

Rhinovirus-induced PBMC responses and outcome of experimental infection in allergic subjects

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Background: The immune response to rhinovirus (RV) infections is considered to contribute to upper respiratory symptoms and may also be an important contributor to lower airway dysfunction in patients with asthma.

Objective: This study was conducted to determine the relationship of RV-specific responses in PBMCs to the outcome of experimentally induced infection with RV16.

Methods: Twenty-two subjects with either allergic rhinitis or asthma were inoculated with RV16: virus-induced proliferation and cytokine production were determined on PBMCs obtained before and then again 7 and 28 days after inoculation.

Results: Several subjects had proliferative responses to RV16 before inoculation, and precold RV-specific proliferative responses were inversely correlated ($r_s = -0.62$, $P < .005$) with RV shedding after inoculation. In addition, there was a negative correlation ($r_s = -0.58$, $P = 0.01$) between precold RV-induced IFN- γ secretion ex vivo and peak RV shedding during the cold.

Conclusions: Certain RV-specific lymphocyte responses before the cold (vigorous proliferation or IFN- γ secretion) were associated with reduced viral shedding after inoculation. These findings suggest that variations in mononuclear cell responses to RV could contribute to the individual variability in viral shedding during experimentally induced, and perhaps naturally acquired, RV infections in subjects with respiratory allergy or asthma. (*J Allergy Clin Immunol* 2000;105:692-8.)

Key words: Rhinovirus, mononuclear cells, proliferation, common cold, blood, allergy, asthma

Viral respiratory infections are important causes of wheezing for many patients with asthma. For children less than 2 years old, respiratory syncytial virus is the primary cause of wheezing. Rhinovirus (RV) infection is

Abbreviations used

PFU:	Plaque-forming unit
r_s :	Spearman's correlation coefficient
RV:	Rhinovirus
SI:	Stimulation index
TCID ₅₀ :	Tissue culture infectious dose ₅₀

most commonly associated with acute wheezing exacerbations in older children and adults.¹⁻³ To establish the mechanisms by which respiratory viruses cause asthma exacerbations, experimental inoculation with RV has been used to investigate mechanisms by which viral infections induce immune responses that are likely to contribute to airway dysfunction and respiratory symptoms.^{4,5}

Interestingly, despite the use of standardized lots of viral inoculum, inoculation techniques, and the enrollment of subjects, there has been great individual variability in the severity of respiratory symptoms after inoculation with a single lot of RV⁶⁻⁸ and also in the amount of virus shed after experimental inoculation.⁹⁻¹¹ Although the level of neutralizing antibody is one host factor that can modulate the severity of respiratory symptoms and viral shedding after experimental inoculation,¹² there are indications that other influential host factors have yet to be identified. Evidence for additional modifying factors includes that fact that viral inoculation produces variable results even when neutralizing antibody cannot be detected⁹⁻¹¹ and also that the quantity of neutralizing antibody does not seem to account for all of the variability in viral shedding after experimental inoculation with RV39.¹³

These observations generated from studies of experimental RV infections suggest that there are host factors other than the quantity of virus-specific antibody that contribute to variability in the outcome of viral respiratory infections. It seems likely that variability in T-cell responses could influence the outcome of viral respiratory infections because previous studies have demonstrated the antiviral and proinflammatory effects of T cells in vitro and in animal models.^{14,15} In addition, there are also data to indicate that the magnitude of changes in T-cell recruitment from the blood to the airway and priming of the function of circulating lymphocytes correlates

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Supported by National Institutes of Health grant No. R29 AI40685.

Received for publication Mar 18, 1999; revised Nov 23, 1999; accepted for publication Nov 23, 1999.

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0091-6749/2000 \$12.00 + 0 1/1/104785

doi: 10.1067/mai.2000.104785

with the severity of cold symptoms or changes in airway responsiveness.^{5,16-18} To test the hypothesis that the quantity or quality of virus-specific T-cell responses can influence the outcome of RV infections in vivo, we conducted a prospective study to analyze virus-induced proliferative and cytokine (IFN- γ , IL-5) responses in PBMCs of allergic and asthmatic subjects before and then again after inoculation with RV16 and examined the relationships among these activities, clinical variables, and viral shedding during the cold.

METHODS

Subjects

Twenty-two consecutive subjects (12 female, 10 male) with mild allergic asthma (n = 17) or allergic rhinitis (n = 5) were enrolled for study (Table I). All subjects were otherwise healthy and had no serum neutralizing antibody specific for RV16 at the time of screening, although low levels of neutralizing antibody were detected in the serum of 7 subjects on the day of inoculation. Asthma was defined by clinical history: (1) physician diagnosis, (2) prescription for asthma medication, (3) history of cough, wheeze, or shortness of breath and relief with appropriate asthma medication, or (4) evidence of a 12% or more improvement in FEV₁ after inhaled β -adrenergic agonist. At the screening visit, all subjects underwent a physical examination, prick skin tests to a range of common aeroallergens (including ragweed, cat, and house dust mite), and spirometry. Use of topical nasal corticosteroids, if any, was suspended for 3 days before inoculation with virus. The experimental protocol was approved by the University of Wisconsin Hospital and Clinics Human Subjects Committee.

Study design

Whole blood was obtained from subjects before RV inoculation and again 7 and 28 days after inoculation. At each of these 3 visits PBMCs were obtained and tested for virus-induced proliferative responses and cytokine secretion (see below). White blood cell differential counts on peripheral blood were done at baseline, during virus inoculation, and 7 days after the cold. Cold symptom scores and recovery of RV16 from nasal secretions were measured around the time of viral inoculation.

RV16 preparation

Human RV16 was kindly provided by Wai-Ming Lee (University of Wisconsin-Madison) and was prepared as previously described.¹⁹ The preparation had a high viral titer (10^{10} plaque-forming units [PFU]/mL) and was stored at -70°C until used.

Virus inoculation

During the "cold phase" of the study subjects were given an experimental RV16 infection. Before each inoculation the subjects underwent nasal lavage, and the fluid was cultured for RV16. The same lot of RV16 was used to inoculate each subject on 2 consecutive days, as previously described.¹¹ Briefly, after an aliquot of RV16 suspension ($\sim 10^3$ tissue culture infectious dose₅₀ [TCID₅₀]/mL) was quick thawed from stocks frozen in liquid nitrogen, 0.25 mL of viral suspension was atomized into each nostril, and an additional 0.25 mL of viral suspension was instilled into each nostril by pipette. Quantitative viral culture of the residual suspension in the atomizer showed 2.9 ± 0.5 log TCID₅₀/mL (geometric mean $\pm$ SD). Cold symptoms were evaluated for the next 7 days with a subject-completed questionnaire. Eight signs and symptoms that included cough, nasal discharge, sneezing, nasal congestion, headache, malaise, chills, and fever at any time during

TABLE I. Study subjects

Subject	Sex	Age (y)	Disease	Fev ₁ *	Antibody titer†
1	M	42	AR	86	2.8
2	M	40	AA	84	1.4
3	M	24	AA	106	0
4	F	25	AA	112	0
5	M	23	AR	111	0
6	M	23	AA	93	0
7	M	21	AA	88	0
8	F	32	AR	130	1.4
9	M	25	AR	110	0
10	M	20	AA	83	0
11	M	18	AA	104	0
12	F	20	AA	78	0
13	F	18	AA	80	0
14	F	21	AA	92	1.0
15	F	21	AA	94	32
16	F	25	AA	107	0
17	F	28	AA	120	0
18	F	21	AR	103	0
19	F	25	AA	99	1.4
20	M	20	AA	92	1.0
21	F	23	AA	104	0
22	F	22	AA	97	0

AA, Allergic asthma; AR, allergic rhinitis.

*Percent predicted.

†None of the subjects had detectable neutralizing antibody to RV16 at the time of the initial screening visit. Serum was also obtained on the day of inoculation, and the titers in this table reflect these values.

each 24-hour time period were evaluated on a 4-point scale (0-3). Severity of colds was defined by the total of the numerical symptom scores (<7 mild, 7-11 moderate, or >11 severe).

Detection of RV16 in nasal lavage fluid

Nasal lavage was performed on each subject both before RV16 inoculation and 1, 2, 3, and 7 days afterward. Five milliliters of prewarmed HBSS with 0.5% gelatin was instilled into each nostril. The lavage fluid was then expelled from each nostril consecutively by the subject blowing gently from one nostril while the other nostril was compressed closed. The lavages were inoculated into human lung fibroblast (HEL) cultures and incubated (33°C , 14 days). If the culture showed characteristic RV-induced cytopathic effects, the RV16 nasal titer was determined by neutralization assay with RV16-specific rabbit antiserum with use of the Karber method.²⁰ In addition to HEL cultures, preinfection lavage samples were also inoculated into rhesus monkey kidney cells and Hep-2 cell cultures (33°C) to exclude the possibility of concomitant respiratory virus infection.

Lymphocyte proliferation assay

Whole blood samples were obtained at a precold visit and 7 and 28 days after RV nasal inoculation. PBMCs were isolated from heparinized whole blood by gradient centrifugation with Ficoll, washed twice with PBS, and resuspended in RPMI 1640 medium (Hyclone, Logan, Utah) supplemented with L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 $\mu\text{g}/\text{mL}$). Concentrations of RV16, tetanus toxoid, and PHA that produced optimal PBMC proliferation were established in preliminary experiments. At each of the 3 visits proliferation assays were set up in quadruplicate in 96-well microtiter plates, with each well containing 10^5 PBMCs in RPMI with 25% autologous serum and either RV16 (10^7 PFU/mL), tetanus toxoid (20

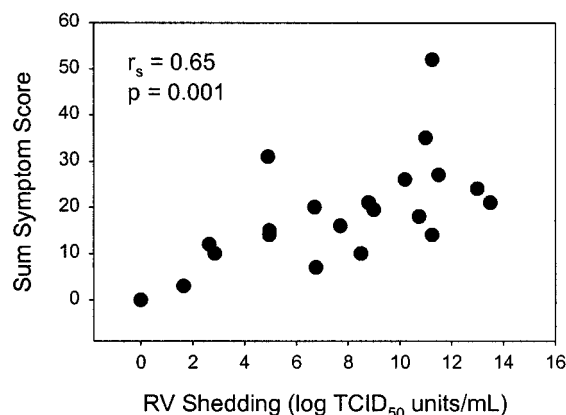


FIG 1. Association between RV shedding in nasal secretions and symptom scores. Cumulative daily quantitative viral titers (log TCID₅₀ units/mL) of nasal secretions from first 3 days after inoculation with RV16 were compared with sum of daily symptom scores over same time period (n = 22).

μg/mL), PHA (1 μg/mL), or medium alone. After 48 hours (PHA) or 6 days (RV and tetanus toxoid), tritiated thymidine (1 μCi/well) was added. Six to 18 hours later, cells were harvested and thymidine uptake was quantitated with use of a scintillation counter. Results were expressed as a stimulation index (SI) where SI = (Stimulus counts per minute)/(Control counts per minute).

RV-stimulated cytokine production

Concurrently with proliferation assays, PBMC cultures were incubated with RV16 to stimulate cytokine production. PBMCs (10⁶ cells/1 mL) in RPMI with 25% autologous plasma were incubated in 5-mL culture tubes with or without RV (10⁷ PFU/mL). After incubation (37°C, 5% carbon dioxide) for 6 days, the cells were pelleted, and supernatants were frozen (−70°C) pending analysis of IL-5 and IFN-γ by ELISA, as previously described.²¹ The sensitivity of the cytokine ELISAs was 3 pg/mL.

Statistical analysis

Values for cell proliferation and cytokine secretion were log transformed before analysis to approximate a normal distribution. Spearman's test (r_s) was used to evaluate correlations between cytokine secretion, cell proliferation, symptom scores, and viral shedding. Changes in cell proliferation over time were analyzed with 1-way repeated ANOVA. Cold-related changes in ex vivo IFN-γ secretion were evaluated with the Wilcoxon signed rank test. All analyses were performed with the aid of computer software (SigmaStat 2.0, Jandel Scientific, San Rafael, Calif).

RESULTS

Clinical effects of RV16 inoculation

Of the 22 subjects who had no detectable antibody at time of screening, 7 had neutralizing antibody (median titer 1.4, Table I) detected immediately before RV experimental infection. Of the 22 people inoculated with RV16, 21 had symptoms of upper respiratory infection and had infection verified by viral culture. The remaining subject did not have cold symptoms or shed virus after inoculation but had an increase in the titer of neutralizing antibody. There were no significant group-related differences in symptom scores, viral shedding, or cytokine responses, so data from subjects with allergic rhinitis and

allergic asthma were pooled for subsequent analyses. Cumulative symptom scores during the acute cold (1-3 days after inoculation) correlated with the amount of virus shed in nasal washes ($r_s = 0.65$, $P = .001$, Fig 1). In addition, peak symptom scores correlated with the maximal amount of virus cultured from nasal secretions ($r_s = 0.43$, $P = .05$, data not shown).

RV effects on PBMC proliferative responses

Proliferative responses of PBMC to RV16, tetanus, and PHA were determined from blood samples obtained just before inoculation and 7 and 28 days after virus inoculation. For the group as a whole, viral infection did not cause significant changes in proliferation to the RV16, tetanus, or PHA. To determine whether high degrees of viral replication promoted more vigorous virus-specific T-cell responses, the peak virus titer obtained from nasal washings was compared with changes in virus-specific PBMC proliferation. There was a significant positive relationship between peak RV shedding in nasal secretions and changes in RV-specific proliferation at 7 and 28 days after viral inoculation (Fig 2, A and B). Interestingly, there was also a significant positive correlation between peak viral shedding and tetanus-specific proliferation 7 days after inoculation, and a similar trend was noted 28 days after inoculation (Fig 2, C and D). There were no significant correlations between peak viral shedding and changes in PHA-induced proliferative responses. These findings suggested that increases in either RV or tetanus-specific proliferation were more likely to increase in subjects with high levels of viral replication after experimental inoculation.

Relationship of PBMC responses before RV inoculation to subsequent viral shedding

RV16-specific proliferation (SI >2) was detected in 5 subjects before they were inoculated with this virus. Furthermore, the level of RV16-specific cell proliferation detected during the precold period was inversely related to the peak amount of RV that was subsequently detected in nasal secretions (Fig 3). To determine whether this inverse relationship was dependent on the level of neutralizing antibody present at the time of inoculation, the data from subjects with undetectable antibody (Fig 3, open circles) were analyzed separately, with similar results ($r_s = -0.715$, $P < 0.01$, n = 14). Peak viral shedding also tended to be lower in subjects with brisk proliferative responses to tetanus and PHA, although these trends were not statistically significant ($r_s = -0.41$ and -0.45 , respectively, $P > .05$ [data not shown]).

Comparison between RV-specific PBMC proliferation and clinical variables

To determine whether RV-specific T-cell proliferation correlated with changes in other clinical variables, we compared antigen-specific PBMC proliferation with clinical symptoms. No statistically significant correlations were found between either RV-, PHA-, or tetanus-specific proliferation and cold symptom scores.

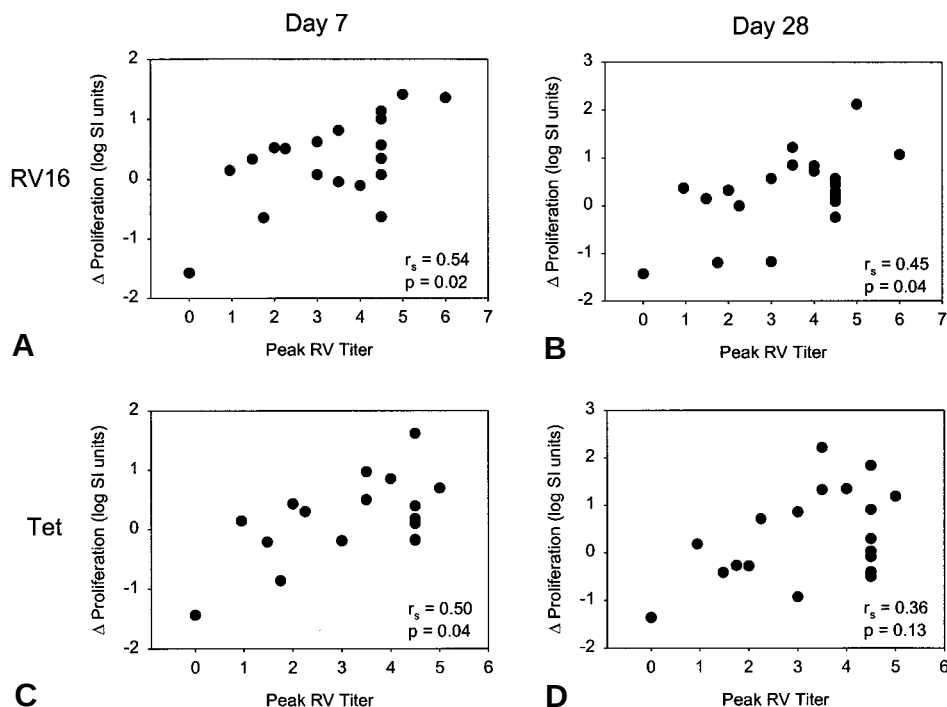


FIG 2. Relationship between peak RV shedding and changes in PBMC proliferative responses. Proliferation of PBMCs to tetanus or RV16 was determined just before and 7 and 28 days after inoculation with RV16. Cold-related changes in proliferative responses to RV16 (**A, B**) and tetanus (**C, D**) were calculated (Δ log [SI units]) for each time period and compared with peak viral shedding for each subject.

RV16-induced cytokine production in PBMCs

To determine the pattern of cytokines secreted by peripheral blood cells in response to RV, PBMCs obtained from subjects before inoculation with RV and again 28 days after inoculation were incubated with RV16 or medium alone, and supernatant fluids were analyzed for IFN- γ and IL-5. RV16 induced large amounts of IFN- γ from cells obtained either before the cold (medians 674 pg/mL vs 95 pg/mL for control cells incubated with medium, Fig 4, A) or 28 days after inoculation (median 495 pg/mL vs 50 pg/mL control cells, Fig 4, A). In contrast, RV did not cause IL-5 secretion from PBMCs (Fig 4, B).

Precold and postcold data for cytokine secretion were then compared to determine whether RV infection enhanced virus-induced cytokine secretion from PBMCs. Although there was no difference in the quantity of cytokine secretion (IFN- γ , Fig 4, A; IL-5, Fig 4, B) in cells obtained before vs after the cold, there was a weak but statistically significant positive correlation between cold-related changes in RV-specific proliferation and RV-induced secretion of IFN- γ ($r_s = 0.56$, $P < .05$, data not shown). Thus subjects who had greater changes in RV-specific proliferative responses also tended to have greater changes in RV-induced IFN- γ secretion.

Association of RV-induced cytokine secretion with outcome of RV infection

The quantity of IFN- γ secreted from RV-stimulated

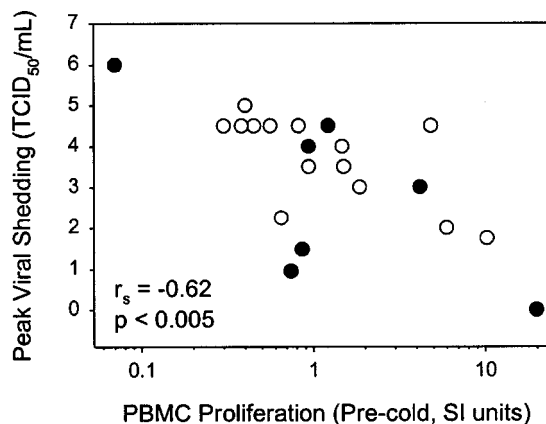


FIG 3. Effect of precold PBMC proliferation on peak viral shedding after inoculation with RV16. Proliferative responses to RV16 were determined in PBMCs isolated from peripheral blood just before inoculation, and these responses were compared with the peak viral shedding measured in nasal lavage fluid after inoculation with RV16. Filled circles, Subjects who had no serologic evidence of prior RV16 infection at screening but had detectable neutralizing activity at inoculation ($n = 7$); open circles, subjects with no detectable neutralizing antibody to RV16 at either point in time ($n = 14$).

PBMCs obtained before the cold was inversely related to the peak virus shedding after inoculation ($r_s = -0.58$, $P = .01$, Fig 5). There was, however, no correlation between RV-induced IFN- γ secretion ex vivo and either cold-related symptom scores or changes in FEV₁.

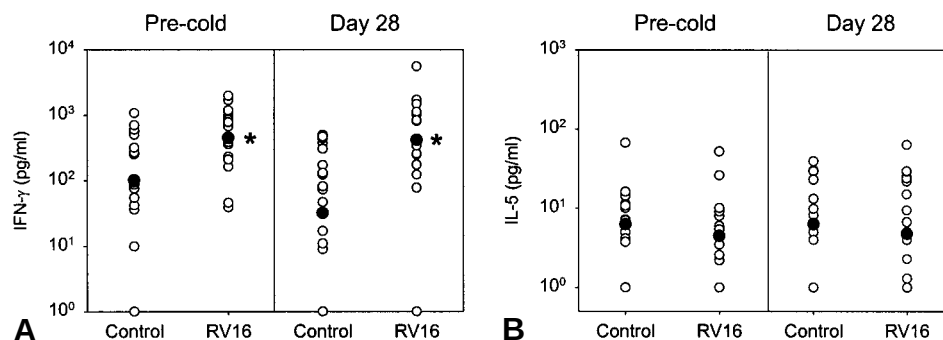


FIG 4. Effect of RV16 on PBMC secretion of IFN- γ and IL-5. PBMCs were obtained from study subjects just before (precold, $n = 17$) and 28 days after (day 28, $n = 22$) inoculation with RV16. PBMCs were incubated ex vivo with RV16 or medium alone for 6 days, and culture supernates were analyzed for IFN- γ (A) and IL-5 (B). Filled circles, Geometric mean values. Asterisk, $P < .001$ versus control samples.

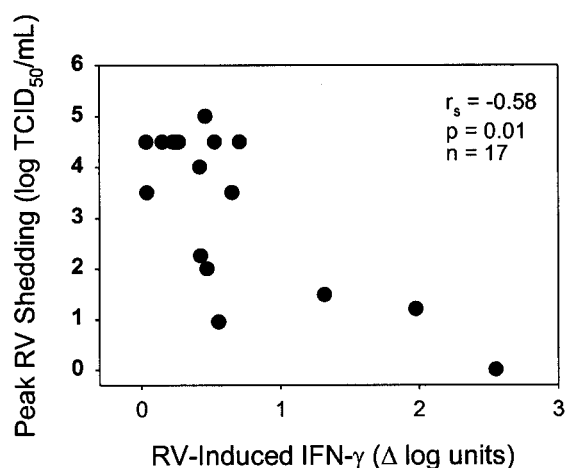


FIG 5. Relationship of precold RV-induced IFN- γ to peak viral shedding after inoculation. PBMCs were obtained before inoculation with RV16, and samples were incubated for 6 days with either RV16 (10^7 PFU/mL) or medium alone as a control. Increases in RV-induced IFN- γ (RV-stimulated samples: control samples, Δ log units) were significantly associated with lower peak viral shedding after inoculation with RV16.

DISCUSSION

This group of experiments provides support for the concept that both host and viral factors affect the course of experimentally induced infections with RV in people with allergies or asthma. For example, the association between the amount of viral shedding in the nasal secretions and cumulative symptom scores over the first 3 days of infection provides evidence that higher levels of RV replication cause more severe colds, and this relationship seems intuitive. However, in this study, as in others, the amount of viral shedding varied considerably among subjects. The 5-log range of peak viral shedding is especially notable because it occurred after inoculation with the same viral strain, infective dose, and technique of inoculation and in subjects who had little or no neutralizing antibody. Because variables related to the virus were carefully controlled, the heterogeneity in viral shed-

ding was likely the result of differences in the host immune response. Our findings suggest that individual variations in the quality of the T-cell response (RV-specific PBMC proliferation and IFN- γ secretion) could be a major determinant of the degree of viral replication.

The observation that the strength of the pre-existing RV16-specific PBMC cell proliferative response was inversely correlated with viral shedding after inoculation raises the question of why, in this study and in others,^{22,23} some subjects have RV-specific responses in PBMC before infection despite the absence of neutralizing antibody. One likely explanation stems from the fact that, although the surface antigens of RV are serotype specific, there is extensive homology (up to 60% to 80%) of protein sequences among different RV serotypes.^{24,25} Thus previous infections with other rhinovirus serotypes could stimulate T cells that recognize epitopes that are shared among serotypes, and in fact cross-reactive T-cell responses have been demonstrated with use of human T-cell clones²⁶ and inferred by analysis of patterns of proliferative responses to different RV serotypes in cells isolated from adenoidal tissue.²⁷ In addition, the inverse relationship between RV16-induced PBMC proliferative responses before the cold and peak viral shedding after inoculation suggests that cross-reactive T cells could help to limit viral replication. Moreover, the concept that T cells can moderate the effects of a picornavirus infection in the absence of antibody is supported by studies in animals: passive transfer of either virus-specific CD4⁺ or CD8⁺ T cells in the absence of neutralizing antibodies were found to protect mice from lethal meningoencephalomyelitis virus infections.^{28,29} Possible mechanisms for this effect in humans or animals remain speculative but may involve earlier and more vigorous secretion of antiviral cytokines (eg, IFN- α) or enhancement of virus-specific antibody production or cytotoxic responses.

Our studies indicate that RV primarily induces high levels of IFN- γ and little or no IL-5 from PBMCs, even from donors with atopic disease. The IFN- γ secreted was in part the result of a virus-specific immune response, as indicated by the weak but significant correlation between cold-related changes in RV-induced proliferation and

IFN- γ secretion. However, PBMCs obtained before the cold or 28 days after inoculation secreted similar amounts of IFN- γ , suggesting that most of the IFN- γ was secreted through activation of innate, and not antigen-specific, immune response pathways and effectors.

Our finding that greater precold induction of IFN- γ was associated with reduced viral shedding raises the possibility that IFN- γ either participates in or is a marker of effective antiviral immune response to RV. Whether the enhanced IFN- γ secretion was a specific response to viral infection or was indicative of a generalized enhancement of IFN- γ secretion was not determined by this study. Although these observations were derived from cell culture *ex vivo* and may not reflect cytokine secretion patterns *in vivo*, these findings suggest that regulated induction of IFN- γ in regional lymph nodes or spleen cells during RV infection could help to mobilize cell-mediated antiviral responses, and this has been demonstrated in animal models of viral infection.^{30,31} In contrast to the protective effects of IFN- γ on cell-mediated immune responses to viral infection, one clinical trial found that administration of topical IFN- γ to the nasal mucosa before inoculation with RV led to enhanced clinical symptoms and a trend toward greater viral shedding in nasal secretions.³² These findings are not necessarily incompatible because the effects of IFN- γ during RV infections could well depend on where it is secreted and when it is secreted relative to host-pathogen interactions (ie, viral binding vs lysis of infected cells).

In contrast to the associations with viral shedding, we found no evidence that either cytokine secretion or proliferation of PBMCs *ex vivo* correlated with severity of respiratory symptoms during the cold. One possible explanation for the lack of correlation is that symptom scores, because of their subjective nature, are an imprecise measure of cold severity. In addition, there may be factors other than the degree of viral replication that contribute to the severity of cold symptoms, and these factors may not be affected by mononuclear cell responses to RV.

An important limitation of this study is in the use of experimental RV inoculation, which only rarely causes significant reductions in pulmonary function and exacerbations of asthma.³³ To determine the relationship between pre-existing cellular responses to virus and exacerbation of asthma will require a study of naturally acquired colds in subjects with asthma. In addition, this study included only atopic individuals. Because there are some allergy-related differences in the cellular immune response to RV infection,²³ it will be important to determine in future studies whether allergic status also affects the relationship between RV-induced cellular responses and reduced viral shedding.

In summary, this study provides evidence that there are factors other than the level of neutralizing antibody that may contribute to the outcome of experimental infection with RV16 in subjects with allergic rhinitis or asthma. In particular, we found that vigorous precold PBMC responses, as marked by RV-induced proliferation and secretion of IFN- γ , were associated with reduced

viral shedding after inoculation. Further investigations into the mechanisms for these effects may provide the basis for new strategies to moderate viral replication during naturally acquired RV infections, a goal that could be of particular importance for patients with asthma.

We thank Dr E. A. Becky Kelly (University of Wisconsin-Madison) for performing the ELISA analyses for IFN γ and IL-5 and Dawn Galagan, RN, for technical assistance and data processing.

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