this, increased levels of the neutrophil activating peptide, interleukin-8, are expressed in the nasal epithelium in the presence of virus.¹⁵ Despite eosinophils' key role in allergic inflammation, the changes in these cells during a viral infection have not been extensively studied. However, there is some evidence for their activation because increased levels of eosinophilic cationic protein are found in the respiratory tracts of children during respiratory syncytial virus-related bronchiolitis.¹⁶

The relationship between viral infection and basophils has been investigated by a number of groups, largely with *in vitro* incubation of virus with cells. Many studies have shown that direct virus-cell interaction results in increased degranulation and histamine release.¹⁷⁻¹⁹ Early studies suggested that interferon may have a role in this enhancement. However, the increased release can occur in the absence of interferon,²⁰ and thus a more complex interplay of mediators is likely. In

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summary, it is possible that pathways common to allergic and virus-induced asthma combine to promote leukocyte accumulation, survival and activation, amplifying the inflammatory signal.

We have focused on the changes seen in circulating basophils at the height of an experimental rhinoviral (human rhinovirus-16 [HRV-16]) infection. We believe that there is no direct interaction between active virus and the peripheral blood basophils and that the systemic effects are brought about by the indirect actions of other cells and production of cytokines. Pertinent to this idea, a parallel study investigated the presence of inflammatory cells within bronchial biopsy specimens obtained from the same HRV-16-infected patients to characterize migration of the cells to the lung and nose.²¹ We are interested in pinpointing events common to inflammation and infection as a clue to the mechanisms of virally induced asthmatic symptoms.

METHODS

Materials

Calcium ionophore A23187, formyl-methionyl-leucyl-phenylalanine (F-Met peptide), ethylenediaminetetraacetic acid, and piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) were all obtained from Sigma Chemical Co. Ltd., Poole, U.K. Dextran T500 and a monoclonal antibody to eosinophilic cationic protein, EG2, were supplied by Pharmacia, Milton Keynes, U.K. Sheep anti-human IgE was obtained from Serotec, Oxford, U.K. The streptavidin-biotin complex detection system, and the murine monoclonal antibodies to mast cell tryptase (AA1), CD3 (UCHT1), CD8, and neutrophil elastase (NP57) were obtained from Dako, Hill Wycombe, U.K. Monoclonal antibody to CD4 was from Becton Dickinson, Cowley, U.K. Glycol methacrylate resin was supplied by Park Sciences Ltd., Northampton, U.K., and DPX resin by BDH, Poole, U.K. Other general laboratory reagents were purchased from Sigma Chemical Co. The HRV-16 inoculum was kindly donated by Drs. E. Dick and W. Busse, Madison, Wisc.

Experimental HRV-16 infection protocol

Informed volunteers were screened for existing antibodies to HRV-16. Those who had negative results were then assessed for atopic status. Two weeks before infection, patients underwent bronchoscopy, and nasal and mucosal biopsy specimens were taken. Subjects were admitted to the infectious diseases unit of Southampton General Hospital 24 hours before inoculation. Blood counts were carried out; nasal lavages and symptom scores also were taken and continued daily.²² On the day of the viral inoculation (designated day 1) the first blood sample was obtained immediately before the patients were nasally administered a total viral dose of 5000 to 10,000 median TCID₅₀. This was repeated

the next day to ensure maximal infection. Ninety-six hours later (i.e., day 5) blood samples, leukocyte counts, bronchoscopy, and the biopsies were repeated. Subjects were discharged on day 6, then returned 6 to 10 weeks later for the final, "convalescent" biopsies and for serologic tests. The presence of viral infection was subsequently confirmed in nasal lavage fluid by measuring serotype-specific neutralizing antibodies. Lavage fluid was also inoculated into HeLa cell cultures, and infection was diagnosed by means of the typical cytopathic effects seen under light microscopy. Further, symptom scores according to the method of Jackson et al.²³ were calculated.

Basophil histamine release experiments

Buffers. All buffers were prepared with a PIPES-based buffer containing 25 mmol/L PIPES, 110 mmol/L NaCl, and 5 mmol/L KCl, adjusted to pH 7.4. Cells were washed in PAG (PIPES-albumin-glucose), which contained 0.003% human serum albumin and 0.1% D-glucose. Histamine release experiments were performed in PAG, which was supplemented with 2 mmol/L CaCl₂ and 1 mmol/L MgCl₂ (PAGCM). All solutions were diluted from stock to appropriate concentrations in PAGCM and were prepared fresh daily.

Basophil isolation and challenge. A mixed leukocyte preparation (containing 1% to 2% basophils) was obtained by dextran sedimentation. Buffy-coat cells were harvested from the upper layer after 1 hour, washed twice in PAG, and resuspended in PAGCM for release experiments. The stimuli were added to the cell suspensions, and histamine release was allowed to proceed for 45 minutes at 37° C. The reaction was halted by centrifugation and the supernatants recovered. Finally, protein was precipitated with 25% trichloroacetic acid and the samples stored at -20° C until they were analyzed for histamine content.

Histamine assay. The histamine content of the samples was assayed with the automated fluorometric technique of Siraganian.²⁴ The supernatants from days 1 and 5 for each subject were analyzed consecutively to minimize variation. Histamine release was expressed as a percentage of total histamine. Values were corrected for spontaneous histamine release, measured in cells incubated with buffer alone. Results were subjected to Wilcoxon signed-rank tests, and a value of $p < 0.05$ was considered to be significant.

Biopsy cell enumeration

Tissue preparation. The biopsy samples were placed into cold acetone containing protease inhibitors and fixed overnight at -20° C. The tissue was then processed into glycol methacrylate resin and stored. Sections (2 µm wide) were cut, floated on 0.2% ammonia solution, and air-dried onto poly-L-lysine-coated slides.

Staining of sections. The techniques for cell staining have been described elsewhere.²⁵ Briefly, endogenous peroxidases were inhibited with 0.3% hydrogen peroxide in 1% sodium azide for 30 minutes, and a blocking

medium was applied. Sections were washed in 0.05 mol/L TRIS-buffered saline solution (pH 7.6). Murine monoclonal antibodies to CD3, CD4, CD8, neutrophil elastase, eosinophilic cationic protein, and mast cell tryptase were added in optimal concentrations and slides were incubated for up to 20 hours. A streptavidin-biotin complex system was employed, and the slides were counterstained with Mayer's hematoxylin. Sections were then mounted in DPX resin. Negative controls were run by omitting each primary antibody, whereas sections of nasal polyp and tonsil provided positive tissue controls.

Cell counting. Cell counts were performed for the epithelial and submucosal layers of each specimen under a light microscope with an eyepiece graticule. For each reading the mean of three sections was taken. Changes between baseline, cold, and convalescence numbers were compared with Wilcoxon ranked-pair analysis for each cell type. Differences between the control and asthmatic groups were analyzed with Mann-Whitney *U* tests. A value of $p \leq 0.05$ was taken as significant.

RESULTS

We investigated the effect of HRV-16 infection on various leukocyte responses. Of the 22 people inoculated with HRV-16, all were successfully infected, as diagnosed by the positive viral cultures or by significant rises in neutralizing antibody to HRV-16. Also, 17 of the patients exhibited a good clinical cold as defined by the symptom scores.

By taking blood samples immediately before the experimental viral inoculation (day 1) and again at the height of infection (day 5), one can compare the activation of basophils via their histamine release. Performing parallel experiments on a control group of asymptomatic donors eliminated any daily variations. Of the 12 donors who gave blood samples, two were found to release less than 5% of their total histamine to an optimum concentration of anti-IgE and therefore were excluded from further analysis. In addition, biopsy specimens were taken from 20 of the patients before the cold, during the cold, and in the convalescent period 6 to 10 weeks later. A total of 43 samples were suitable for immunohistochemical analysis. These biopsy specimens were stained and numbers of mast cells, neutrophils, T lymphocytes, and eosinophils within the tissue were counted.

Fig. 1 shows the effect of HRV-16 infection on basophil histamine release in comparison with the control group. A number of stimuli, both physiologic and nonphysiologic, were employed to challenge the cells. Unchallenged cells (incubated

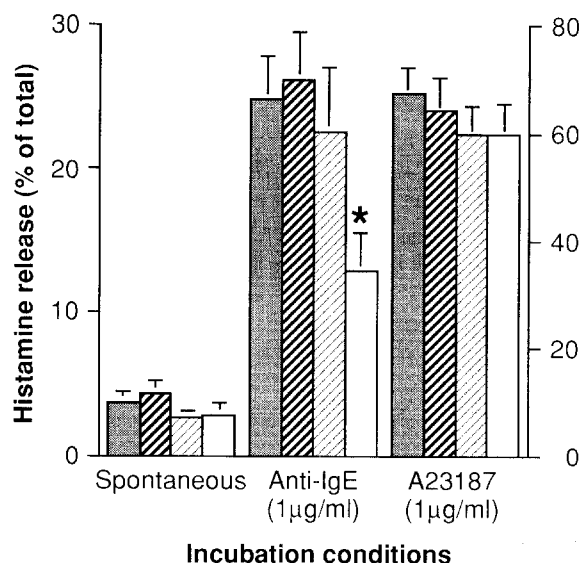


FIG. 1. Effect of HRV-16 on histamine release from human basophils. Basophils from patients infected with HRV-16 ($n = 10$) were challenged with range of different stimuli both before inoculation with virus (day 1, *lightly hatched bars*) and at height of symptoms (day 5, *open bars*). There was significant decrease in response to anti-IgE in patients infected with HRV-16. Control group of uninfected donors were processed in parallel, and there was no change in anti-IgE-induced response from day 1 (*shaded bars*) and day 5 (*heavily hatched bars*). Spontaneous histamine release was consistently less than 5% in both HRV-16 and control groups. Response to A23187 was not significantly altered in either group. Values are expressed as mean $\pm$ SEM. Asterisk indicates $p < 0.05$.

with buffer alone) typically gave histamine release values of less than 5% of total histamine. The spontaneous release by cells from the HRV-16 group was not significantly altered by infection, with percentage histamine release values of $3\% \pm 1\%$ on day 1 and $3\% \pm 1\%$ on day 5. Similarly, the unchallenged cells of the control group of donors showed no change in response, with values of $4\% \pm 1\%$ on day 1 and $4\% \pm 1\%$ on day 5.

The basophils were also stimulated with anti-IgE ($1 \mu\text{g/ml}$) to activate the IgE-dependent pathway of degranulation. Control donors showed some day-to-day variation in their responses to anti-IgE, although their average histamine release was virtually unaltered from $25\% \pm 3\%$ on day 1 to $26\% \pm 3\%$ on day 5. However, an unexpected decrease in IgE-induced histamine release was seen in the HRV-16 group at the height of infection. Histamine release was $23\% \pm 4\%$ on day 1, before infection, but dropped to $13\% \pm 3\%$ on day 5 when donors were symptomatic ($p < 0.05$).

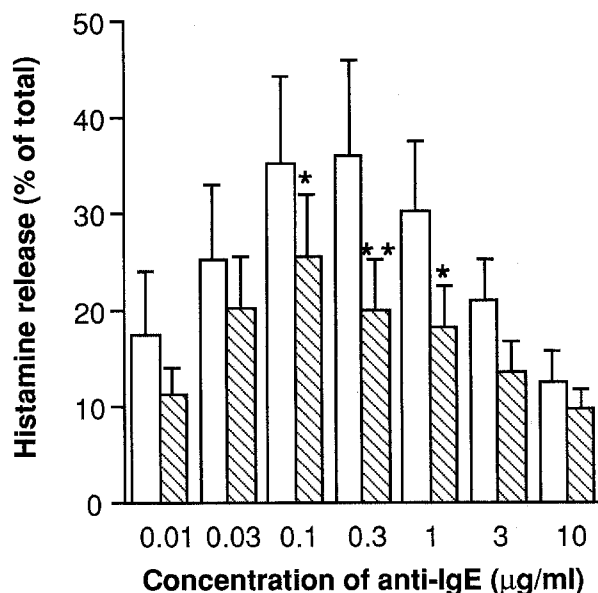


FIG. 2. Effect of HRV-16 infection on anti-IgE dose-response curve. Basophils from donors infected with HRV-16 were challenged with range of different concentrations of anti-IgE before inoculation with the virus (open bars) and 96 hours later, at height of symptoms (hatched bars). Values are expressed as mean $\pm$ SEM. Asterisk indicates $p < 0.05$.

Basophil counts showed no corresponding decrease in cell number. Furthermore, the total amount of histamine measured within the basophil samples was not significantly altered by HRV-16 colds (data not shown).

Calcium ionophore A23187 (1 μ g/ml) was used as a nonphysiologic stimulus to evaluate the basophils responsiveness. There was no significant change in the histamine release caused by A23187 in either the control group (68% $\pm$ 5% on day 1, 64% $\pm$ 7% on day 5) or the HRV-16 group (60% $\pm$ 5% on day 1, 60% $\pm$ 6% on day 5). Although not represented in Fig. 1, F-met peptide (1 μ mol/L) was also employed as an IgE-independent physiologic stimulus. Again, histamine release remained essentially constant in the HRV-16 donor group from day 1 (39% $\pm$ 4%) to day 5 (36% $\pm$ 3%). Likewise, there were no differences in histamine release between days 1 and 5 (45% $\pm$ 5%, 44% $\pm$ 7%) in the control group.

The anti-IgE dose-response curve is bell shaped in human basophils, and thus one possible explanation for the decrease histamine release is a shift in the optimum concentration of anti-IgE for release. Fig. 2 illustrates the effect of HRV-16 infection on histamine release from basophils

challenged with concentrations of anti-IgE ranging from 0.01 to 10 μ g/ml. At all concentrations of anti-IgE used there is a visible decrease in histamine release during the cold. The suppression is greatest for the doses at the peak of the curve. There was no such alteration in the dose-response curves of the control donors.

The cell population in the peripheral blood was characterized by differential cell counts before, during, and after infection. There was a consistent decrease in the total number of lymphocytes in the blood during the cold, dropping from 2.4 ± 0.7 (10^9 /L) to 2 ± 0.6 (10^9 /L) ($p < 0.05$). Examination of bronchial biopsy specimens revealed an increase in the number of eosinophils located in the epithelial layer during the cold ($p < 0.05$) compared with either the precold or the convalescent biopsy specimen (Fig. 3). There was considerable variation between donors; the asthmatic patients had higher levels of tissue eosinophils before the cold. Eosinophil levels in the epithelial layer returned to preinfection levels in nonasthmatic subjects during the convalescent period. However, in asthmatic patients eosinophil levels remained significantly elevated up to 6 weeks after the infection ($p < 0.05$). Examination of the submucosal layer failed to show any significant increase in the number of tissue eosinophils during infection with HRV-16.

The epithelial cell layer also showed increases in CD3⁺, CD4⁺, and CD8⁺ lymphocyte levels at the time of infection, although these were not statistically significant (Fig. 3, lower panel). However, the subsequent decrease from cold to convalescent levels was significant in both nonasthmatic and asthmatic patients. Again, no differences between control and asthmatic patients were obvious. The submucosal cell counts showed an increase in the number of lymphocytes during HRV-16 infection, particularly in the CD3⁺ subgroup of cells, which increased between baseline and cold and decreased again during convalescence ($p < 0.05$) (Fig. 3, lower panel). This pattern was mirrored by the CD8⁺ cells and to a lesser extent by the CD4⁺ group (data not shown). No changes in the ratios of these cells were detected, and no apparent differences existed between control and asthmatic groups.

There were no changes in the numbers of other cells at any time point for the epithelial layer. Intriguingly, the number of mast cells in the bronchial submucosa declined between the cold and convalescent periods, but there was no initial

increase caused by the cold so the significance of this is unclear. There were no detectable changes in other cell types in the mucosa.

DISCUSSION

We assessed the effect of HRV infection on some aspects of leukocyte function. The experimental infection program has a number of advantages over the study of wild-type infections. Patients were inoculated with a known serotype of rhinovirus, HRV-16, and the presence of infection could then be demonstrated, ruling out any "false" colds. The infection process proved to be very efficient, with all subjects having positive viral culture results and the majority having a clinical cold as defined by symptom scores. The specificity of the HRV serotype minimized variations in symptoms and time course of the cold such as would be found between different infective agents. Further, isolation of the patient ensured that contact with different viruses and other environmental influences was restricted. The study protocol also allowed biopsy specimens and blood samples to be collected at well-defined time points.

There has been increasing evidence linking respiratory viruses to the aggravation of asthmatic symptoms and enhancement of airway reactivity, but the precise mechanisms behind this are not known. Many features of a cold are similar to those of an allergic response, so it is possible that the two responsible pathways converge to produce an excessive response to the virus. We investigated the activity of the basophil during the infection, because this cell and the mediators it releases have been implicated in allergy and inflammation. This study has provided us with an excellent *in vivo* model of rhinoviral infection with which to work. There is some indirect evidence that rhinovirus infection penetrates further than the upper airways, but this is rather inconclusive⁸ and may occur only in unusual circumstances such as when the patient is immunocompromised or when the temperature of the airways is lowered.⁹ We believe that there is no direct contact between the virus and basophils within the circulation. This promotes the idea of transmission of the virus' effects via mediators such as cytokines.

A large proportion of the work in this field to date has looked at *in vitro* models whereby leukocyte preparations are directly incubated with live virus. Ida et al.¹⁷ first demonstrated that

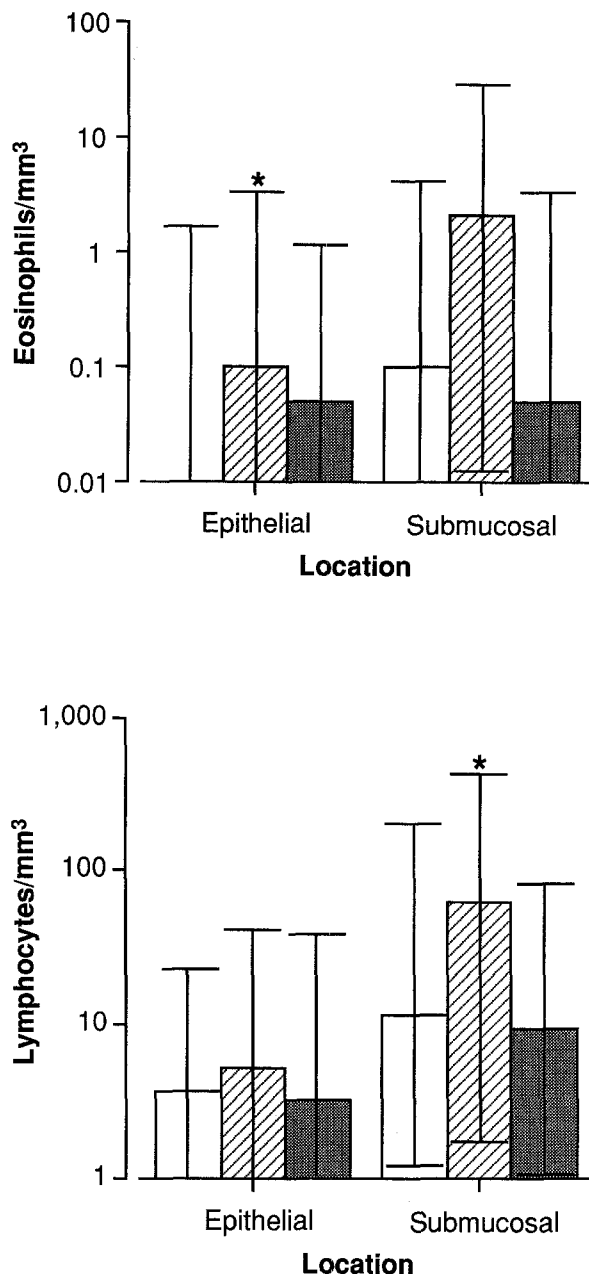


FIG. 3. Effect of HRV-16 infection on leukocyte populations in bronchial biopsy specimens. Biopsy specimens were obtained from patients at baseline (open bars), at height of symptoms (hatched bars) and during convalescent period (shaded bars). Specimens were fixed with acetone at -20°C and processed into glycol methacrylate resin and stored. Sections $2\text{ }\mu\text{m}$ thick were cut and stained with murine monoclonal antibodies to CD3 and eosinophilic cationic protein. Cell counts were performed for epithelial and submucosal layers of each specimen, and changes between baseline, cold, and convalescence numbers were compared with Wilcoxon ranked-pair analysis for each cell type. Value of $p \leq 0.05$ was taken as significant. Values are medians, with range of responses shown by lines extending from bars.

viruses were capable of inducing histamine release from basophils and suggested that interferon was responsible. Paramyxoviruses incubated with leukocytes were shown to increase degranulation of basophils in an IgE-dependent manner, suggested to be via direct glycoprotein interactions on the cell surface.¹⁸ However, Busse et al.¹⁹ reported increases in both IgE-dependent and IgE-independent histamine release from *in vitro* viral cultures and have implicated a leukocyte-derived mediator in the process. In contrast, our study demonstrated that the amount of histamine released actually decreased during the HRV-16 infection in response to anti-IgE. Cell counts demonstrated that the decrease was not due to any fall in basophil number. Further, the total histamine content of the cells was not altered. Spontaneous histamine release was not affected, indicating no overall change in responsiveness. Also, degranulation evoked by F-met peptide, a receptor-mediated stimulus, and by the calcium ionophore A23187, remained constant. Together, these data indicate that this decrease occurs by an IgE-specific mechanism and may be due to changes in an early part of the signal transduction in basophils.

A decrease in the IgE-induced histamine release of peripheral basophils appears to be inconsistent with earlier studies and with the notion of a heightening of inflammatory mechanisms during infection. However, a number of possibilities can explain this observation in the context of an *in vivo* model. *In vitro* models in which virus is incubated directly with basophils may not reflect the systemic effects of a cold.

Basophils accumulate in the airways during an asthma attack,²⁶ and there is some evidence that respiratory viruses increase basophil migration into the lungs. This is supported by reports that interferon acts as a basophilic chemotactic factor.²⁷ Because many respiratory viruses, including rhinoviruses, are potent inducers of interferon, this could result in activated basophils moving from the blood to the tissue. This migratory response, similar to the events in the development of a late-phase response, is more likely to occur in the presence of a RTI.²⁸ Thus the most activated basophils could be those residing in the lungs. These tissue basophils may be releasing greater amounts of inflammatory mediators locally and hence contribute to bronchial reactivity.

The IgE dose-response curve in human basophils is bell shaped with a marked decrease in histamine release at superoptimal concentrations.

Therefore we also considered the possibility that the presence of a viral infection increased the sensitivity of the basophils to IgE crosslinking and that the alteration of an optimal response to a superoptimal response could explain our apparent decrease. There is some precedent for such a shift in the dose-response curve, although most studies have focused on an increase in histamine release. Mast cells isolated from an area of active inflammation are more sensitive to IgE crosslinking than are their counterparts from uninvolved tissue.²⁹ Similarly, BAL mast cells obtained from patients with asthma during a late-phase response are more responsive to IgE crosslinking than are mast cells from patients without asthma.³⁰ However, the evidence for a shift in the IgE dose-response curve in basophils from patients with a viral infection is not conclusive and suggests a general decrease in responsiveness across the entire dose-response curve.

We also investigated the changes in cell populations in bronchial tissue in response to the HRV-16 infection. Changes in the numbers and ratios of cells can significantly alter the balance of cytokines and can have profound effects on the surrounding tissue. There is a significant fall in a T-cell subset during rhinoviral infection, altering the ratio of CD4⁺ to CD8⁺ in favor of the suppressor T cells.¹¹ Our present study has confirmed the decrease in peripheral lymphocytes and has shown an accumulation of both submucosal and epithelial lymphocytes at the height of HRV-16 infection, supporting the idea of a concerted movement of the T cells from the bloodstream into the tissue to cause a local rather than a systemic reaction. Huftel et al.³¹ demonstrated that T-cell depletion attenuates the enhanced histamine release caused by *in vitro* incubation with influenza A. This fits with the idea of distinct responses between the basophils in the lung and in the blood, because of their respective environments. Basophils may be primed in the airways where T cells are accumulating, but not in the circulation, because T-cell number is depleted. Production of interferon- γ by lymphocytes during *in vitro* RV-16 infection has been detected and may contribute to local basophil accumulation and histamine release, further implicating interferon in the pathogenesis of virally induced asthma.³²

The importance of neutrophils in RTI is becoming an area of interest; these cells have been found in increased numbers in HRV-infected nasal biopsy specimens.¹³ Nasal lavages also show elevated

numbers of neutrophils in clinical colds.¹⁴ Further, expression of interleukin-8 (neutrophil-activating peptide) is increased in nasal epithelial cells when stimulated with respiratory syncytial virus.¹⁵ Because interleukin-8 is a potent chemotactic agent for neutrophils,³³ it may well be acting as a signal for the accumulation of these inflammatory cells. However, we found no significant changes in the number of neutrophils measured in the biopsy specimens. This may be due to the very rapid elimination of airway material. Thus the cells are moved too quickly through the mucosa and expelled. The contribution of the neutrophil to virally provoked inflammation is not yet known. Increases in neutrophil superoxide production³⁴ and thromboxane B₂ release³⁵ are found after incubation with influenza and a respiratory syncytial virus antibody complex, respectively. If a similar activation is seen in the airways of rhinovirus-infected persons, release of toxic products and inflammatory mediators could lead to further bronchial tissue damage in patients with asthma.

The number of eosinophils in the bronchial epithelium increased in the group with HRV-16 infection, and the levels remained elevated in the asthmatic group whereas they declined to baseline levels in the normal group. This may be an important difference because the patients with asthma tend to have increased numbers of eosinophils because of their asthma. Although there has been little information on the role of eosinophils in viral infections, this cell is a key player in inflammatory events in the lung, where they accumulate and release cytokines, cytotoxic granule contents, oxygen metabolites, and lipid mediators. Incubation of leukocytes with either influenza or rhinovirus in vitro has led to an increase in the percentage of low-density eosinophils.³⁶ These hypodense cells appear to be more metabolically active and have increased releasability in comparison with eosinophils of normal density. Further, children with bronchiolitis related to respiratory syncytial virus have increased airway eosinophil degranulation caused by the virus, as measured by levels of nasopharyngeal eosinophilic cationic protein.¹⁶ This suggests that eosinophilic products may be released into the respiratory tract and could contribute significantly to airway disease.

In summary, we characterized bronchial cell infiltration in HRV-16 infection and observed the effect of the virus on systemic basophils. The results support the hypothesis that viral infection promotes indirect actions on the circulation to

produce accumulation and activation of inflammatory cells in the lung, resulting in local airway effects. This suggests that mechanisms common to inflammation and viral infection combine and amplify signals, giving rise to virus-induced asthmatic symptoms in susceptible persons.

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Cytokine control of eosinophils in pulmonary diseases

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Pulmonary eosinophilia is found in asthma, allergic bronchopulmonary aspergillosis (ABPA) and some granulomatous vasculitides (including Churg-Strauss syndrome) as a response to certain drugs (e.g., nitrofurantoin) and parasites (*Ascaris* and *Wuchereria*), and as part of systemic eosinophilic syndromes.¹⁻⁵ Cytokines are important in eosinophil generation, localization, and function.