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Research Article

Airway inflammation is thought to play an important role in the pathogenesis of asthma. We have used in situ hybridization and an immunoassay to determine whether granulocyte macrophage colony-stimulating factor (GM-CSF) (a cytokine capable of eosinophil activation) is present in the airway of asthmatics (n = 6) who have 37.0 +/- 15.1% airway eosinophilia after endobronchial allergen challenge. Levels of immunoreactive GM-CSF (less than 4 pg/ml pre-allergen versus 180.5 +/- 46.9 pg/ml post-allergen) increased significantly 24 h after endobronchial allergen stimulation. The cellular source of bronchoalveolar lavage (BAL) GM-CSF, as determined by in situ hybridization and immunoperoxidase staining, was derived predominantly from UCHL-1 positive BAL lymphocytes, as well as from a smaller population of alveolar macrophages. Before local endobronchial allergen challenge, less than 1% of lymphocytes and alveolar macrophages recovered by BAL expressed GM-CSF mRNA, whereas after allergen stimulation 92.6 +/- 3.4% of lymphocytes and 17.5 +/- 22.7% of alveolar macrophages expressed GM-CSF mRNA. This study provides evidence that in an experimental model of allergen-induced asthma, activation of the immune and inflammatory response (BAL lymphocyte and alveolar macrophage production of GM-CSF) is temporally associated with an inflammatory cell influx of eosinophils into the airway.

Endobronchial Allergen Challenge in Asthma

Demonstration of Cellular Source of Granulocyte Macrophage Colony-stimulating Factor by In Situ Hybridization

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Abstract

Airway inflammation is thought to play an important role in the pathogenesis of asthma. We have used in situ hybridization and an immunoassay to determine whether granulocyte macrophage colony-stimulating factor (GM-CSF) (a cytokine capable of eosinophil activation) is present in the airway of asthmatics ($n = 6$) who have 37.0±15.1% airway eosinophilia after endobronchial allergen challenge. Levels of immunoreactive GM-CSF (< 4 pg/ml pre-allergen versus 180.5±46.9 pg/ml post-allergen) increased significantly 24 h after endobronchial allergen stimulation. The cellular source of bronchoalveolar lavage (BAL) GM-CSF, as determined by in situ hybridization and immunoperoxidase staining, was derived predominantly from UCHL-1 positive BAL lymphocytes, as well as from a smaller population of alveolar macrophages. Before local endobronchial allergen challenge, < 1% of lymphocytes and alveolar macrophages recovered by BAL expressed GM-CSF mRNA, whereas after allergen stimulation 92.6±3.4% of lymphocytes and 17.5±22.7% of alveolar macrophages expressed GM-CSF mRNA. This study provides evidence that in an experimental model of allergen-induced asthma, activation of the immune and inflammatory response (BAL lymphocyte and alveolar macrophage production of GM-CSF) is temporally associated with an inflammatory cell influx of eosinophils into the airway. (*J. Clin. Invest.* 1991. 88:1048–1053.) Key words: granulocyte macrophage colony-stimulating factor • lymphocyte • alveolar macrophage • eosinophil • asthma • UCHL-1

Introduction

Airway inflammation is thought to play an important role in the pathogenesis of asthma (1). Endobronchial allergen challenge in asthmatics has provided an important in vivo experimental model to study this inflammatory response to a specific allergen. Several studies using endobronchial allergen challenge have demonstrated that the local inflammatory response is associated with mast cell activation (2) and the recruitment of eosinophils (3) into the airway. More recently, investigators have focused attention on the potential interaction of the immune response (i.e., T cell activation) (4–6) and the inflammatory response (i.e., eosinophil recruitment and activation) in the pathogenesis of asthma. In this regard, cytokines derived from activated T cells (e.g., granulocyte macrophage colony-stimulating factor [GM-CSF],[1] IL-5, IL-3, tumor necrosis factor [TNF]) (7–11) and alveolar macrophages (12) have been demonstrated in vitro to upregulate eosinophil effector function. We have therefore sought to determine whether one such cytokine (i.e., GM-CSF) is secreted into the airway of asthmatics by lymphocytes and/or alveolar macrophages after endobronchial allergen stimulation.

Methods

Study subjects. Informed consent was obtained from study subjects in a protocol approved by the University of California San Diego (UCSD) Human Subjects Committee. Six subjects with mild asthma (four males and two females) who had a mean age of 21.3±3.1 yr were studied. Study subjects who required medications other than inhalation beta agonists and antihistamines to adequately control symptoms of asthma and associated allergic rhinitis were not enrolled into the study. The subjects were defined as having mild asthma on the basis of symptoms (no hospitalization or emergency room visits for treatment of asthma in the year before the study), pulmonary function studies (baseline forced expiratory volume 1 s (FEV_1) > 75% of predicted at the time of study), and minimal current asthma medication requirements to control symptoms (restricted to use of beta agonist metered dose inhaler as needed). Only atopic asthmatics with a current history of wheezing after exposure to cats, dogs, or house dust were recruited. Laboratory confirmation of respiratory allergy to cat, dog, or house dust mite was demonstrated using immediate hypersensitivity skin tests and inhalation allergen challenge. The study subjects had a history and physical examination, baseline spirometry, immediate hypersensitivity skin tests, and methacholine challenge performed at visit 1, diluent inhalation challenge at visit 2, inhalation allergen challenge at visit 3, and endobronchial allergen challenge at visit 4.

Methacholine inhalation challenge. The concentration of methacholine that caused a 20% decrease in FEV_1 (PC_{20} FEV_1) was calculated by having the study subject inhale five breaths of increasing concentrations of methacholine (0.015–25 mg/ml of methacholine) delivered as an aerosol generated by a nebulizer (646; DeVilbiss Co., Somerset, PA) attached to a Rosenthal-French dosimeter (Rosenthal-French, Baltimore, MD) (13).

Diluent inhalation challenge. Study subjects underwent an 8-h diluent inhalation challenge to exclude subjects who had either a significant immediate response to diluent inhalation challenge (> 10% decrease in

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; FEV_1, forced expiratory volume one second; GM-CSF, granulocyte macrophage colony-stimulating factor; TNF, tumor necrosis factor.

FEV_1) or a spontaneous late phase response (> 10% decrease in FEV_1 2–8 h after inhalation of diluent).

Allergen inhalation challenge. Before inhalation of allergen, subjects had immediate hypersensitivity prick puncture skin tests to house dust mite (*Dermatophagoides farinae* and *Dermatophagoides pteronyssinus*), cat, dog, histamine (1 mg/ml), and saline (negative control). A wheal diameter of > 3 mm (compared to the negative control) was interpreted as a positive skin test. Subjects with a history of wheezing on exposure to cat, dog, or house dust, as well as an associated positive skin test, underwent inhalation allergen challenge. The concentration of allergen that caused a 20% decrease in FEV_1 (PD_{20} FEV_1) was calculated by having the study subject inhale five breaths of increasing concentrations (1:500,000 to 1:500 wt/vol of cat, dog, or house dust mite allergen (Hollister-Stier Laboratories, Spokane, WA). Results are expressed as inhalation units/five breaths, where one inhalation unit is defined as one breath of a 1/500 wt/vol dilution of allergen (14). Only subjects who had both an immediate (> 20% decrease in FEV_1) as well as a late phase response (> 15% decrease in FEV_1 2–8 h later) were continued in the study.

Endobronchial allergen challenge. Endobronchial allergen challenge was performed at least 2 wk, and in most cases 4–6 wk after inhalation allergen challenge. Subjects (admitted to the UCSD Clinical Research Center) were premedicated with atropine 0.6 mg i.m. and received supplemental O_2 during the bronchoscopy. Topical anesthesia of the upper and lower airways was achieved with 0.45% tetracaine. A preallergen challenge bronchoalveolar lavage (BAL) with a total vol of 100 ml sterile 37°C normal saline was used to lavage the right middle lobe using a flexible bronchoscope (Model 19D; Pentax Precision Instrument Corp., Orangeburg, NY). This was followed by installation of 1 ml of a 10% solution (vol/vol) of the PD_{20} FEV_1 concentration of allergen (as determined at the inhalation allergen visit) into the posterior segment of the right lower lobe, and 1 ml of diluent (negative control) instilled into the anterior segment of the right lower lobe. A repeat bronchoscopy with 100 ml saline lavage in both the right lower lobe posterior segment (site of allergen challenge) and right lower lobe anterior segment (site of control diluent challenge) was performed 24 h after the endobronchial instillation of allergen or diluent. BAL samples were aspirated with gentle suction, collected in polyethylene tubes on ice, passaged through a single layer of gauze, and processed immediately to separate cells from the lavage fluid by centrifugation at 300 *g* for 10 min at 4°C. Aliquots of cell-free lavage fluid were concentrated 20-fold using a centriprep concentrator (Amicon-10; Amicon Corp., Danvers, MA) and stored at −70°C before GM-CSF immunoassay analysis. Cells pelleted by centrifugation were resuspended in buffered PBS to 2×10^5 cells/ml and 100-μl aliquot of this suspension was used to prepare a set of cytocentrifuge slides by spinning the aliquots at 450 rpm for 4 min in a cytospin (Shandon Inc., Pittsburgh, PA). An aliquot of 10^6 cells was separated into adherent and nonadherent cell populations by incubation on culture chamber slides (Nunc Inc., Naperville, IL) containing 500 μl cell culture medium for 45 min at 37°C. Nonadherent cells were washed in PBS and cytocentrifuge slides prepared. After air drying, unfractionated, adherent, and nonadherent cell slides were either Wright-Giemsa stained for cell differential counts, or fixed in 4% paraformaldehyde for 4 min at room temperature and stored in 70% ethanol at 4°C before in situ hybridization.

In situ hybridization. In situ hybridization with ^{35}S-labelled single stranded GM-CSF sense or antisense RNA probes (kindly provided by Dr. Ken Kauschansky, University of Washington, Seattle, WA) was performed as previously described in this laboratory (15). The identity and orientation of the GM-CSF probes were confirmed by restriction endonuclease mapping and by hybridization to the appropriately sized band in Northern blot analyses (16). In situ hybridized slides were counterstained with hematoxylin, permanently mounted, and coded. For each slide, the number of grains over the cytoplasm of 400 individual cells was determined by counting 100 cells in each of 4 randomly selected fields. Cells were considered positive for GM-CSF mRNA if > 15 grains were localized over the cytoplasm. Most GM-CSF antisense positive cells had > 50 grains/cell and were easily distinguished from background or control GM-CSF sense staining of 0–5 grains/cell.

Immunoperoxidase staining. UCHL-1 (DAKO Corp., Carpenteria, CA), which recognizes the 180-kD component of the leukocyte common antigen on T cells (17), was used in preference to other T cell markers because of its superior staining of paraformaldehyde fixed cells when using the immunoperoxidase technique (15). Immunoperoxidase staining with UCHL-1 was performed as previously described (15).

GM-CSF immunoassay. The GM-CSF ELISA (Genzyme Corp., Cambridge, MA) used a mouse monoclonal anti-human GM-CSF antibody and a rabbit polyvalent antibody to human GM-CSF. The assay can detect 4–512 pg/ml GM-CSF and there is no cross-reactivity in the assay with G-CSF, M-CSF, IL-3, IL-6, TNF α, or gamma interferon. Addition of known amounts of recombinant GM-CSF to asthma BAL fluid resulted in recovery of 89.8% of GM-CSF added to samples.

Statistics. Statistical analysis was performed using the Student's *t* test.

Results

Asthma study subjects. The asthma study subjects (Table I) all had mild asthma, as evidenced by a baseline FEV_1 of 3.74±0.61 liters (85.2±6.4% of predicted FEV_1). All were atopic and had positive immediate hypersensitivity skin test and biphasic (immediate and late phase) response to inhalation allergen challenge (two challenged with cat allergen, one with dog allergen, and three with *Dermatophagoides pteronyssinus*).

Table I. Asthma Study Subject BAL GM-CSF Levels

Study subject	Age	Sex	Baseline FEV_1		Methacholine PC_{20}	Allergen	BAL GM-CSF	
							Preallergen	Postallergen
	yrs	*M:F*	*liters*	*% predicted*	*mg/ml*			
1	21	M	3.92	(80%)	0.62	Cat	<4	201.0
2	20	F	3.43	(90%)	0.31	Cat	<4	132.2
3	22	M	3.84	(83%)	2.50	Dog	<4	250.4
4	27	M	4.79	(95%)	5.00	House dust mite	<4	123.8
5	20	F	3.03	(78%)	0.15	House dust mite	<4	192.6
6	18	M	3.43	(85%)	0.15	House dust mite	<4	182.7
Mean±SEM	21.3±3.1 yr	4M 2F	3.74±0.61 liters	(85.2±6.4%)	1.46±2.0 mg/ml		<4 pg/ml	180.5±46.9 pg/ml

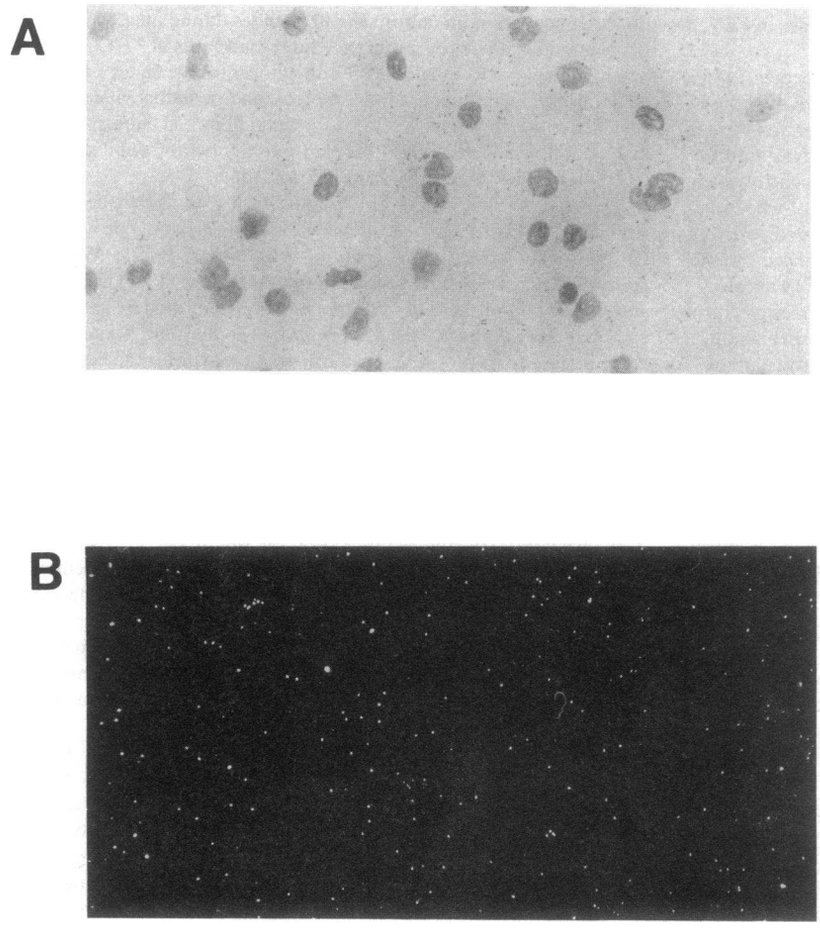


Figure 1. Pre-allergen challenge BAL cells hybridized to antisense GM-CSF probe. No GM-CSF positive cells are detected by either (*A*) light field or (*B*) dark field views of the same photographic field.

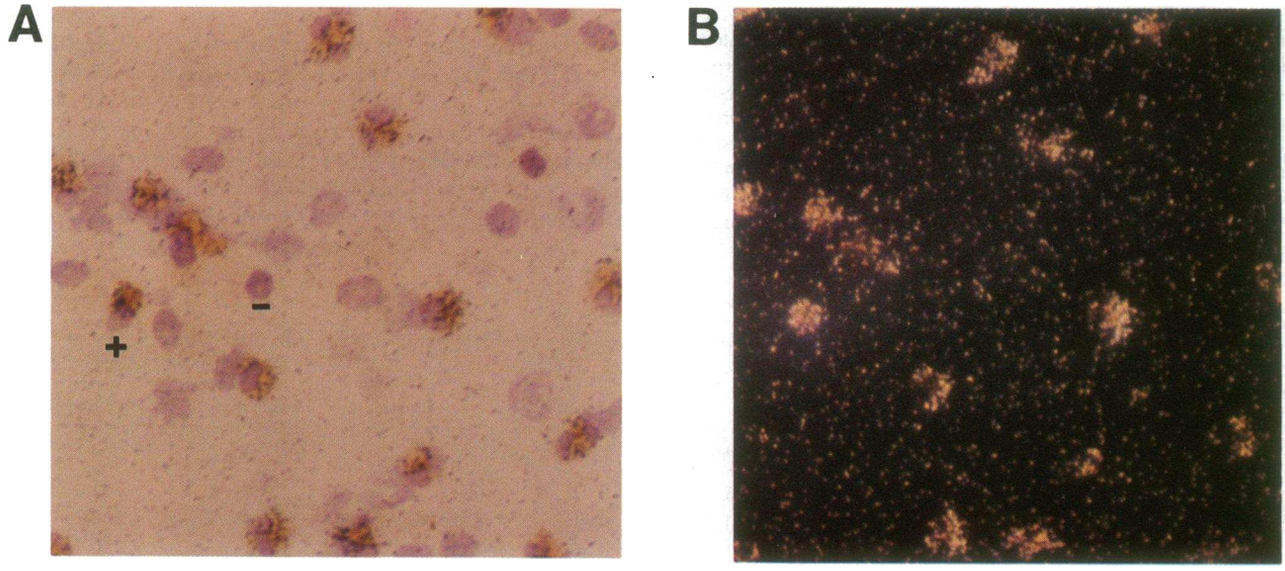
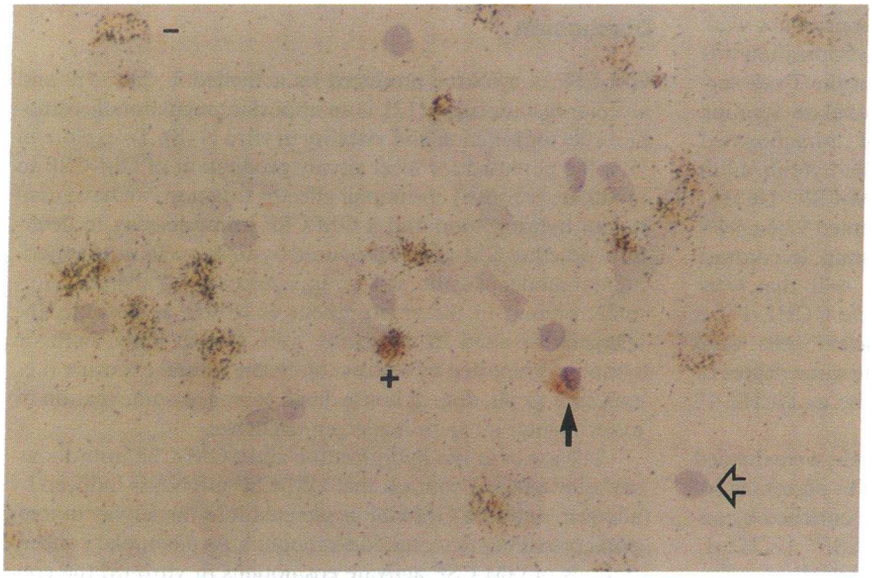


Figure 2. Post-allergen challenge BAL cells were hybridized with the GM-CSF antisense probe, and then stained for UCHL-1 using the immunoperoxidase method. (*A*) Light field and (*B*) dark field view of the same photographic field. The UCHL-1$^+$ cells (golden brown cytoplasm) are virtually all GM-CSF$^+$ (silver grains over cytoplasm). Hematoxylin counterstains nuclei of all cells purple. Examples of GM-CSF$^+$, UCHL-1$^+$ cells are labelled + and GM-CSF$^-$, UCHL-1$^-$ cells are labelled -.

No subject had a spontaneous immediate or late phase response to diluent inhalation challenge. The concentration of methacholine which caused a 20% decrease in FEV_1 was 1.46±2.0 mg/ml, a level characteristic of asthmatics who generally have a $PC_{20} < 8$ mg/ml (13, 14). The PD_{20} concentration of allergen was 13.6±13.8 inhalation units (range 2.5–37 inhalation units).

BAL cells. The lavage volume recovered (from 100 ml saline instilled) did not differ significantly in the pre-allergen (58.8±12.4 ml) versus post-allergen (46.4±12.6 ml) site. Local allergen challenge induced a significant increase in BAL eosinophils recovered 24 h post-allergen (37.0±15.1% of total BAL cells) from pre-allergen challenge levels (1.0±1.5% of total BAL cells) ($P = 0.0001$). In contrast, control diluent challenge did not induce a significant change in BAL eosinophils recovered 24 h post-diluent challenge (0.8±0.8%) compared to pre-diluent challenge levels (1.0±1.5%). Comparison of BAL total cell counts and differential cell counts obtained pre- and post-allergen showed a statistically significant increase in total cell count (6.0±3.3 × 10^6 cells versus 10.8±5.2 × 10^6 cells) ($P = 0.04$), and increases in the percent eosinophils (1.0±1.5% versus 37.0±15.1%) ($P = 0.0001$), neutrophils (0.3±0.2% versus 18.8±13.6%) ($P = 0.003$), but not lymphocytes (15.3±8.3% versus 13.5±2.2%). Although the percentage of alveolar macrophages was less in BAL fluid obtained post compared to pre-allergen challenge (31.3±11.9% versus 84.0±7.9% of total BAL cells) ($P = 0.00002$), the difference in the absolute numbers of alveolar macrophages pre- versus post-allergen challenge (4.9±2.9 × 10^6 cells versus 3.5±2.0 × 10^6 cells) ($P = 0.15$) was not significant.



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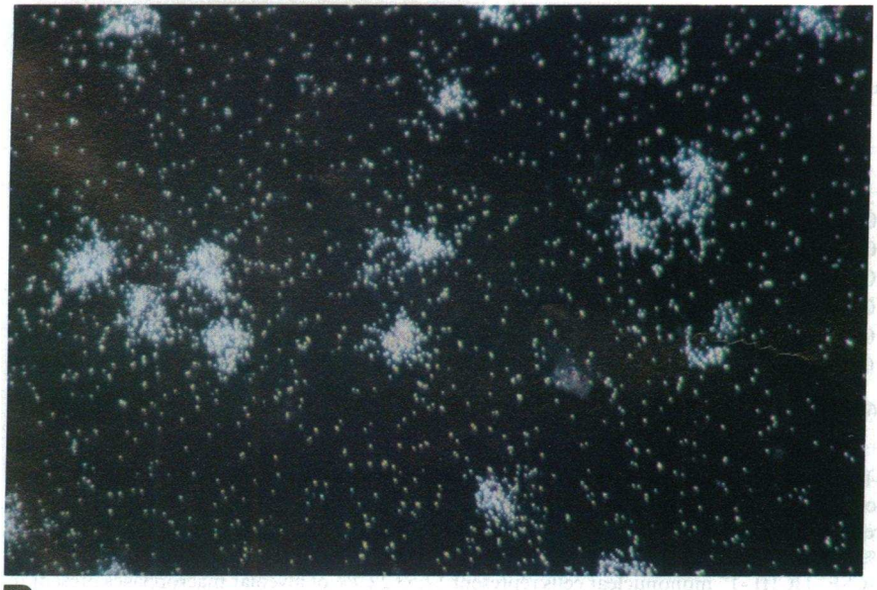

B

Figure 3. Post-allergen challenge BAL cells were hybridized with the GM-CSF antisense probe, and then stained for UCHL-1 using the immunoperoxidase method. (*A*) Light field and (*B*) dark field view of the same photographic field. The BAL cells in this figure are derived from study subject 3 (Table II) who had 12.2% GM-CSF$^+$ UCHL-1$^+$ and 12.1% GM-CSF$^+$ UCHL-1$^-$ cells. Hematoxylin counterstains only nuclei of immunoperoxidase negative cells. Examples of GM-CSF$^+$ UCHL-1$^+$ cells are labelled + and GM-CSF$^+$ UCHL-1$^-$ cells are labelled -. A GM-CSF$^-$ UCHL-1$^+$ mononuclear cell is indicated with a closed arrow, and a GM-CSF$^-$ UCHL-1$^-$ mononuclear cell with an open arrow.

GM-CSF immunoassay. Levels of GM-CSF in BAL fluid 24 h post-allergen challenge (180.5±46.9 pg/ml range 123.8–250.4 pg/ml) were significantly elevated compared to pre-allergen challenge levels (< 4 pg/ml) (P = 0.000002) (Table I). In contrast, there was no significant elevation of GM-CSF (< 4 pg/ml) in control airway sites challenged with diluent.

In situ hybridization (Table II). Pre-allergen challenge the GM-CSF antisense probe hybridized to 0.8±0.3% of BAL cells (Fig. 1), whereas 24 h post-allergen challenge 16.7±4.5% of BAL cells expressed GM-CSF mRNA (P = 0.00003) (Figs. 2 and 3). In both pre- and post-allergen challenge BAL cell populations, the control GM-CSF sense probe bound to < 0.1% of cells. In control airway sites challenged with diluent, there was no significant difference in the percent of GM-CSF mRNA positive cells pre- versus 24 h post-diluent challenge (< 1% versus < 1%).

To determine the identity of the BAL cells hybridizing to the GM-CSF antisense probe, two different approaches were used. In the first series of experiments, in situ hybridization was combined with immunoperoxidase staining for the T cell surface marker UCHL-1 (Fig. 2). Immunoperoxidase staining with UCHL-1 confirmed that the majority of unfractionated BAL cells that were GM-CSF positive by in situ hybridization were also UCHL-1 positive (Table II). The GM-CSF$^+$ UCHL-1$^+$ cells (12.5±2.2% of total BAL cells) represented 92.6±3.4% of lymphocytes identified by Wright-Giemsa stain. In contrast to the majority of GM-CSF$^+$ mononuclear cells that were UCHL-1$^+$ (77.3±13.4% of GM-CSF$^+$ cells were UCHL-1$^+$), a smaller population of GM-CSF$^+$ mononuclear cells were UCHL-1$^-$. These UCHL-1$^-$ cells exhibited the same degree of hybridization to the GM-CSF antisense probe as UCHL-1$^+$ cells.

In the second series of experiments, BAL cells were divided into adherent and nonadherent populations. The percentage of GM-CSF$^+$ UCHL-1$^-$ mononuclear cells were enriched by adherence (11.8±11.7% of adherent cells GM-CSF$^+$ UCHL-1$^-$ versus 4.2±3.9% of total BAL cells GM-CSF$^+$ UCHL-1$^-$). Several lines of evidence suggested that the GM-CSF$^+$ UCHL-1$^-$ mononuclear cells were alveolar macrophages. This included the adherence properties of the GM-CSF$^+$ UCHL-1$^-$ mononuclear cells, the limited number of mononuclear cell types present in BAL fluid (alveolar macrophages, lymphocytes, and mast cells), and the percentage of GM-CSF$^+$ UCHL-1$^-$ mononuclear cells. Since the percentage of GM-CSF$^+$ UCHL-1$^-$ mononuclear cells (4.2±3.9% of unfractionated BAL cells) exceeded the sum of either GM-CSF$^-$ lymphocytes (1.0% of unfractionated BAL cells) and mast cells (0.1% of unfractionated BAL cells), the alveolar macrophage would be the only other mononuclear cell present in sufficient numbers (31.3±11.9% of unfractionated post-allergen challenge BAL cells) to account for the GM-CSF$^+$ UCHL-1$^-$ mononuclear cells. In ^{35}S-labelled in situ hybridization slides counterstained with hematoxylin, the multilobed neutrophil nucleus is readily distinguished from the single lobed nucleus of mononuclear cells.

Discussion

GM-CSF, a cytokine produced by activated T cells (18) and alveolar macrophages (12), is an important regulator of eosinophil effector function and viability in vitro (7–8). To explore in vivo the potential for local airway production of GM-CSF to modulate recruited eosinophil effector function, we have used in situ hybridization and a GM-CSF immunoassay to determine whether GM-CSF is produced by airway cells in an experimental model of asthma. To mimic exposure to allergens normally present in the environment as closely as possible, the allergens we used in this study were chosen based both on symptoms reported by study subjects on natural exposure (i.e., exposure to cat, dog, or house dust), as well as confirmation by experimental inhalation allergen challenge.

The use of in situ hybridization and a GM-CSF immunoassay allowed us to confirm that GM-CSF mRNA is induced by allergen, and that GM-CSF is secreted into the airway in temporal proximity to recruited eosinophils. As picomolar concentrations of GM-CSF activate eosinophils in vitro (8) the concentrations of GM-CSF detected in the airway are sufficient to

*Table II. GM-CSF mRNA Expression by Asthma BAL Cells**

Study subject	Preallergen			Postallergen		
	Total of GM-CSF POS cells‡	UCHL-1§		Total of GM-CSF POS cells	UCHL-1	
		POS	NEG		POS	NEG
1	0.3%	0.3%	0.0%	17.9%	14.8%	3.1%
2	0.5%	0.4%	0.1%	12.5%	10.1%	2.4%
3	1.2%	0.9%	0.3%	24.3%	12.2%	12.1%
4	0.9%	0.7%	0.2%	11.9%	9.8%	2.1%
5	0.8%	0.6%	0.2%	17.9%	14.5%	3.5%
6	0.9%	0.9%	0.0%	15.8%	13.7%	2.1%
Mean±SEM	0.7±0.3%	0.6±0.3%	0.1±0.1%	16.7±4.5%	12.5±2.2%‖	4.2±13.9%¶

* The percentage of cells hybridizing to the ^{35}S-labelled GM-CSF antisense RNA probe are indicated in the table. Less than 0.1% of either UCHL-1 stained or unstained cells hybridized to the control ^{35}S-labelled RNA sense probe. ‡ The total number of GM-CSF$^+$ BAL cells = % GM-CSF$^+$ UCHL-1$^+$ + % GM-CSF$^+$ UCHL-1$^-$ cells for each study subject. § The percentages of UCHL-1$^+$ and UCHL-1$^-$ cells were determined by immunoperoxidase staining. ‖ The 12.5±2.2% of GM-CSF$^+$ UCHL-1$^+$ cells represent 92.6±3.4% of lymphocytes present in BAL fluid post-allergen challenge. ¶ The 4.2±13.9% of GM-CSF$^+$ UCHL-1$^-$ mononuclear cells represent 17.5±22.7% of alveolar macrophages present in BAL fluid post-allergen challenge.

influence eosinophil effector function and viability in vivo. If cytokines such as GM-CSF were able to enhance eosinophil viability in vivo, as previously shown in vitro (7, 8), the local generation of GM-CSF in the airway could theoretically prolong the duration of eosinophil effector function in asthma. As eosinophils contain proinflammatory mediators such as major basic protein in cytoplasmic granules and are able to generate leukotriene C4 and platelet activating factor (3), the enhanced viability and functional properties of GM-CSF primed eosinophils could prolong airway inflammation in patients with asthma. Using a combination of in situ hybridization, immunoperoxidase staining with UCHL-1 (Table II), and adherence properties, we have identified the lymphocyte and the alveolar macrophage as the cellular sources of GM-CSF in asthma. The lymphocyte (77.3±13.4% of GM-CSF$^+$ cells) appears to be the predominant, but not the sole source of GM-CSF (alveolar macrophage, 22.7±13.4% of GM-CSF$^+$ cells). The monoclonal antibody UCHL-1, which recognizes the 180-kD component of the cell surface CD 45 family (17), has been used to distinguish "naive" (UCHL-1$^-$) and "memory" (UCHL-1$^+$) T cells. The ability of mononuclear UCHL-1$^+$ non-adherent cells in asthmatic airway to produce GM-CSF in response to allergen, suggests that the UCHL-1$^+$ cells might be memory (defined as T cells which have previously been activated or "primed" by antigen). The cellular source of GM-CSF in asthma differs from other immune mediated diseases such as rheumatoid arthritis where macrophages, but not UCHL-1$^+$ T cells, are the source of GM-CSF (16). Whether detection of GM-CSF in the airway is important to the pathogenesis of asthma, or useful as an index of T cell/alveolar macrophage activation in asthma, will require further investigations using recombinant GM-CSF and neutralizing anti-GM-CSF antibodies in animal and human models of asthma.

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