IL-9 Stimulates Release of Chemotactic Factors from Human Bronchial Epithelial Cells

Frédéric F. Little, William W. Cruikshank, and David M. Center

The Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts

Interleukin (IL)-9 is a T helper 2 cytokine implicated as a candidate gene and contributor to human asthma. We hypothesized that the inflammatory potential of bronchial epithelium is affected by its local environment and explored this hypothesis with respect to the effect of IL-9 on bronchial epithelium. We investigated the response of primary and immortalized human bronchial epithelial cells to IL-9 stimulation with respect to the release of T-cell chemoattractant factors. In response to IL-9, the HBE4-E6/E7 cell line, but not BEAS-2B cells, released the T-cell chemoattractants IL-16 and regulated on activation, normal T cells expressed and secreted (RANTES) in a dose-dependent fashion. We found a similar dose response to IL-9 in primary cells from bronchial brushings of healthy subjects and that nearly all of the T-cell chemoattraction was attributable to IL-16 and RANTES. Reverse transcriptase/polymerase chain reaction of BEAS-2B, HBE4-E6/E7, and primary cells from two subjects revealed messenger RNA for IL-9 receptor (IL-9R) α but not in BEAS-2B cells. Fluorescence-activated cell sorter analysis of HBE4-E6/E7 and primary cells confirmed surface expression of the IL-9 receptor. Costimulation of both cell types with IL-9 and antibody to either γ-common chain or IL-9Rα completely blocked the release of T-cell chemoattractant activity, confirming the primary role of a functioning IL-9 receptor for IL-9 signaling in HBE4-E6/E7 and primary bronchial epithelial cells. We conclude that IL-9 is a stimulus for airway epithelial cell release of T-cell chemoattractant factors, which in turn may modulate the immune response in allergic airway inflammation.

Interleukin (IL)-9 is a T helper (Th) 2 cytokine that has been proposed as a candidate gene and contributor to human asthma (1, 2). Linkage analyses of critical phenotypic asthmatic characteristics in families, such as presence of wheeze, bronchial hyperresponsiveness (BHR), and total serum immunoglobulin (Ig) E led to the identification of candidate genes clustered on chromosome 5q3.1-3.5, including IL-4, IL-5, IL-9, IL-13, and granulocyte macrophage colony-stimulating factor (3, 4). Parallel studies in mice strains with differing degrees of BHR have demonstrated the corresponding region on chromosome 13 linked to BHR to contain Th2 cytokine genes, including IL-9. Correlating lung tissue IL-9 levels with degree of BHR in background strains and their crossbred progeny has elucidated this finding (1). Certain aspects of the asthmatic phenotype absent in certain mouse strains, such as elevated serum IgE and airway eosinophilia, can be reconstituted by the intratracheal instillation of IL-9 (5). These findings are supported by the presence of increased antigen-induced bronchoalveolar lavage eosinophilia, BHR, and total serum IgE in an IL-9 transgenic mouse with markedly increased baseline lung IL-9 levels (6), and the recent demonstration that IL-9 stimulation induces eotaxin expression in cultured mouse bronchial epithelial cells (7). These interventional studies in mice suggest that IL-9 is at least sufficient to elicit certain important characteristics of the asthmatic phenotype.

In contrast to considerable recent evidence supporting a key function for IL-9 in experimental murine allergic disease, its function in human inflammatory disorders, including asthma, has yet to be determined. In normal peripheral mononuclear cells, IL-9 expression can be elicited by phytohemagglutinin stimulation or T-cell receptor activation with anti-CD3 antibody and is primarily seen in Th2 cells (8). IL-9 has been identified as a stimulus of mucin gene transcription in tracheal aspirates from asthmatics intubated for respiratory failure (9). Recently, IL-9 and IL-9 receptor (IL-9R) immunoreactive cells have been identified in bronchial biopsies of atopic asthmatics (10). The IL-9R consists of two chains, IL-9Rα and the γ-common chain (γ-c) of the IL-2, IL-4, IL-7, and IL-15 receptors. In a COS cell transfection system, effective signaling depends on heteromerization of these two components, whereas native IL-9R activation in bronchial epithelial cells has not been studied (11).

As an interface between the external environment and the circulating immune system, the bronchial epithelium is poised to play a role in the generation and perpetuation of the inflammatory response that defines the asthmatic phenotype. The evolving role of IL-9 in the development of asthma-related inflammation led us to explore the potential of IL-9 to induce T-cell chemoattractants from bronchial epithelial cells, with special attention to the contribution of two well-described T-cell chemotactic factors, IL-16 (12–14) and regulated on activation, normal T cells expressed and secreted (RANTES) (15). We describe a pattern of elaboration of chemotactic factors by immortalized and primary human bronchial epithelial cells stimulated with recombinant human IL-9 and provide evidence that the IL-9 stimulus is mediated in these cells by the IL-9R complex. We propose that these findings have implications for the role of airway epithelial contribution to bronchial inflammation in asthma.

(*Received in original form August 23, 2000 and in revised form April 27, 2001*)

Address correspondence to: Dr. Frédéric F. Little, Pulmonary Center R-304, Boston University School of Medicine, 715 Albany St., Boston, MA 02118. E-mail: flittle@lung.bumc.bu.edu

Abbreviations: bronchial hyperresponsiveness, BHR; bovine serum albumin, BSA; complementary DNA, cDNA; ethylenediaminetetraacetic acid, EDTA; fluorescein-activated cell scanner, FACS; gamma-common chain, γ-c; enzyme-linked immunosorbent assay, ELISA; immunoglobulin, Ig; interleukin, IL; interleukin-9 receptor, IL-9R; messenger RNA, mRNA; phosphate-buffered saline, PBS; polymerase chain reaction, PCR; phycoerythrin, PE; regulated on activation, normal T cells expressed and secreted, RANTES; reverse transcriptase/polymerase chain reaction, RT-PCR; standard error of the mean, SEM; T helper, Th.

Am. J. Respir. Cell Mol. Biol. Vol. 25, pp. 347–352, 2001
Internet address: www.atsjournals.org

Materials and Methods

Cell Culture

Immortalized human bronchial epithelial cells. HBE4-E6/E7 (16) and BEAS-2B cells (17) were obtained from the American Type Culture Collection (no. CRL-2078 and 9609; Manassas, VA). Both cell lines are nontumorigenic in nude mice.

HBE4-E6/E7 cells were grown in tissue culture–treated dishes (Corning, Corning, NY) and maintained in keratinocyte–serum-free medium (GIBCO-BRL, Grand Island, NY) with additives: 5 μg/liter human recombinant epidermal growth factor (EGF), 50 mg/liter bovine pituitary extract (GIBCO-BRL), and 10 ng/ml cholera toxin (List Biological, Campbell, CA). BEAS-2B cells were grown in tissue culture–treated dishes (Corning) further coated with a mixture of 10 μg/ml human plasma fibronectin (GIBCO-BRL), 30 μg/ml collagen (Vitrogen-100; Collagen Corp., Palo Alto, CA), and 0.01 mg/ml bovine serum albumin (BSA) (Intergen, Purchase, NY) in growth medium. The excess mixture was removed, the dish air-dried in a sterile hood for 4 to 6 h, and rehydrated with warm sterile phosphate-buffered saline (PBS) (GIBCO-BRL) before use. The growth medium was bronchial epithelial cell basal medium (BEBM) (Clonetics, Walkersville, MD) to which were added: 50 mg/liter bovine pituitary extract, 5 mg/liter insulin, 0.5 mg/liter hydrocortisone, 50 mg/liter gentamicin, 50 μg/liter amphotericin-B, 0.1 μg/liter retinoic acid, 10 mg/liter transferrin, 6.5 μg/liter triiodothyronine, 0.5 mg/liter epinephrine, and 0.5 μg/liter human recombinant EGF.

Before reaching 80% confluence, cells were passed 1:4 to 1:6 using 0.05% trypsin–ethylenediaminetetraacetic acid (EDTA) (GIBCO-BRL) with 0.5% polyvinylpyrrolidone (Sigma, St. Louis, MO) to detach cells, thereafter neutralizing with 0.1% soybean trypsin inhibitor (GIBCO-BRL) and 0.1% BSA in growth medium. Cells were fed every 2 to 3 d and passed weekly.

Primary bronchial epithelial cells. Bronchoscopy of healthy volunteers was performed after informed consent and with approval from the Boston Medical Center Institutional Review Board. Briefly, after anesthesia with topical 2% lidocaine (Lederle, Cherry Hill, NJ), a standard cytology brush was passed under bronchoscopic visualization into the right middle lobe bronchus. After brushing and withdrawal from the bronchoscope, the brush was vortexed in 7 ml cold sterile growth medium (bronchial epithelial growth medium [BEGM]:BEBM plus additives; *see* previous section) and rinsed with sterile PBS before repeat sampling. Cells were immediately centrifuged, resuspended in 37°C medium, counted, and aliquoted into tissue culture dishes precoated with a mixture of 10 μg/ml human plasma fibronectin, 30 μg/ml collagen, and 0.01 mg/ml BSA in growth medium. The initial culture medium was supplemented with 4× filtered Nystatin and 2× penicillin/streptomycin solutions (GIBCO-BRL); BEGM was used alone for subsequent medium changes and passage. Cells were fed every 3 to 4 d and passed every 7 to 10 d in an identical fashion as were HBE4-E6/E7 cells (*see* previous section). At the end of this culture period, the cells had a homogenous morphology identical to HBE4-E6/E7 cells in culture. No other cell types could be identified.

Cell Culture Stimulation

In specified experiments, the following reagents were added to the cell cultures: recombinant human IL-9 (rhIL-9), murine monoclonal antihuman IL-9Rα, 2 μg/ml (CD129) (R&D Systems, Minneapolis, MN); rat monoclonal antihuman IL-2 receptor gamma, 1 μg/ml (CD132; γ-c) (Pharmingen, San Diego, CA); murine IgG isotype control, 2 μg/ml (Immunotech, Marseille, France); rat IgG_{2b}-κ isotype control, 1 μg/ml (Pharmingen). Cytokines and antibodies were kept sterile and stored at the manufacturers' recommended temperature and concentration.

A full exchange with fresh medium was made 24 h before cell stimulation. Concentrated reagents were aliquoted directly into culture dishes to achieve the desired final concentrations, the dishes swirled gently, and returned to 37°C. Supernatants were harvested 24 h later into triplicate aliquots and stored at −20°C to minimize protein degradation before chemotaxis assay.

T-Cell Chemotaxis and Coincubation with Blocking Antibodies

T cells were isolated from healthy volunteers by Ficoll-Paque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), followed by passage through a sterile nylon wool column to purify nonadherent T cells. This method yields a > 95% pure T-cell population. After isolation, cells were maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) with 1% BSA, 200 U/ml penicillin G, 0.2 mg/ml streptomycin, and 24 mM Hepes (GIBCO-BRL) in standard tissue culture conditions.

To determine the nature of secreted chemotactic substances, antihuman cytokine monoclonal antibodies (anti-RANTES [R&D Systems]) and anti–IL-16 (clone 14.1) used at neutralizing concentrations (10 μg/ml) were coincubated at room temperature with supernatants for 30 min before assaying. Parallel supernatants, as noted, were coincubated with an equal volume of antibody diluent alone (PBS). Chemotactic bioactivity was assayed in a microchemotaxis chamber by the ability of cell supernatants to stimulate migration of T cells into 8-μm pore nitrocellulose filters (Neuroprobe, Cabin John, MD), as previously described (18–20). All supernatants were assayed in duplicate wells. After assembly and addition of T cells, the chamber was incubated at 37°C for up to 2 h. Filters were subsequently fixed in ethanol, stained with hematoxylin, dehydrated by sequential washes in ethanol, propanol, and xylene, then mounted on glass slides with Permount (Fisher, Fair Lawn, NJ). T-lymphocyte migration is expressed as a percent of control migration elicited by appropriate unconditioned growth medium. Routinely, 10 to 15 cells were counted per high-power field under these control conditions. Results expressed are from duplicate or triplicate experiments. Two-tailed unpaired Student's *t* tests were performed with the aid of StatviewSE + Graphics software (Abacus Concepts, Berkeley, CA), with type I error probabilities (*P*) noted in figure legends.

Enzyme-Linked Immunosorbent Assay of Cell Culture Supernatants

Enzyme-linked immunosorbent assay (ELISA) of supernatants for RANTES (Biosource, Camarillo, CA) and IL-16 were performed according to the manufacturer's recommendations and our own protocol (21), respectively. Data are expressed as mean ± standard error of the mean (SEM).

Reverse Transcriptase/Polymerase Chain Reaction

Total RNA from epithelial cells was extracted by the phenol/chloroform method per manufacturer's recommendations (Tri-Reagent; Molecular Research Institute, Cincinnati, OH). Thereafter, the RNA was incubated for 5 min at 70°C in the presence of 100 μg/ml oligo dT (Promega, Madison, WI) and 1× first strand buffer (GIBCO-BRL). A total of 1 mM deoxynucleotide triphosphate (dNTP), reverse transcriptase (RT) (Moloney murine leukemia virus; GIBCO-BRL), and RNAsin (Promega) were then added and reverse transcription carried out by incubating the reaction mixture for 60 min at 42°C, then 5 min at 95°C.

The following intron-spanning primer pairs were used for amplification of specific complementary DNA (cDNA) sequences: IL-9Rα, 5′TGTGCACCCAGAGATAGTT and 5′GAAATTGT CAGATGGCACGAG (expected product length, 710 bp); actin, 5′ATGCCATCCTGCGTCTGGA and 5′CACATCTGCTGGA

AGGTGG (expected product length, 540 bp). The following reagent concentrations were used in each polymerase chain reaction (PCR): PCR buffer without Mg^{2+} (1×), MgCl (1.5 mM), Taq polymerase (2.5 U/100 μl) (all GIBCO-BRL), dNTP, cDNA, and primers. The following reaction parameters were implemented for 30 cycles: denature, 94°C for 30 s; anneal, 55°C for 60 s; polymerize, 72°C for 90 s.

Equal volumes of PCR products were electrophoresed using standard techniques and stained with ethidium. Bands represented are of predicted product length noted previously.

Fluorescein-Activated Cell Scanning and Analysis

For flow cytometric analysis of surface molecules, epithelial cells were harvested with 100 mM EDTA in 1× PBS, pH 7.40, washed in cold PBS, centrifuged, and resuspended in an appropriate volume of fluorescein-activated cell scanner (FACS) staining buffer: 0.01% NaN_3, 0.02% BSA in PBS, pH 7.40, 0.22 μm filtered. Cells were incubated in antibodies for 30 min in the dark at 4°C and washed twice in cold staining buffer between incubations. The following antibody types, concentrations, and sources were employed to detect surface IL-9R: unconjugated mouse IgG_1, 10 μg/ml (isotype control); unconjugated mouse antihuman IL-9Rα (IgG_1), 10 μg/ml (both R&D Systems); phycoerythrin (PE)-conjugated rat antimouse Ig κ light chain, 5 μg/ml (Pharmingen). The following antibody types, concentrations, and sources were employed to detect surface γ-c of the IL-2R: PE-conjugated rat IgG_{2b}-κ, 4 μg/ml (isotype control); PE-conjugated rat antihuman γ-c (IgG_{2b}-kappa), 4 μg/ml (both Pharmingen). Before FACS scanning, cells were fixed in isotonic 10% formalin.

FACS scanning and analysis were carried out using the Becton Dickinson FACScan and accompanying CELLQuest software (San Jose, CA) per manufacturer's guidelines. A total of 10×10^3 events was acquired, and data were expressed as representations of all (ungated) events.

Results

Immortalized and Primary Bronchial Epithelial Cell Release of T-Cell Chemotactic Factors

First, we stimulated HBE4 cells with a panel of Th2 cytokines. There was no induction of chemoattractant activity in response to IL-4. There was a small, but statistically significant response to IL-13 (data not shown). In contrast, after stimulation of HBE4-E6/E7 cells with rhIL-9, there was a significant dose-dependent increase in release of T-cell chemoattractant factors at 24 h (Figure 1). Conditioned medium alone from a parallel untreated dish elicited no T-cell chemotactic activity, nor did stimulation of BEAS-2B cells for 24 h with a range of IL-9 doses (50 to 500 ng/ml) (data not shown). For HBE4-E6/E7 cells, a threshold for release of lymphocyte chemotactic activity occurs between 2.5 to 25 ng/ml of IL-9. Significantly greater increases in chemotactic activity (more than 225% of control migration) were not observed in response to supernatants from HBE4-E6/E7 cells stimulated with higher concentrations of IL-9. IL-9 alone at a wide range of concentrations was not chemotactic for T cells (data not shown).

To confirm that the examined response to IL-9 was not unique to the HBE4-E6/E7 cell line and that this cell line is an appropriate model for the IL-9 response in airway epithelial cells, the response of primary bronchial epithelial cells from two healthy subjects to a dose range of IL-9 stimulation is demonstrated in Figure 2. There is notable similarity in the dose response to IL-9 stimulation between

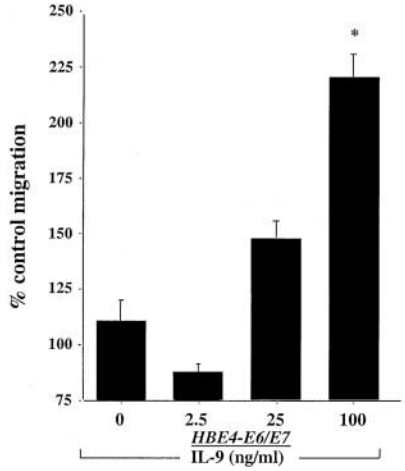

Figure 1. Release of T-cell chemoattractant activity by IL-9–stimulated immortalized human bronchial epithelial cells. Supernatants were harvested 24 h after stimulation of HBE4-E6/E7 cells with varying concentrations of IL-9 or diluent alone. Isolated human T-lymphocytes from healthy volunteers were used as target cells in a microchemotaxis chamber. Total T-cell migration is expressed as percentage of migration (mean ± SEM) of that elicited by unconditioned growth medium. $*P < 0.05$ versus conditioned medium from unstimulated cells.

HBE4-E6/E7 cells and primary cells, with a mean lymphocyte chemotactic activity from stimulated cells of 114, 164, and 205% of control migration at 10, 50, and 100 ng/ml, respectively, of IL-9 stimulation for 24 h.

In order to elucidate the nature of the T-cell chemotactic activity released by IL-9–stimulated HBE4-E6/E7 and primary bronchial epithelial cells, supernatants were coincubated with neutralizing concentrations of anticytokine antibodies before the chemotaxis assay. IL-16 and RANTES were targeted because of their known association with airway epithelium and their preferential effect on $CD4^+$ T cells (14, 15, 22–25). Coincubation with anti–IL-16 antibody significantly reduced the mean migratory response

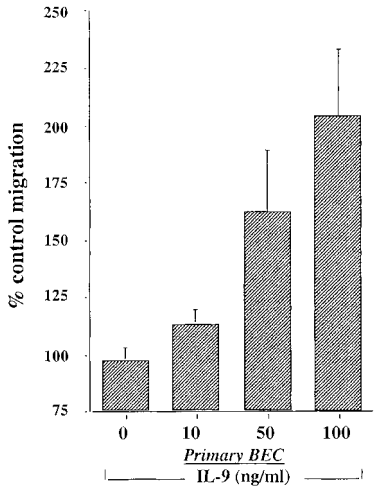

Figure 2. Dose response of T-cell chemoattractant activity released from IL-9–stimulated primary bronchial epithelial cells. Airway cells from two individuals were obtained by bronchoscopy and cytologic brushing of large airways and expanded in tissue culture. At approximately 80% confluence, cells were stimulated with IL-9 in a concentration range of 10 to 100 ng/ml. T-cell migration is expressed as percentage of migration (mean ± SEM) of that elicited by unconditioned growth medium.

induced by IL-9–stimulated HBE4-E6/E7 supernatants from 221 to 129% over that elicited by medium alone. Neutralization of RANTES decreased the chemotactic response to 173% over medium alone in the same supernatants (Figure 3A). Neutralization with both antibodies reduced T-cell chemoattraction to control levels, suggesting that the majority of T-cell chemoattractant activity released by HBE4-E6/E7 cells after 24 h of stimulation with IL-9 is attributable to IL-16 and RANTES.

A similar pattern was observed in primary bronchial epithelial cells stimulated with IL-9 (Figure 3B). In contrast to HBE4-E6/E7 cells, RANTES appeared to contribute less to the total lymphocyte chemotactic activity induced in primary cells by IL-9. Furthermore, coincubation of supernatants with both anti–IL-16 and anti-RANTES antibodies did not reduce chemotaxis to control levels. This is likely due to the elaboration of other chemoattractant cytokines that were not identified in this study.

A dose response of the T-cell chemoattractants IL-16 and RANTES is illustrated in Figure 3C for comparison of the magnitude of the chemotactic effect observed in epithelial cell supernatants. Protein analysis by ELISA of IL-9–stimulated HBE4-E6/E7 and primary cell supernatants for IL-16 was below detectable limits (< 15 pg/ml). RANTES levels from HBE4-E6/E7 cells were 136 ± 55 pg/ml. By contrast, primary cells secreted levels of RANTES at the lower limit of detection for the assay (3.6 ± 1.2 pg/ml). These data are consistent with our prior observations that chemotactic activity of IL-16 can be identified at concentrations that are below levels detectable by ELISA (21, 24). Also, the ELISA data correlate with our chemotaxis assays

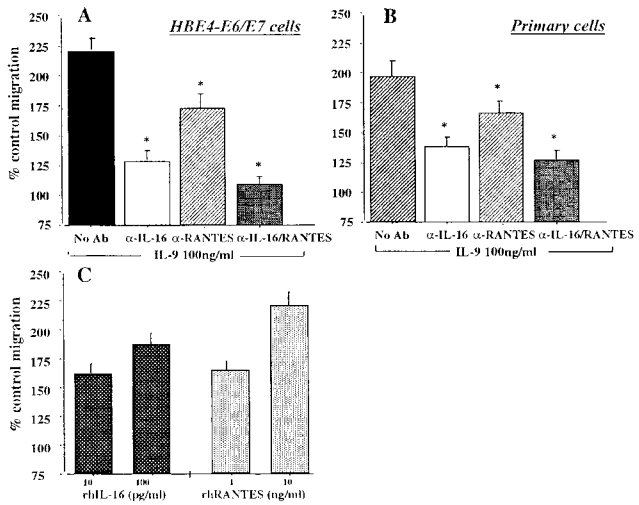

Figure 3. Characterization of T-cell chemoattractant activity released from IL-9–stimulated HBE4-E6/E7 and primary bronchial epithelial cells. Supernatants from HBE4-E6/E7 (*A*) and primary cells ($n = 6$) (*B*) stimulated for 24 h with IL-9 were coincubated with diluent (*solid bars*), anti–IL-16 antibody (*open bars*), anti-RANTES antibody (*hatched bars*), or both antibodies (*gray bars*) at neutralizing concentrations for 30 min before chemotaxis assay. T-cell migration is expressed as percentage of migration (mean ± SEM) of that elicited by unconditioned growth medium. $^*P < 0.05$ versus supernatant from IL-9–stimulated cells coincubated with diluent alone.

in that more RANTES is elaborated by HBE4-E6/E7 cells than by primary cells in response to IL-9 stimulation.

Presence of IL-9R Messenger RNA and Protein in Bronchial Epithelial Cells

To investigate the presence of IL-9R on our immortalized and primary bronchial epithelial cells, we performed RT-PCR to identify messenger RNA (mRNA) of the unique α component of the IL-9R. The primary cells were passage 2 in culture. Bands of 700 bp were amplified by IL-9Rα–specific primers in RT-PCR reactions of RNA from both primary and HBE4-E6/E7 cell preparations. No PCR product was identified when RNA from BEAS-2B cells was similarly analyzed (Figure 4A).

FACS analysis of HBE4-E6/E7 cells confirmed the presence of the two components of the functioning IL-9R complex. Surface expression of IL-9Rα and of the γ-c of the IL-2 receptor in these cells is demonstrated in Figure 4B. FACS analysis to investigate the presence of surface IL-9Rα on primary bronchial epithelial cells was confirmatory, as depicted in Figure 4C.

Proximal Mechanism of IL-9 Responsiveness of Bronchial Epithelial Cells

We next demonstrated the specific contribution of the IL-9R complex to the IL-9–induced release of T-cell chemotactic factors by costimulation of HBE4-E6/E7 and primary bronchial epithelial cells with IL-9 in combination with antibodies to the IL-9Rα and γ-c. The results are depicted in Figure 5. Antibody to either the α or γ-c components of the IL-9R completely abrogated release of IL-9–generated T-cell chemoattraction. Costimulation with isotype control antibodies for both anti–IL-9Rα and anti–γ-c had no effect on epithelial cell IL-9 responsiveness.

Discussion

We have demonstrated that IL-9 directly stimulates bronchial epithelial cells in culture to secrete the T-cell chemoattractant factors IL-16 and RANTES. In both primary bronchial epithelial cells and an immortalized cell line, cells are stimulated via the IL-9R complex, with both components being necessary for IL-9–stimulated release of T-cell chemoattractant bioactivity. This is the first description of a direct effect of IL-9 on airway epithelial cell secretion of T-cell chemotactic factors and complements other studies that implicate IL-9 as a contributor to the chronic airway inflammatory phenotype typical of murine experimental and human clinical asthma.

The current study demonstrates the release of T-cell chemotactic factors by IL-9–stimulated bronchial epithelial cells. Similar studies in mice have demonstrated IL-9 induction of eosinophil active chemokines from cultured bronchial epithelial cells (7). Incubation of cultured human airway epithelial cells with tracheal aspirates from intubated asthmatic patients significantly increased MUC5A gene expression; further investigation of this observation attributed this gene upregulation to IL-9 (9). These observations supplement the *in vivo* demonstration of IL-9–stimulated mucous glycoprotein expression in murine airways and *in vitro* mucous glycoprotein production in hu-

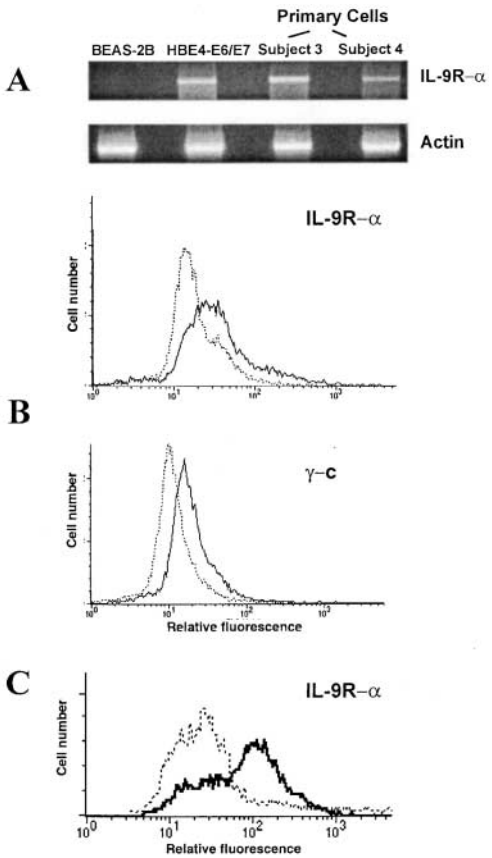

Figure 4. Presence of IL-9R on HBE4-E6/E7 and primary bronchial epithelial cells. (*A*) RT-PCR for IL-9Rα. Total RNA was isolated from immortalized bronchial epithelial cell lines and primary epithelial cells from two healthy subjects for the generation of cDNA by reverse transcription. Primary cells were obtained in an identical fashion as described in Figure 2. Products of reverse transcription were amplified by IL-9Rα and actin-specific primers, and subjected to agarose gel electrophoresis. Bands represented are 0.7 and 0.5 kb for IL-9Rα and actin, respectively. There was no detectable amplified cDNA for IL-9Rα in BEAS-2B cells, whereas both HBE4-E6/E7 and primary epithelial cells contain amplified cDNA for IL-9Rα. This is a representative gel from three separate experiments. (*B*) FACS analysis of HBE4-E6/E7 bronchial epithelial cells for surface expression of IL-9R components. After detachment with 100 mM isotonic EDTA, cells were washed and labeled with either antibody to IL-9Rα followed by a secondary PE-conjugated antibody or with PE-conjugated anti–IL-2 receptor γ (γ-common chain) antibody. Cells were incubated for 30 min in the dark at 4°C before washing and FACS analysis. Isotype control antibody labeling is depicted by the dotted curve; IL-9Rα and γ-c–specific labeling are indicated by the solid curves. (*C*) FACS analysis of primary bronchial epithelial cells for surface expression of IL-9Rα. Cells were prepared and stained in an identical fashion to *B*. These are representative curves from three separate experiments.

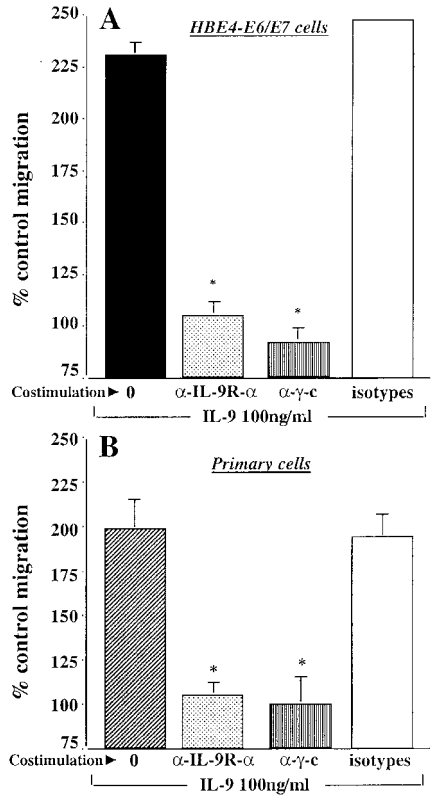

Figure 5. Effect of antibodies to components of the IL-9R on IL-9–stimulated release of T-cell chemoattractant activity by HBE4-E6/E7 and primary bronchial epithelial cells. In addition to IL-9 (100 ng/ml), HBE4-E6/E7 cells ($n = 4$) (*A*) and primary cells ($n = 3$) (*B*) were coincubated with diluent, anti–IL-9Rα (2 μg/ml), anti-γ-c (1 μg/ml), or both isotype control antibodies, as described in MATERIALS AND METHODS. Supernatants were harvested at 24 h and subjected to chemotaxis assay. T-cell migration is expressed as percentage of migration (mean ± SEM) of that elicited by unconditioned growth medium: IL-9 alone (*solid bar*), IL-9 and anti–IL-9Rα (*light gray bars*), IL-9 and anti-γ-c (*vertically hatched bars*), IL-9 and isotypes (*open bars*). $^*P < 0.05$ versus supernatant from IL-9–stimulated cells coincubated with diluent alone.

man cell culture (26). By immunohistochemistry and *in situ* hybridization, bronchial biopsies of asthmatic subjects have a markedly higher number of IL-9–positive cells than those of healthy subjects (10). Interestingly, biopsies from nonasthmatic atopics had a number of IL-9–positive cells intermediate to the other two groups. The IL-9–positive cells were primarily in the submucosa, 70% of which were $CD3^+$ T cells with the remainder made up of eosinophils and neutrophils. Taken together, these studies demonstrate both the presence of lymphocyte-derived IL-9 in asthmatic airways and clearly defined epithelial cell IL-9 responsiveness. These data further complement our findings of the capacity of human bronchial cells to secrete IL-16 and RANTES in response to IL-9 stimulation *in vitro* and suggest that IL-9 alone is sufficient to induce this response.

The observation of significantly greater numbers of IL-9–positive cells in the airways of asthmatic subjects compared with nonasthmatic subjects (10) combined with our findings of IL-9 responsive release of IL-16 from bronchial epithelium may help explain the prior *in vivo* demonstration of IL-16 in the bronchial epithelium of atopic asthmatics but not in atopic nonasthmatics (22). In addition, human bronchial epithelium can be directly stimulated to release IL-16 in response to allergen and histamine *in vivo* (21, 24). It

has also been noted that experimental interference with T-cell activation in allergen-stimulated asthmatic bronchial biopsy explants abrogated IL-16 release (25). The IL-16 from these bronchial explants could either be directly released from mucosal T cells or follow IL-9 release from activated T cells, which in turn stimulates epithelial IL-16 generation.

There is direct evidence for the presence of IL-9Rs on murine and human airway epithelium. We have confirmed their presence by RT-PCR and FACS analysis, and further, have demonstrated that functionality in the airway epithelial cell requires expression of both the IL-9Rα and IL-2 receptor γ-c. The absence of IL-9 responsiveness in BEAS-2B cells is consistent with our inability to detect IL-9R mRNA in these cells by RT-PCR. A recent investigation also found BEAS-2B cells to be unresponsive to IL-9 stimulation alone (27). IL-9R transcripts have been detected by RT-PCR in primary murine (7), immortalized human, and primary human airway epithelial cells (9) in culture. These findings have been matched by immunoblots of immortalized cell lysates (9) and with histologic data from endobronchial biopsies demonstrating human epithelial IL-9R mRNA by *in situ* hybridization (10) and protein by immunohistochemistry (26).

The recently reported heterogeneity in epithelial IL-9R expression between asthmatic and nonasthmatic subjects (10, 28) has potential implications for the *in vivo* responsiveness of bronchial epithelial cells to IL-9. This heterogeneity could be due to several influences: increased IL-9R expression due to the local microenvironment, differences in baseline expression at the transcriptional level, and *in vivo* upregulation of IL-9R in response to IL-9 itself. We are currently investigating the modulation of IL-9R expression on bronchial epithelial cells by Th2 cytokine stimulation. In addition, the recent observations by Pinsonneault and coworkers (29) that IL-16 inhibits IL-5 secretion from T cells suggest that epithelial cell–derived IL-16 may play an immunomodulatory role in Th2 cytokine–related lung inflammation.

Acknowledgments: The authors thank Drs. Thomas Ryan and Martin Joyce-Brady for their assistance with RT-PCR experiments. This study was supported in part by grant AI35680 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health. F.F.L. is the recipient of a fellowship training award from the American Lung Association. W.W.C. is a Career Investigator of the American Lung Association.

References

1. Nicolaides, N. C., K. J. Holroyd, S. L. Ewart, S. M. Eleff, M. B. Kiser, C. R. Dragwa, C. D. Sullivan, L. Grasso, L. Y. Zhang, C. J. Messler, T. Zhou, S. R. Kleeberger, K. H. Buetow, and R. C. Levitt. 1997. Interleukin-9: a candidate gene for asthma. *Proc. Natl. Acad. Sci. USA* 94:13175–13180.
2. Soussi-Gounni, A., M. Kontolemos, and Q. Hamid. 2001. Role of IL-9 in the pathophysiology of allergic diseases. *J. Allergy Clin. Immunol.* 107:575–582.
3. Postma, D. S., E. R. Bleecker, P. J. Amelung, K. J. Holroyd, J. Xu, C. I. Panhuysen, D. A. Meyers, and R. C. Levitt. 1995. Genetic susceptibility to asthma: bronchial hyperresponsiveness coinherited with a major gene for atopy. *N. Engl. J. Med.* 333:894–900.
4. Doull, I. J., S. Lawrence, M. Watson, T. Begishvili, R. W. Beasley, F. Lampe, S. T. Holgate, and N. E. Morton. 1996. Allelic association of gene markers on chromosomes 5q and 11q with atopy and bronchial hyperresponsiveness. *Am. J. Respir. Crit. Care Med.* 153:1280–1284.
5. Levitt, R. C., M. P. McLane, D. MacDonald, V. Ferrante, C. Weiss, T. Zhou, K. J. Holroyd, and N. C. Nicolaides. 1999. IL-9 pathway in asthma: new therapeutic targets for allergic inflammatory disorders. *J. Allergy Clin. Immunol.* 103:485–491.
6. McLane, M. P., A. Haczku, M. van de Rijn, C. Weiss, V Ferrante, D. MacDonald, J.-C. Renauld, N. C. Nicolaides, K. J. Holroyd, and R. C. Levitt. 1998. Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. *Am. J. Respir. Cell Mol. Biol.* 19:713–720.
7. Dong, Q., J. Louahed, A. Vink, C. Sullivan, C. J. Messler, Y. Zhou, A. Haczku, F. Huaux, M. Arras, K. J. Holroyd, J.-C. Renauld, R. C. Levitt, and N. C. Nicolaides. 1999. IL-9 induces chemokine expression in lung epithelial cells and baseline airway eosinophilia in transgenic mice. *Eur. J. Immunol.* 29:2130–2139.
8. Renauld, J. C., A. Goethals, F. Houssiau, H. Merz, E. van Roost, and J. van Snick. 1990. Human P40/IL-9: expression in activated CD4+ T cells, genomic organization, and comparison with the mouse gene. *J. Immunol.* 144:4235–4241.
9. Longphre, M., D. Li, M. Gallup, E. Drori, C. L. Ordonez, T. Redman, S. Wenzel, D. E. Bice, J. V. Fahy, and C. Basbaum. 1999. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *J. Clin. Invest.* 104:1375–1382.
10. Shimbara, A., P. Christodoulopoulos, A. Soussi-Gounni, R. Olivenstein, Y. Nakamura, R. C. Levitt, N. C. Nicolaides, K. J. Holroyd, A. Tsicopoulos, J. J. Lafitte, B. Wallaert, and Q. A. Hamid. 1999. IL-9 and its receptor in allergic and nonallergic disease: increased expression in asthma. *J. Allergy Clin. Immunol.* 104:108–115.
11. Bauer, J. H., K. D. Liu, Y. You, S. Y. Lai, and M. A. Goldsmith. 1998. Heteromerization of the γc chain with the interleukin-9 receptor α subunit leads to STAT activation and prevention of apoptosis. *J. Biol. Chem.* 273:9255–9260.
12. Center, D. M., H. Kornfeld, T. C. Ryan, and W. W. Cruikshank. 2000. Interleukin-16: implications for CD4 functions and HIV-1 progression. *Immunol. Today* 21:273–279.
13. Cruikshank, W. W., H. Kornfeld, and D. M. Center. 1999. Structure and function of interleukin-16. *Curr. Trends Immunol.* 2:99–109.
14. Cruikshank, W. W., H. Kornfeld, and D. M. Center. 2000. Interleukin-16. *J. Leukoc. Biol.* 67:757–766.
15. Bacon, K. B., B. A. Premack, P. Gardner, and T. J. Schall. 1995. Activation of dual T cell signaling pathways by the chemokine RANTES. *Science* 269:1727–1730.
16. Viallet, J., C. Liu, J. Emond, and M. S. Tsao. 1994. Characterization of human bronchial epithelial cells immortalized by the E6 and E7 genes of human papillomavirus type 16. *Exp. Cell Res.* 21:36–41.
17. Reddel, R. R., Y. Ke, B. I. Gerwin, M. G. McMenamin, J. F. Lechner, R. T. Su, D. E. Brash, J. B. Park, J. S. Rhim, and C. C. Harris. 1988. Transformation of human bronchial epithelial cells by infection with SV40 of adenovirus-12 SV40 hybrid virus, or transfection via strontium phosphate coprecipitation with a plasmid containing the SV40 early region genes. *Cancer Res.* 48:1904–1909.
18. Mashikian, M. V., T. C. Ryan, A. Seman, W. Brazer, D. M. Center, and W. W. Cruikshank. 1999. Reciprocal desensitization of CCR5 and CD4 is mediated by IL-16 and macrophage-inflammatory protein 1β, respectively. *J. Immunol.* 163:3123–3130.
19. Van Drenth, C., A. Jenkins, L. Ledwich, T. C. Ryan, M. V. Mashikian, W. Brazer, D. M. Center, and W. W. Cruikshank. 2000. Desensitization of CXC chemokine receptor 4, mediated by IL-16/CD4, is independent of $p56^{lck}$ enzymatic activity. *J. Immunol.* 165:6356–6363.
20. Cruikshank, W. W., J. S. Berman, A. C. Theodore, J. Bernardo, and D. M. Center. 1987. Lymphokine activation of $T4^+$ T lymphocytes and monocytes. *J. Immunol.* 138:3817–3822.
21. Cruikshank, W. W., A. Long, R. E. Tarpy, H. Kornfeld, M. P. Carroll, L. Teran, S. T. Holgate, and D. M. Center. 1995. Early identification of interleukin-16 (lymphocyte chemoattractant factor) and macrophage inflammatory protein 1-α (MIP1-α) in bronchoalveolar lavage fluid of antigen-challenged asthmatics. *Am. J. Respir. Cell Mol. Biol.* 13:738–747.
22. Laberge, S., P. Ernst, O. Ghaffar, W. W. Cruikshank, H. Kornfeld, D. M. Center, and Q. Hamid. 1997. Increased expression of interleukin-16 in bronchial mucosa of subjects with atopic asthma. *Am. J. Respir. Cell Mol. Biol.* 17:193–202.
23. Fahy, J. V., D. J. Figueroa, H. H. Wong, J. T. Liu, and J. S. Abrams. 1997. Similar RANTES levels in healthy and asthmatic airways by immunoassay and *in situ* hybridization. *Am. J. Respir. Crit. Care Med.* 155:1095–1100.
24. Mashikian, M. V., R. E. Tarpy, J. J. Saukkonen, K. G. Lim, G. D. Fine, W. W. Cruikshank, and D. M. Center. 1998. Identification of IL-16 as the lymphocyte chemotactic activity in the bronchoalveolar lavage fluid of histamine-challenged asthmatic patients. *J. Allergy Clin. Immunol.* 101:786–792.
25. Hidi, R., V. Riches, A. Musa, W. W. Cruikshank, D. M. Center, S. T. Holgate, and R. Djukanovic. 2000. Role of B7-CD28/CTLA-4 costimulation and NF-kappaB in allergen-induced T cell chemotaxis by IL-16 and RANTES. *J. Immunol.* 164:412–418.
26. Louahed, J., M. Toda, J. Jen, Q. Hamid, J.-C. Renauld, R. C. Levitt, and N. C. Nicolaides. 2000. Interleukin-9 upregulates mucus expression in the airways. *Am. J. Respir. Cell Mol. Biol.* 22:649–656.
27. Yoshida, N., M. Arima, G. Cheng, E. Fukiko, H. Hirata, K. Honda, F. Fukushima, and T. Fukuda. 2001. Interleukin (IL)-4/IL-9 and exogenous IL-16 induce IL-16 production by BEAS-2B cells, a bronchial epithelial cell line. *Cell. Immunol.* 207:75–80.
28. Bhathena, P. R., S. A. Comhair, K. J. Holroyd, and S. C. Erzurum. 2000. Interleukin-9 receptor expression in asthmatic airways *in vivo. Lung* 178:149–160.
29. Pinsonneault, S., S. El Bassam, B. Mazer, W. W. Cruikshank, and S. Laberge. 2001. IL-16 inhibits IL-5 production by antigen-stimulated T cells in atopic subjects. *J. Allergy Clin. Immunol.* 107:477–482.