Different profiles of T-cell IFN-γ and IL-12 in allergen-induced early and dual responders with asthma

Makoto Yoshida, MD, Richard M. Watson, BSc, Tracy Rerecich, BSc, and Paul M. O'Byrne, MB, FRCP(C) *Hamilton, Ontario, Canada*

Background: IFN-γ and IL-12 are anti-inflammatory cytokines released from various cells, including T cells. Allergen inhalation by atopic subjects with asthma results in 2 bronchoconstrictor phenotypes, termed *isolated early* and *dual responders*. Persistence of allergen-induced airway response and inflammation is a distinctive feature of dual responders.
Objective: To evaluate the roles of IFN-γ and IL-12 in resolving allergen-induced airway inflammation by comparing T lymphocytes ($CD4^+$ and $CD8^+$ cells) producing these cytokines in isolated early and dual responders.
Methods: Twenty-four subjects with asthma (12 isolated early and 12 dual responders) were challenged with inhaled allergen. Peripheral blood and induced sputum were taken before and 1 day, 3 days, and 7 days after challenge. Frequency of IFN-γ, IL-12, IL-4, and IL-13 producing $CD4^+$ and $CD8^+$ cells was assessed by using flow cytometry.
Results: After allergen, both $CD4^+$ and $CD8^+$ IFN-γ positive cells in peripheral blood significantly decreased in dual responders only, whereas $CD4^+$ and $CD8^+$ IFN-γ positive cells in induced sputum significantly increased in isolated early responders only. By contrast, IL-12 positive cells in peripheral blood significantly increased after allergen challenge only in isolated early responders. The ratio of $CD4^+$ and $CD8^+$ IL-4/IFN-γ positive cells in peripheral blood significantly decreased in isolated early responders by 3 days and had recovered by 7 days.
Conclusion: These results suggest that contrasting profiles of IFN-γ and IL-12 production may be responsible for different time courses of allergen-induced airway responses between isolated early and dual responders. (J Allergy Clin Immunol 2005;115:1004-9.)

Key words: *Asthma, allergen challenge, T_H1 and T_H2 cytokines*

Allergen inhalation provokes rapid bronchoconstriction in atopic subjects with asthma. This reaction occurs within half an hour, peaks within 2 hours after the exposure, and is called the *allergen-induced early asthmatic response*.[1] In some subjects with asthma, slowly progressive and persistent bronchoconstriction begins 3 to 7 hours after allergen inhalation,[1] the *allergen-induced late asthmatic response*. The late asthmatic response is associated with airway hyperresponsiveness and eosinophilic airway inflammation,[2] which can persist for as long as 1 week.

Abbreviations used
DR: Dual responder
IER: Isolated early responder

T_H lymphocytes help orchestrate allergen-induced airways inflammation via production of specific cytokines. Among these, T_H2 cytokines such as IL-4, IL-5, and IL-13 are thought to play a pivotal role.[3] There is evidence that T_H1 cytokines such as IFN-γ and IL-12 are capable of counteracting T_H2 responses and vice versa.[4-6] This has suggested that an imbalance between T_H1 and T_H2 lymphocytes and their respective cytokine production is thought to underlie allergic responses. Furthermore, we have recently reported that treatment with IFN-γ protein reversed ongoing allergic airway responses in IFN-γ–deficient mice.[7] Although less is known about IFN-γ and IL-12 in asthma, results from one study did not demonstrate any clinical benefit in subjects with severe asthma from treatment with IFN-γ,[8] whereas another could not demonstrate attenuation of allergen-induced late responses after treatment with IL-12.[9]

The objective of the current study was to measure T lymphocytes producing IFN-γ and IL-12 in peripheral blood and induced sputum from atopic subjects with asthma by flow cytometry before and after allergen inhalation and to compare the changes between isolated early responders (IERs) and dual responders (DRs) in relation to airway inflammation and airway responsiveness. We hypothesized that the profiles of IFN-γ and IL-12 would be different between IER and DR, which may relate to different time course of allergic responses in these groups of subjects with asthma. We also investigated the profiles of IL-4 and IL-13 as important T_H2 cytokines.

METHODS

Subjects

Twenty-four subjects with mild atopic asthma (12 DR and 12 IER) who fulfilled the American Thoracic Society criteria for asthma[10] were studied (see Table E1 in the Journal's Online Repository at www.mosby.com/jaci). All subjects gave signed consent before

From the Firestone Institute for Respiratory Health, Department of Medicine, McMaster University.
Supported by an operating grant from the Canadian Institutes for Health Research.
Received for publication November 3, 2004; revised February 1, 2005; accepted for publication February 7, 2005.
Available online March 23, 2005.
Reprint requests: Paul M. O'Byrne, MB, Department of Medicine, McMaster University, Health Sciences Centre, Room 3W10, 1200 Main St W, Hamilton, Ontario L8N 3Z5, Canada. E-mail: obyrnep@mcmaster.ca.
0091-6749/$30.00

doi:10.1016/j.jaci.2005.02.003

participating in the study, which was approved by the Ethics Committee of McMaster University Health Sciences Center. All were nonsmokers, and none had a respiratory infection for at least 4 weeks before the study. Subjects required only intermittent use of inhaled β_2-agonist, with baseline FEV_1 values >70% predicted. Medication was withheld for at least 8 hours before each study day. Atopic status was defined by positive skin prick test responses. Subjects were classified as DR if they developed both early and late asthmatic responses as defined by a greater than 15% drop in FEV_1 from baseline, or as IER if they developed only an early fall in FEV_1 greater than 15% from baseline.

Study design

Subjects attended the laboratory during 2 study periods. The first period was a screening period to document either an early or a dual airway response to inhaled allergen. The second study period lasted for 9 days. On the first day of this period, preallergen blood samples were obtained. This was followed by spirometry, methacholine inhalation challenge, and induced sputum. On the next day, subjects underwent an allergen challenge. Spirometry was measured until 7 hours after allergen inhalation to evaluate the early and late asthmatic responses. Sputum samples were obtained 7 hours after allergen. The third, fourth, and fifth visits to the laboratory occurred 24 hours, 3 days, and 7 days after allergen inhalation, respectively. At these visits, postallergen blood samples were obtained. This was followed by spirometry, methacholine inhalation challenge, and sputum sampling.

Methacholine inhalation challenge

Methacholine inhalation challenge was performed by using the method described by Cockcroft et al[11] (see Methods in the Online Repository at www.mosby.com/jaci). The concentration of methacholine required to achieve a decrease in FEV_1 of 20% (methacholine PC_{20}) was calculated through linear interpolation of percent fall in FEV_1 against the log-transformed methacholine concentration.

Allergen inhalation challenge

Allergen challenge was performed according to the method described by O'Byrne et al[1] (see Methods in the Online Repository at www.mosby.com/jaci). The early response was taken to be the largest percent fall in FEV_1 within 2 hours after allergen inhalation, and the late response was taken to be the largest percent fall in FEV_1 in the period beginning 3 hours and ending 7 hours after allergen inhalation.

Sputum analysis

Sputum was induced and processed according to the method described by Pizzichini et al[12] (see Methods in the Online Repository at www.mosby.com/jaci). Differential cell counts were obtained from the mean of 2 slides with 400 cells counted per slide stained with Diff-Quik (American Scientific Products, McGaw Park, Ill).

mAbs

All mAbs were purchased from PharMingen (San Diego, Calif). Phycoerythrin-conjugated antihuman IFN-γ mAb, B27 (mouse IgG_1), antihuman IL-4 mAb, 8D4-8 (mouse IgG_1), isotype control mouse IgG_1, antihuman IL-12 (p70) mAb, 20C2 (rat IgG_1) antihuman IL-13 mAb, and isotype control rat IgG_1 were used for the study. In nonpermeabilized cells, positive staining for IFN-γ decreased to 20% of the paired sample with permeabilization, showing that approximately 80% of the positive signal was of intracellular origin. Fluorescein isothiocyanate–conjugated anti-CD4 mAb, RPA-T4 (mouse IgG_1, κ), and CyChrome (BD Biosciences, Mississauga, Ontario, Canada)–conjugated anti-CD8 mAb, RPA-T8 (mouse IgG_1, κ) were used for identifying each T-cell subset. Percentages of $CD4^+$ and $CD8^+$ positive lymphocytes in PBMC was not altered by treatment with 0.1% dithiothreitol compared with control PBMC without treatment (data not shown).

Cell cultures

Isolation and cultures of both peripheral blood mononuclear and sputum cells were performed as described (see Methods in the Online Repository at www.mosby.com/jaci).

Staining

Cells were washed in simple PBS and resuspended at a density of 1×10^6 cells in 100 μL PBS with 0.1% sodium azide and 5% normal mouse serum (Sigma, St Louis, Mo), incubated for 10 minutes, followed by surface staining with anti-CD4 mAb and anti-CD8 mAb for 30 minutes in the dark. For intracellular cytokine staining, the cells were washed once in simple PBS and then fixed in 100 μL fixation buffer (Caltag, Burlingame, Calif) containing 4% paraformaldehyde for 20 minutes. After an additional wash with simple PBS, cells were resuspended in 100 μL permeabilization buffer containing 0.1% saponin (Caltag) with 5% normal mouse serum or normal rat serum (Sigma) for 10 minutes, and then added with anti–IFN-γ mAb, anti–IL-4 mAb, isotype control mouse IgG_1, anti–IL-12 mAb, anti–IL-13 mAb, or isotype control rat IgG_1 for 30 minutes in the dark. After a final wash in simple PBS, cells were resuspended in PBS with 1% paraformaldehyde and kept in the dark at 4°C until flow-cytometric evaluation.

Flow cytometric analysis

A FACScan flow cytometer (Becton Dickinson, San Jose, Calif) equipped with a 15-mA argon ion laser and filter settings for fluorescein isothiocyanate (530 nm; FL-1), phycoerythrin (585 nm; FL-2), and CyChrome (650 nm; FL-3) was used. The detailed methods are described in the Online Repository (www.mosby.com/jaci). The frequency of true-positive cells was obtained by subtracting the value of isotype control from the value of sample stained with anti–IFN-γ, anti–IL-4, anti–IL-12, or anti–IL-13 mAb (see Fig E1 in the Online Repository at www.mosby.com/jaci).

Statistical analysis

Statistica software, version 5 (StatSoft, Inc, Tulsa, Okla), was used to analyze the data. Data were expressed as the means ± SEMs. PC_{20} methacholine measurements were log_2-transformed to normalize the data and are reported as geometric means and geometric SEM. Comparisons between IER and DR were made by using 2-factor repeated-measures ANOVA to analyze the effect of the 2 independent variables, types of asthmatic responses, and time on the outcome variables. Appropriate post hoc testing was performed by using the Duncan test to assess for significant effects while controlling for multiple comparisons. All comparisons were 2-tailed, and P values <.05 were considered significant.

RESULTS

Bronchoconstrictor responses

The mean maximal percent falls in FEV_1 during the early response were 27.9% ± 7.1% in IER and 29.3% ± 6.7% in DR ($P = .683$). The maximal percent falls in FEV_1 during the late response were 7.1% ± 5.0 in IER and 22.7% ± 6.5% in DR ($P < .01$; Fig 1, *A*).

Airway hyperresponsiveness

Methacholine airway hyperresponsiveness developed in both DR and IER 24 hours after allergen challenge.