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*J Immunol* 1998; 160:1279-1284; ;
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Eosinophils Bind Rhinovirus and Activate Virus-Specific T Cells

Zeev T. Handzel,* William W. Busse,† Julie B. Sedgwick,* Rose Vrtis,* Wai Ming Lee,‡ E. A. B. Kelly,* and James E. Gern†‡

Episodes of virus-induced exacerbations of asthma are accompanied by increased eosinophils (EOS) in respiratory secretions and evidence of EOS degranulation. Although rhinoviruses (RV) are the viruses most often implicated in exacerbations of asthma in both children and adults, little is known about the immune response to this group of viruses and, in particular, EOS-RV interactions. To define such interactions, we incubated human rhinovirus type 16 (RV16), a serotype using ICAM-1 as a receptor, with EOS purified from PBMC, and measured EOS-RV binding, EOS-mediated Ag presentation and T cell activation, and EOS cell surface marker expression and superoxide production. Significant RV16 binding occurred to EOS that were pretreated with granulocyte-macrophage CSF, and this binding was inhibited by anti-ICAM-1 mAb. EOS also presented viral Ags to RV16-specific T cells, causing T cell proliferation and secretion of IFN-γ. RV16 induced a significant shift from CD18dim to CD18bright, but did not affect EOS expression of CD54, CD69, or HLA-DR. Finally, RV16 did not induce superoxide production from peripheral blood EOS. These findings suggest that RV16 also binds to airway EOS, which resemble granulocyte-macrophage CSF-treated blood EOS in terms of high expression of ICAM-1. Furthermore, our findings suggest that EOS could participate in RV-induced immune responses through Ag presentation and T cell activation. By activating RV-specific T cells, EOS may play an important role in the initiation of antiviral T cell responses, and these effects could also contribute to enhanced airway inflammation and increased asthma symptoms in susceptible individuals. The Journal of Immunology, 1998, 160: 1279–1284.

Infections with respiratory viruses, and RV in particular, are potent triggers of acute exacerbations of asthma (1–5). Epidemiologic studies utilizing sensitive PCR detection methods demonstrate that respiratory viruses are present in the nasal secretions of 80% of children (5) and 44% of adults (1) with acute asthma exacerbations, and RV accounts for about two-thirds of the viruses detected. Although the precise mechanisms by which viral infections promote asthma attacks are not known, there is experimental evidence that viral infections augment allergic inflammation by increasing EOS recruitment and activation. For example, Calhoun and colleagues experimentally infected adults with allergic rhinitis with RV type 16 (RV16), and compared lower airway responses to allergen before and during viral infection (6). EOS recruitment to the lower airway after segmental allergen challenge was enhanced significantly by RV infection. In addition, Fraenkel and colleagues found that experimental RV infection increased EOS in bronchial epithelium, and that the epithelial eosinophilia persisted for 6 to 10 wk in asthmatic, but not normal, subjects (7). Furthermore, Trigg et al. reported increased numbers of EOS in the bronchial epithelium of both normal and atopic individuals with symptoms of an acute upper respiratory infection, although viruses were only detected in 8 of the 20 subjects in this study (8). Finally, EOS granular proteins are elevated in nasal secretions of children with RV or respiratory syncytial virus infections, providing evidence that EOS are activated during viral infection (9–11). Taken together, these studies suggest that RV-mediated augmentation of eosinophilic inflammation could be an important factor in the pathogenesis of increased asthma symptoms during RV infection.

To define interactions between RV and EOS, we performed the following experiments to identify binding of RV16 to EOS, and to measure the effects of RV16 on expression of cell surface activation markers and superoxide production. In addition, we have explored the possibility that EOS could also play an indirect role in RV-induced inflammation by presenting viral Ags to and thus activating RV-specific T cells.

Materials and Methods

Human subjects

EOS were isolated from peripheral blood of subjects ages 22 to 54 yr with either allergic rhinitis (n = 13) or atopgia (n = 8), including four subjects with both disorders. All were skin-prick positive to at least one environmental allergen, most commonly house-dust mite, ragweed pollen, or cat dander. Inhaled β2-agonists or corticosteroids were taken as needed, but none of the subjects was taking systemic medications. The study protocol was approved by University of Wisconsin Human Subjects Committee (Madison, WI), and informed consent was obtained from all subjects before entry into the study.

mAb and reagents

Mouse anti-human CD54, CD69, HLA-DR, and mouse IgG1 labeled with either phycocerythrin or fluorescein were obtained from Becton Dickinson (San Jose, CA). Anti-CD16-coated magnetic microbeads and steel-meshed columns, type c, were purchased from Miltenyi-Biotech (Auburn, CA). A blocking anti-ICAM-1 mAb (C78.4A) was kindly provided by Dr. J. Greve (Miles Pharmaceutical Division, West Haven, CT). A pair of anti-IFN-γ

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0022-1767/98/$02.00
mAb used for ELISA was purchased from Endogen (Cambridge, MA). PMA, FMLP, superoxide dismutase, and horse heart ferricytochrome c (type VI) were obtained from Sigma Chemical Co. (St. Louis, MO).

**Rhinovirus suspensions**

RV16 was kindly supplied by Dr. Elliot Dick (Department of Preventive Medicine, University of Wisconsin). For experiments in which the effects of RV16 on EOS activation were measured, the virus was purified by centrifugation through a sucrose density gradient, as previously described (12), to remove soluble factors of HeLa cell origin. Radiolabeled (13)RV16 was prepared as previously described (12), and was used to measure binding of RV16 to EOS. Viral infectivity was expressed in tissue culture infective units (TCID50), and all virus stocks were stored at −70°C until needed.

**Cell separation**

EOS were purified from peripheral blood using the method developed by Hansel (13), with modifications. Briefly, heparinized whole blood was centrifuged (700 × g, 20 min) over a Percoll gradient (density 1.090 g/ml; Pharmacia Biotech, Piscataway, NJ) to separate mononuclear cells from granulocytes. After removal of the mononuclear cell band, RBC were lysed by twice incubating (30 s) with sterile deionized water. The remaining white blood cells were incubated with anti-CD16-coated microbeads (100 μl/2 × 10⁶ cells) for 40 min and were then passed through steel mesh columns that had been previously washed with 2% newborn calf serum. The cells in the eluent were stained (Diff Quik; Baxter, Miami, FL), and 400 cells were examined microscopically; greater than 99% of the cells were EOS, and the few remaining cells were either neutrophils or mononuclear cells.

**Binding of 35S-RV16 to EOS**

EOS (3–4 × 10⁶) suspended in 100 μl PBS (pH 7.4), with 0.1% BSA, 0.01% CaCl₂, and 0.01% MgCl₂, were incubated (room temperature, 60 min) in 5-ml polypropylene tubes on a rocking platform with 35S-RV16 (4000 viral particles/cell). Some EOS samples were preincubated (overnight, 37°C) with GM-CSF (100 pM) to increase expression of ICAM-1, the cellular receptor for major group RV such as RV16. To block RV16/EOS binding, additional samples were incubated with either mAb specific for ICAM-1 (200 μg/ml), or an equivalent amount of an isotype control IgG1. After the initial incubation, the cells were washed twice in PBS and the supernatants were discarded. Cell pellets were then suspended in 4 ml of scintillation fluid, and cell-associated radioactivity (cpm) was measured in a standard scintillation counter. All samples were run in duplicate.

**Stimulation of RV-specific T cell clones by EOS**

RV16-specific CD4⁺ T cell clones were prepared from the peripheral blood of a healthy RV16-seropositive donor using Ag stimulation and limiting dilution (14). T cell clones (1 × 10⁵/well) were incubated (48 h, 37°C, 5% CO₂) in round-bottom 96-well plates with either RV16 (10 TCID₅₀ APC) or control fluid (CF, medium from uninfected HeLa cells) in the presence of either 1 × 10⁵ autologous irradiated (5000 rad) PBMC, 1 × 10⁵ autologous EOS, 1 × 10⁵ allogeneic EOS, or no APC. The cells were then incubated for an additional 18 h with trinitiated thymidine (1 μCi/well), and thymidine uptake was quantitated in a scintillation counter. Results are expressed as stimulation indexes (SI), calculated by dividing cpm of stimulated samples by cpm of cells incubated with medium alone.

**IFN-γ ELISA**

Cell-free supernatants from cultured EOS were stored at −80°C for determination of IFN-γ levels by a two-step sandwich ELISA assay, as previously described (15). The sensitivity of the assay was 12 pg/ml, and coefficient of variation <10%.

**Flow cytometry**

EOS (2 × 10⁶/ml) were cultured overnight in RPMI with 10% FCS, glutamine, and penicillin/streptomycin, in the presence or absence of purified RV16 (10 TCID₅₀/cell). Cells incubated with GM-CSF (100 pM) and TNF-α (100 ng/ml) were used as positive controls for the induction of cell surface markers. For some experiments, EOS were incubated with GM-CSF (100 pM), TNF-α (100 ng/ml), or both of these cytokines to increase ICAM-1 expression; the cells were then washed, and incubated (37°C, 5% CO₂) for an additional 24 h with RV16 (10 TCID₅₀/cell). The following morning, aliquots of EOS (1–2 × 10⁵ cells/100 μl sample) were washed in PBS and incubated (30 min on ice) with 5 μl of phycoerythrin-conjugated mAbs specific for CD54, HLA-DR, CD69, or phycoerythrin-conjugated mouse IgG2a (isotype control), and expression of these surface proteins was analyzed using flow cytometry, as previously described (15). Measurements of CD54, HLA-DR, and CD69 expression were expressed as median fluorescent units. CD18 expression, which had a bimodal distribution, was expressed in terms of percentage of CD18bright or percentage of CD18dim cells.

**Superoxide anion (O₂⁻) generation**

Assessment of O₂⁻ generation was performed by superoxide dismutase-inhibitable cytochrome c reduction as previously described (16). EOS were either freshly isolated, or for some experiments, incubated overnight with GM-CSF (100 pM) ± TNF-α (100 ng/ml) to increase the expression of ICAM-1 and enhance binding of RV16. The EOS (1 × 10⁵ cells/well) were then suspended in HBSS/0.1% gelatin and added to 96-well plates along with 100 nM cytochrome c. Controls included cells activated with PMA (1 ng/ml), a strong inducer of superoxide (O₂⁻) production, and cells incubated in buffer alone to measure spontaneous release. Duplicate samples of EOS were incubated (37°C) with either purified RV16 (10 TCID₅₀/cell), an equivalent amount of CF, or medium alone, and absorbance (550 nM) was measured for 2 h. O₂⁻ generation was calculated using an extinction coefficient of 21.1 × 10⁻⁵ M⁻¹ cm⁻¹, and results were calculated as nanomols of reduced cytochrome c/5 × 10⁵ cells, minus spontaneous O₂⁻ generation.

**Statistical analysis**

Data were analyzed using SYSTAT software (version 5; Systat, Evanston, IL). Analysis of variance was performed after normalization of the data on O₂⁻ generation and flow cytometry. Paired data were evaluated with Fisher’s least significant test and the paired Student’s t test, and a p value of ≤0.05 was considered significant.

**Results**

**EOS bind RV16 via ICAM-1**

To determine whether RV binds to EOS, 35S-RV16 was incubated with EOS, and cell-associated radioactivity was measured. After a 60-min incubation with radiolabeled RV16, there was little radioactivity associated with unstimulated EOS, and this binding was not sensitive to anti-ICAM-1 mAb (Fig. 1A). In contrast, EOS pretreated with GM-CSF, which induces ICAM-1 expression on EOS (17), bound more 35S-RV16, and RV binding was inhibited by anti-ICAM-1 (Fig. 1B). These findings demonstrate that RV16, a major group RV, binds to EOS, and that GM-CSF increases this binding, probably by increasing expression of ICAM-1.
EOS present viral Ags to RV16-specific T cell clones

To determine whether EOS can participate in RV-induced immune responses by serving as APC, we measured virus-induced proliferation of RV16-specific T cell clones in the presence of either autologous iPBMC, autologous EOS, allogeneic EOS, or no APC. Of the nine RV16-specific T cell clones tested, eight proliferated vigorously (SI = 15) in the presence of RV16 and autologous EOS (Fig. 2A), or autologous iPBMC (SI = 12, Fig. 2B), but not with allogeneic EOS (SI = 1.5), or in the absence of APC (SI = 0.8). Filled symbols represent median cpm for each condition, and the numeric values represent the median SI for each condition.

**Figure 2.** EOS Ag presentation to RV16-specific T cell clones. RV-specific T cell clones (10⁴/well) were incubated for 3 days with either medium alone or RV16 (10 TCID₅₀/cell), along with either: A, iPBMC (10⁵/well); B, autologous EOS (10⁵/well); C, allogeneic EOS (10⁵/well); or D, no APC. Vigorous Ag/T cell proliferation ([³H]Tdr incorporation, cpm) occurred in the presence of autologous EOS (A, SI = 15), or autologous iPBMC (B, SI = 12), but not with allogeneic EOS (C, SI = 1.5), or in the absence of APC (D, SI = 0.8). Filled symbols represent median cpm for each condition, and the numeric values represent the median SI for each condition.

**Table I.** Comparison of Ag-induced IFN-γ secretion by RV-specific T cell clones using EOS vs iPBMC as APC

<table>
<thead>
<tr>
<th>T Cell Clones</th>
<th>IFN-γ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No APC</td>
</tr>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>16.8</td>
<td>35</td>
</tr>
<tr>
<td>16.19</td>
<td>44</td>
</tr>
<tr>
<td>16.26</td>
<td>33</td>
</tr>
<tr>
<td>No clone</td>
<td>67</td>
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</table>

**Figure 3.** Effect of iPBMC concentration on proliferation of RV16-specific T cells. RV16-specific T cells were incubated for 3 days with either medium alone or RV16 in the presence of 0, 10⁴, 3 × 10⁴, or 10⁵ iPBMC, and T cell proliferation was determined by measuring incorporation of tritiated thymidine. The results of triplicate samples are expressed as mean cpm ± SEM.

**Effect of RV16 on the expression of surface molecules by EOS**

EOS that are functionally up-regulated express increased amounts of several cell surface markers, including ICAM-1 (CD54), CD69,
HLA-DR, and CD18 (18). We incubated blood EOS with RV16 (10 TCID<sub>50</sub>/cell), and then measured effects on these surface proteins using flow cytometry. RV16 did not affect the surface expression of ICAM-1, CD69, or HLA-DR (Fig. 4). Furthermore, although preincubation with GM-CSF (100 pM), TNF-α (100 ng/ml), or the combination of these cytokines induced increased expression of these cell surface markers, sequential incubation (24 h, 37°C) with these cytokines followed by RV16 did not cause additional increases (Table II). In fact, RV16 reduced ICAM-1 detection by about 50% on cells preincubated with cytokine(s), probably by competing for the same binding site as the detection Ab.

RV16 did cause, however, a small but statistically significant shift in CD18 expression. At baseline, CD18 expression on peripheral blood EOS was bimodal (Fig. 5), and 17% of the cells were CD18<sup>dim</sup> (mean, n = 3). Incubation with RV16 shifted the distribution of CD18 so that only 5% of cells were CD18<sup>dim</sup> (mean, n = 3, p < 0.01), and cytokine (TNF-α and GM-CSF) incubation had similar effects.

**Effect of RV16 on O<sub>2</sub>− generation by EOS**

To determine whether RV binding to EOS caused cell activation, peripheral blood EOS were incubated overnight with GM-CSF to increase ICAM-1 expression and enhance RV16 binding, and then EOS O<sub>2</sub>− production was evaluated at 0 to 120 min after incubation with RV16 (10 TCID<sub>50</sub>/cell), PMA (1 ng/ml), or CF. In contrast to the vigorous superoxide production induced by PMA, RV16 had no effect on O<sub>2</sub>− generation by EOS (Fig. 6). Additional experiments performed with freshly isolated EOS, or EOS treated with both GM-CSF and TNF-α to maximally up-regulate ICAM-1 expression, yielded similar results (data not shown).

**Discussion**

The EOS is a key effector cell in the pathophysiology of asthma, and there is growing evidence that EOS recruitment and activation are enhanced during RV-induced exacerbations of asthma. In this study, we have begun to define specific interactions between RV and EOS, and the results presented here provide new insights into the mechanisms by which RV may contribute to asthma exacerbations.

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**Table II. Effects of cytokines and RV16 on expression of EOS cell surface markers**

<table>
<thead>
<tr>
<th>Cell Surface Marker</th>
<th>RV16</th>
<th>GM-CSF (100 pM)</th>
<th>TNF-α (100 ng/ml)</th>
<th>GM-CSF + TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM-1 (CD54)</td>
<td>No</td>
<td>0</td>
<td>39</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0</td>
<td>12</td>
<td>31</td>
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<tr>
<td></td>
<td></td>
<td>93</td>
<td>174</td>
<td>151</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Yes</td>
<td>105</td>
<td>193</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>174</td>
<td>14</td>
</tr>
<tr>
<td>CD69</td>
<td>No</td>
<td>185</td>
<td>25</td>
<td>251</td>
</tr>
</tbody>
</table>

*p* Cell surface marker data were calculated as median fluorescent units for the treatments described above less values for corresponding isotype controls.
and EOS in terms of direct binding. Ag presentation, and EOS activation. Peripheral blood EOS, which express minimal amounts of ICAM-1, bound little RV16. However, when the EOS were treated with GM-CSF, RV16 binding was increased. This increase was probably due to increased ICAM-1 expression, as indicated by inhibition of binding with anti-ICAM-1 mAb. Since airway EOS express higher levels of ICAM-1 than blood EOS (19), it is likely that airway EOS bind substantial amounts of RV during natural infections.

In addition to binding RV, EOS also presented viral Ag to RV16-specific T cells, inducing T cell proliferation and IFN-γ secretion. T cell proliferation was mediated by autologous, but not allogeneic EOS, proving that this was an MHC-restricted process. Although there were small numbers (<1%) of mononuclear cells and neutrophils present in the EOS preparations, titration of iPBMC as APC demonstrated that these few cells could not have accounted for the observed T cell proliferation. Although there are previous reports that EOS can present purified protein Ags (20, 21), allergens (17), or superantigens (22) to specific T cells, this is the first report that EOS may participate in virus-induced immune responses by processing and presenting complex viral proteins and/or intact viral particles.

Our data indicate that EOS have the potential to activate RV-specific T cells, and this could have several effects on airway physiology. First, activation of virus-specific T cells is likely to contribute to antiviral responses, leading to clearance of virus and resolution of symptoms. On the other hand, virus-specific T cell responses could also augment pre-existing airway inflammation, and thereby potentiate respiratory symptoms (23). In particular, virus-induced IFN-γ could increase adhesion molecule expression, leading to increased recruitment of cells to the airway, and through the induction of ICAM-1 (24, 25), IFN-γ could also potentiate RV binding to epithelial cells and airway inflammatory cells.

While we found little evidence that RV directly activates EOS, it is important to consider that the process of Ag presentation is associated with bidirectional cellular signals and activation (26), and that EOS could be activated during the process of Ag presentation. Specifically, the engagement of cell surface receptors during T cell/EOS binding, along with the secretion of cytokines by the activated T cell, could augment EOS inflammatory functions. In fact, it has been demonstrated that activated T cells bind to either resting or PMA-treated EOS via VLA-4 and CD18, and that this binding causes EOS activation, as indicated by increased expression of CD11b and CD66b surface markers (27). Furthermore, we found that RV incubation enhanced EOS CD18 expression, which could lead to greater T cell/EOS binding. When considered together, these findings suggest that RV could initiate a bidirectional activation of both cell types.

The presence of increased numbers of EOS in the airway, as occurs in asthma, may counteract the suppressive effects of alveolar macrophages, which are not effective APC, and can suppress activation of T cells under some conditions (28). Enhancement of T cell proliferation and cytokine secretion in asthma could cause increased inflammation, and ultimately, produce greater airway obstruction, bronchial hyper-responsiveness, and clinical symptoms (23). Thus, the availability of increased numbers of EOS in such subjects to interact with RV and activate virus-specific T cells could, in part, explain the more severe clinical manifestations of RV infection that occur in asthmatic vs normal individuals.

Our data suggest that RV produces small changes in EOS phenotype, as indicated by a significant shift from CD18<sup>dim</sup> to CD18<sup>bright</sup> expression, but major changes in cell surface markers were not observed. Likewise, RV did not trigger superoxide release from EOS, even after priming with cytokines (GM-CSF ± TNF-α) to enhance ICAM-1 expression. Thus, these observations suggest that the EOS activation observed during natural RV infection may not be due to direct effects of the virus on EOS. These results need to be tempered by the fact that there are functional and phenotypic differences between primed peripheral blood EOS and airway EOS (29), and additional experiments to examine the effects of RV on airway EOS are now in progress in our laboratory.

There are several other events in the airway biology during RV infection that are more likely to influence EOS function. For example, RV infection induces RANTES secretion in vivo (30), and inoculation of epithelial cells with RV induces secretion of cytokines such as GM-CSF in vitro (31); both of these cytokines have profound effects on EOS recruitment and function. RANTES is a chemoattractant for EOS and memory T cells, both of which are regarded as key cells in asthmatic airway inflammation (32, 33). Furthermore, GM-CSF is a potent activator of EOS survival and adhesion molecule expression, and is a cofactor for EOS superoxide production and degranulation (18, 34, 35). These limited data suggest that EOS activation during RV infections is more likely to occur as a consequence of T cell or epithelial cell activation than as a result of direct activation of EOS by RV.

In conclusion, to explore features of the immune response to RV infections that may be unique to patients with allergies and/or asthma, we have examined effects of RV on EOS phenotype and function in vitro. Our findings suggest that EOS could contribute to RV-induced immune responses by binding viral particles and functioning as APC. EOS-mediated T cell activation could initiate both antiviral responses and proinflammatory effects that could augment pre-existing airway inflammation in diseases such as asthma. We found little evidence that RV directly potentiates EOS inflammatory functions; rather, it is likely that EOS activation in RV infections in vivo is mediated through RV actions on activated airway T cells, epithelial cells, or other inflammatory cells, leading to the generation of cytokines and other mediators that promote EOS inflammatory function.

Acknowledgments

We thank Dr. Ronald Sorkness for his help in performing the statistical analysis of the data, and Dawn M. Galagan and Janelle Luedke for technical assistance.
References


