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# Allergen-stimulated interleukin-4 and interferon-γ production in primary culture: responses of subjects with allergic rhinitis and normal controls

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## SUMMARY

The balance of interleukin-4 (IL-4) to interferon-γ (IFN-γ) production that is induced following exposure to common environmental antigens is believed to be instrumental in determining whether hypersensitivity or clinical unresponsiveness results to that antigen. To date, evaluation of cytokine (protein) production has been based predominately on allergen-reactive CD4 T-cell clones or activation of fresh unselected peripheral blood mononuclear cell (PBMC) populations with non-physiological stimuli such as phorbal myristate acetate (PMA) and calcium ionophore, phytohaemagglutinin (PHA), anti-CD3 or anti-CD2/anti-CD28 monoclonal antibodies (mAb). Here, ultrasensitive IL-4 and IFN-γ assays were optimized to allow direct analysis of antigen-stimulated cytokine production by fresh human PBMC. Primary cultures of cells from grass pollen-sensitive allergic rhinitis subjects and non-atopic controls were stimulated using a range of grass pollen allergen concentrations in the absence of exogenous cytokines or polyclonal activators. The majority of subjects (45 of 52) exhibited chloroquine-sensitive, CD4-dependent cytokine production in allergen-stimulated, short-term primary culture. Median IL-4 production was substantially greater among atopics (13·0 pg/ml versus < 1 pg/ml, Mann–Whitney *U* test, $P < 0{\cdot}000001$) and IFN-γ was lower ($P = 0{\cdot}008$), providing direct evidence for an imbalance in both IL-4 and IFN-γ production among circulating, pollen-reactive cells in individuals with seasonal allergic rhinitis. The distinction in the allergen-driven cytokine responses elicited from normal and atopic donors was underscored by examination of the ratios of IFN-γ : IL-4 synthesis. Non-atopic individuals exhibited intense IFN-γ dominance of the T-cell response, in marked contrast to that observed among grass pollen-sensitive individuals (median IFN-γ : IL-4 ratios of 14·0 versus 0·096, $P = 0{\cdot}000002$). The observation that essentially all individuals produced IFN-γ (± IL-4) following antigen stimulation *in vitro* argues that the most relevant consideration in determining susceptibility to immediate hypersensitivity versus clinical tolerance to environmental allergens is not a genetically defined capacity to recognize the antigen (i.e. if allergen-reactive T cells are present in that individual) but the nature of the cytokine response.

## INTRODUCTION

Since the demonstration that interleukin-4 (IL-4) and interferon-γ (IFN-γ) reciprocally regulate polyclonally stimulated murine IgE production *in vitro*,[1] an intense effort has been devoted to determining if human immediate hypersensitivity is associated with an imbalanced production of these cytokines (reviewed in refs 2–4). Direct analysis of human cytokine protein production by fresh peripheral blood mononuclear cells (PBMC) has been a difficult challenge because the levels of IL-4 and IFN-γ produced following antigen-specific stimulation in primary culture have proven to be undetectable. Consequently, research has focused on derivation and characterization of T-cell lines and clones,[4–7] polyclonal activation of fresh PBMC populations[8,9] and analysis of cytokine mRNA levels.[9–11]

Data from these three approaches have led to the current consensus that allergic diseases such as immediate hypersensitivity, and broadly related disorders like atopic dermatitis and hyperIgE syndrome, are associated with expression of inappropriate, T-helper type-2 (Th2)-like, cytokine responses. However, careful examination of the literature reveals conflicting conclusions as to the nature of the specific defect in cytokine synthesis. In many studies, IFN-γ production was reported to be essentially normal in atopic subjects,[12–15] whereas in others it was markedly reduced[16–19] or elevated.[20,21] IL-4 production was usually,[4,12,18,22] but not always,[23] reported as substantially higher. In some studies, neither IL-4 nor IFN-γ production was different,[15,23] whereas in others synthesis of both cytokines was affected, with IFN-γ

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inhibited and IL-4 elevated[18,19] relative to the responses elicited in normal controls. Some of these discrepancies may reflect different aetiologies for related but distinct hypersensitivity states (i.e. atopic dermatitis versus allergic rhinitis). However, the variation frequently observed by different groups studying the same disease may also stem from the fact that many studies have been carried out with very small numbers of subjects, that data obtained from low numbers of T-cell clones derived from any given individual were extrapolated to the entire allergen-specific T-cell repertoire, and that potentially unphysiological stimuli such as phorbal myristate acetate (PMA) plus ionomycin were used as surrogates for major histocompatibility complex (MHC)-restricted, antigen-driven activation of cytokine production. Analysis of cytokine protein production by fresh, unselected cell populations in response to physiological stimulation (i.e. antigen) remains an important goal.

Here, we examined IL-4 and IFN-$\gamma$ synthesis by mononuclear cell populations freshly derived from peripheral blood. Twenty-six grass pollen-sensitive, allergic rhinitis subjects and an equal number of normal, non-atopic controls were studied. Ultrasensitive IL-4 and IFN-$\gamma$ assays allowed examination of cytokine production following short-term, antigen-driven stimulation. These primary cultures of 2–6 days duration were used to evaluate (1) the proportion of atopic and normal subjects capable of producing detectable levels of IL-4 and/or IFN-$\gamma$ in response to these common environmental antigens, and (2) the intensity of their cytokine responses to physiological stimulation.

The data obtained provide direct evidence for an imbalance in both IL-4 and IFN-$\gamma$ production by circulating, pollen-reactive cells in individuals with seasonal allergic rhinitis. Strikingly, 19 of 26 clinically tolerant, 'normal' subjects also demonstrated cytokine responses following short-term stimulation with these antigens. Cytokine production was CD4 T-cell dependent and chloroquine sensitive.

## MATERIALS AND METHODS

*Patients and clinical evaluation*

This study, approved by the University of Manitoba Faculty Committee on the Use of Human Subjects in Research (Winnipeg, Canada), was conducted with 26 grass pollen-allergic subjects (nine female), and 26 normal non-atopic control subjects (13 female) as controls. All participants were between 18 and 35 years of age. Allergic subjects were recruited based on (1) a history of seasonal allergic rhinitis of at least 2 years duration, severe enough to warrant regular daily treatment with a topical corticosteroid and/or histamine receptor antagonist, and (2) positive epicutaneous tests (wheal diameter at least 4 mm greater than negative control) to the grass pollen mix used for *in vivo* and *in vitro* testing (Grass mix 1649, consisting of June/Kentucky Blue grass, Timothy grass, Orchard grass, Brome grass and Red-top grass; Hollister-Stier/Miles, Toronto, Ontario, Canada). Normal subjects had no history of allergic rhinitis, asthma or other allergic disorders, and exhibited negative epicutaneous tests to the grass pollen mixture used in this study. Blood was collected within 90 days of the peak of the local grass pollen season.

*Cell and antigen preparation*

Freshly obtained whole blood (20 ml/subject) was diluted with 1 ml of sterile 2·7% EDTA immediately upon collection. PBMC were obtained by centrifugation with Histopaque-1077 (Sigma Chemical Co., St Louis, MO), carried out within 30 min of blood collection. Cells collected from the interface were washed twice in serum-free phosphate-buffered saline (PBS), counted (>99% viability as determined by trypan blue exclusion) and used for bulk culture as described below.

*Primary bulk culture*

Cells were cultured at $1{\cdot}5 \times 10^6$/ml (2 ml/well) in 24-well plates (Corning Science Products, Rochester, NY) using RPMI-1640 supplemented with 10% fetal calf serum, 10 mM L-glutamine, $2 \times 10^{-5}$ M 2-mercaptoethanol (2-ME), and antibiotic antimycotic (Gibco BRL, Burlington, Ontario, Canada) (complete medium). Following initial optimization of antigen concentration and culture duration, three short-term cultures were set up in duplicate for each subject in the absence and presence (200 and 2000 $\mu$g/ml) of grass pollen extract. The same allergen extract was used for skin testing and for *in vitro* culture. Culture supernatants were harvested at three to four time-points/subject between 2 and 6 days and stored at −20° until analysed for IL-4 and IFN-$\gamma$. The peak cytokine production elicited in response to antigenic stimulation was reported for each subject.

*Effects of chloroquine on antigen presentation*

Two approaches were used to determine the chloroquine sensitivity of grass pollen-stimulated cytokine production. In the first, $3{\cdot}3 \times 10^5$ PBMC/well were added to 96-well round bottom plates and incubated at 37° for 30 min. Chloroquine (Sigma) in RPMI solution was added to yield a final concentration of 0, 20 or 100 $\mu$M. After 30 min further incubation, 50 $\mu$l of grass pollen antigen (to yield a final concentration of 1000 $\mu$g/ml), or medium alone, was added to each well. Following 3 hr incubation at 37°, the plates were washed three times with complete medium to remove chloroquine and antigen. The cells were then cultured for 5 days in medium alone or, as a control, fresh antigen. Supernatants were harvested at 3, 4 and 5 days. IFN-$\gamma$ levels were determined by enzyme-linked immunosorbent assay (ELISA). In the second approach, PBMC were plated and chloroquine was added at the indicated concentrations, followed 30 min later by antigen (1000 $\mu$g/ml). These cultures were maintained at 37° without washing to remove chloroquine or antigen. Supernatants were harvested and analysed at 3, 4 and 5 days.

*Cytokine assays*

*IL-4.* The CT.h4S cell line [provided by Dr W. Paul, NIAID, National Institutes of Health (NIH), Bethesda, MD] was used in a MTT assay, modified essentially as described previously[24] for CT.4S. Under the conditions used, this assay detected 0·25–1·0 pg/ml IL-4, quantifying amounts above 1–2 pg/ml (see the Results). All samples were evaluated at least twice, with the concentration of cytokine in each supernatant calculated from a minimum of three points falling on the linear portion of an internal standard generated with recombinant human (rh)IL-4 (specific activity $1{\cdot}8 \times 10^7$ U/mg; Sterling Winthrop Inc., Collegeville, PA), serially diluted on each plate. Assay specificity was assessed by analysis of replicate supernatants in the presence of rabbit anti-human IL-4 neutralizing antibody (40 ng/ml; Sterling Winthrop). CT.h4S did not respond to concentrations of rhIL-2 below 666 pg/ml

(our unpublished data), IL-1, IL-3, IL-5, IL-6, tumour necrosis factor-α (TNF-α), granulyte-macrophage colony-stimulating factor (GM-CSF) nor IFN-γ.[25] Standard deviations for this assay were < 5%, frequently 1–3%.

*IFN-γ*. A sandwich ELISA was developed using purified polyclonal rabbit anti-human IFN-γ as capture reagent, and a purified, biotinylated monoclonal antibody (mAb) cocktail (MIF 3009 and MIF3125, recognizing spatially distinct epitopes, generated by Dr F. Jay) in combination with streptavidin–alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA). Internal standards of an IFN-γ-containing, phytohaemagglutinin (PHA)-stimulated (Difco, Detroit, MI) PBMC supernatant, calibrated against hIFN-γ reference reagent Gg 23-901-530 (1 NIH unit = 115 pg; provided by Dr C. Laughlin, NIAID, NIH, Bethesda, MD), were included in each ELISA. The lower limit of detection was typically 0·08–0·15 U/ml, with quantitative measurement of IFN-γ beginning from 0·15 to 0.30 U/ml (see the Results).

*Statistical analysis*

Statistical significance was determined using the Mann–Whitney *U*-test.

## RESULTS

### Assay sensitivity

The major problem in previous attempts to quantify cytokine production by fresh PBMC has been assay insensitivity. Most IL-4 assays have been capable of detecting the presence of this cytokine only at rather high concentrations (> 25 to > 100 pg/ml),[26–30] with accurate quantification requiring at least twofold higher levels. This has led to strong reliance on derivation of T-cell clones or, alternatively, stimulation of PBMC in primary culture with polyclonal activators such as PHA, immobilized anti-CD3 or PMA and $Ca^{2+}$ ionophore. We therefore optimized assays developed for detection of IL-4 and IFN-γ protein. These assays readily enabled us to quantify cytokine in culture supernatants at levels above 1 pg/ml IL-4 (Fig. 1) and 0·15 U/ml IFN-γ (Fig. 2). They provided exquisitely sensitive detection systems, permitting analysis of cytokine protein production by allergen-stimulated, normal cell populations in response to short-term stimulation. The low standard deviations observed (typically 2–3%) were particularly useful in differentiating positive and negative cultures at levels of IL-4 synthesis that would be undetectable in most commercially available ELISA.

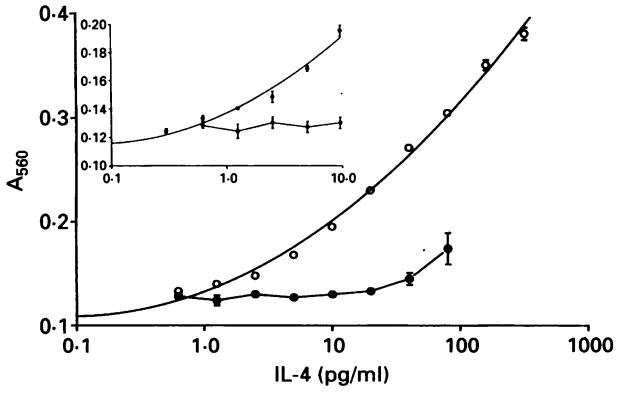


**Figure 1.** Sensitivity of the MTT-based CT.h4S bioassay for IL-4. CT.h4S (5000/well, in triplicate) was cultured with rhIL-4 at the indicated concentrations (open symbols) prior to MTT addition, as described in the Materials and Methods. Assay specificity was determined by parallel analysis in the presence of a neutralizing anti-IL-4 at 40 ng/ml (closed symbols). Data shown are mean absorbances (560 nm–690 nm) ± SD. As used, this assay detected 0·25–1·0 pg/ml rIL-4, quantifying amounts above 1–2 pg/ml. (Inset shows absorbance.)

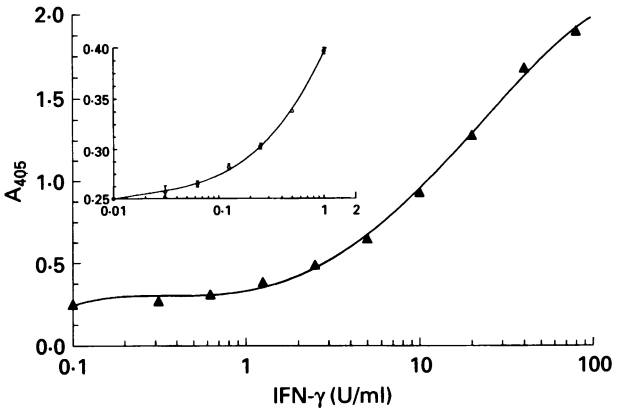


**Figure 2.** Sensitivity of IFN-γ ELISA. Purified polyclonal rabbit anti-human IFN-γ was used in combination with a biotinylated mAb cocktail in a streptavidin–alkaline phosphatase-based ELISA. Data shown are means ± SD. The detection limit (background + 3 SD) was typically < 0·1 U/ml, with quantitative measurement of IFN-γ at concentrations ≥ 0·2 U/ml. (Inset shows absorbance.)

### Detection and analysis of IL-4 protein synthesis in antigen-stimulated primary culture

Using the conditions described above, we evaluated the capacity of fresh PBMC from 26 grass pollen-allergic subjects and 26 normal non-atopic controls to respond to allergen-specific *in vitro* stimulation. For each subject, duplicate cultures were established using 0, 200 and 2000 μg/ml grass pollen allergen. No exogenous cytokines or polyclonal activators were added. Aliquots of culture supernatant were harvested at 24-hr intervals between 2 and 6 days. IL-4 and IFN-γ levels were determined for each time-point and antigen concentration, for each individual.

### Allergen-stimulated IL-4 production was readily detected

The data in Fig. 3a,b demonstrated that fresh PBMC from all but one atopic subject produced IL-4 in response to short-term grass pollen stimulation. Peak IL-4 production, which varied somewhat between individual subjects as to the optimal antigen concentration or duration of culture, is shown. In contrast, cultures from 24 of 26 non-allergic subjects failed to produce detectable IL-4 in response to antigen. Median IL-4 production was 13·0 pg/ml among atopic individuals, whereas that among normal subjects was < 1 pg/ml (Mann–Whitney *U* test, $P < 0{\cdot}000001$). In the absence of antigen stimulation, cultures of atopic subjects produced IL-4 at or below 2 pg/ml. All of the normal controls failed to make detectable levels of IL-4 (data not shown).

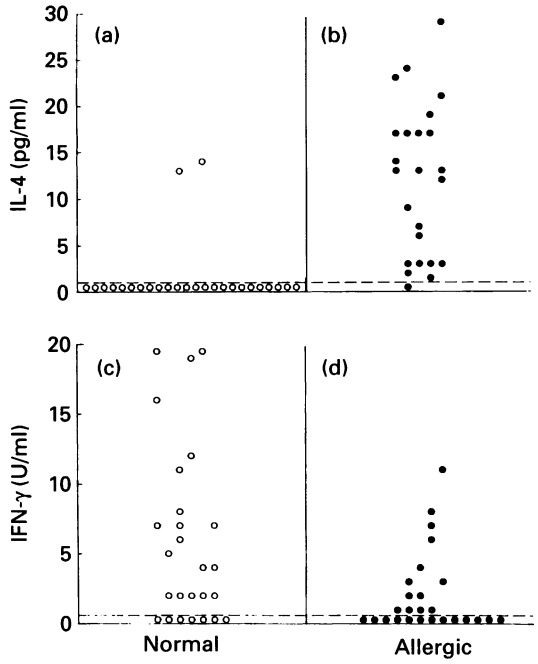


**Figure 3.** Subjects with allergic rhinitis exhibited elevated IL-4 ($P < 0{\cdot}000001$; Mann–Whitney *U*-test) and decreased IFN-γ ($P = 0{\cdot}008$) responses in antigen-driven, short-term bulk culture. PBMC from grass pollen-allergic (closed symbols) and non-atopic (open symbols) volunteers were cultured for 2–6 days as described in the Materials and Methods. IL-4 (a,b) and IFN-γ (c,d) production in culture supernatants was determined as described in Figs 1 and 2. Data shown represent peak mean cytokine concentrations observed, typically at days 3–5. Dotted lines indicate assay sensitivity limits.

### Allergen-stimulated IFN-γ production

In contrast to the data above, where responsiveness was virtually confined to the atopic population, the majority of primary cultures established from both normal and atopic subjects responded to grass pollen stimulation with IFN-γ production. Nineteen of 26 non-allergic subjects, and 13 of 26 allergic subjects, exhibited detectable levels of IFN-γ in bulk culture. Median IFN-γ production was approximately three-fold more intense in normal subjects than in subjects with allergic rhinitis (Fig. 3c,d; Mann–Whitney, $P = 0{\cdot}008$). In the absence of antigen stimulation, IFN-γ production in culture supernatants was undetectable or just above the limits of detection in both normal and atopic groups (Tables 1 and 2).

For each of the subjects examined, antigen-stimulated IL-4 and IFN-γ levels had declined by day 6 of culture. No clear difference emerged between normal and atopic subjects in terms of the kinetics of production of either of the cytokines studied (our unpublished data). Antigen concentrations between 200 and 2000 μg/ml led to similar, optimal stimulation of IL-4 and IFN-γ, whereas concentrations substantially above or below these elicited weaker cytokine levels.[31] For each of the three cytokines examined, cultures set up in the absence of antigen, or stimulated with an irrelevant antigen (normal rat immunoglobulin at 50 and 500 μg/ml), led to very low or undetectable levels of cytokine synthesis (Table 2 and data not shown).

### Grass pollen allergen-stimulated cytokine production is CD4 dependent and chloroquine sensitive

Most grass pollen-allergic subjects exhibit multiple sensitivities, an observation that led us to utilize the mixture of pollen allergens used in this study. However, given the high frequency of responsiveness that was found, we considered the possibility that these responses were not truly antigen-driven but instead reflected mitogenic stimulation. Three independent approaches were used to determine if grass pollen-stimulated cytokine production was due to polyclonal or antigen-driven T-cell activation.

In the first, the presence of putative mitogenic components in the antigen preparation used in this study was evaluated by culturing spleen cells of naive C57Bl/6 mice for 10 days with grass pollen (5, 50, 500, 2500 and 5000 μg/ml) or, as a positive control, concanavalin A (Con A; 5 μg/ml). Murine IL-4 and IFN-γ synthesis was evaluated in culture supernatants obtained at 24-hr intervals over the culture period. Although Con A stimulated strong synthesis of both cytokines, grass pollen failed to stimulated cytokine production or proliferation (data not shown).

**Table 1.** Grass pollen-stimulated IFN-γ production is CD4 dependent

| | | IFN-γ (U/ml)† | | | |
|---|---|---|---|---|---|
| Subject | Stimulus* | None | Grass pollen | Grass pollen, OKT4 | Grass pollen, normal mouse IgG |
| Non-atopic | | <0·3 | 16·9 | 1·3 | 19·0 |
| Non-atopic | | 0·35 | 20·4 | 0·5 | 14·8 |
| Grass pollen sensitive | | <0·15 | 3·1 | <0·15 | 5·3 |
| Grass pollen sensitive | | <0·15 | 7·4 | 0·85 | 7·2 |

* Primary cultures from two normal and two atopic subjects were set up at $1{\cdot}5 \times 10^6$ PBMC/ml ± grass pollen extract (1000 μg/ml) in the presence and absence of fourfold concentrated OKT4 culture supernatant or purified normal mouse IgG (50 μg/ml).

† Peak IFN-γ production in supernatants harvested after 4–5 days of culture by ELISA. SEM were < 10% in most instances.

**Table 2.** Grass pollen antigen-stimulated IFN-γ synthesis is chloroquine sensitive

| | Approach 1* IFN-γ production following antigen/chloroquine pulse: | | | | |
|---|---|---|---|---|---|
| | Chloroquine pulse | | Antigen pulse | Antigen in 5-day culture | |
| | 20 μM | 100 μM | (1000 μg/ml) | None | Grass pollen |
| | – | – | + | 28·8 (± 3·1) | 32·2 (± 2·6) |
| | + | – | + | 5·8 (± 1·2) | 11·5 (± 1·0) |
| | – | + | + | 1·1 (± 0·56) | 20·3 (± 1·8) |
| | Approach 2† IFN-γ production (U/ml) following continuous culture with | | | | |
| Subject | No Antigen | Antigen | Antigen + 20 μM chloroquine | Antigen + 100 μM chloroquine | |
| N15 | <0·15 | 31·6 (±1·5) | 7·5 (±1·5) | <0·15 | |
| N16 | <0·15 | 1·0 (±0·09) | <0·15 | <0·15 | |
| N29 | <0·15 | 17·1 (±2·5) | 0·23 (±0·08) | <0·15 | |
| N31 | <0·15 | 2·2 (±0·3) | <0·15 | <0·15 | |

* PBMC were chloroquine/antigen pulsed as described in the Materials and Methods, washed and then cultured (1) in fresh antigen-free medium or (2) in the presence of additional grass pollen extract (1000 μg/ml) for 5 days. IFN-γ production in the resulting supernatants was determined by ELISA. Unstimulated cultures produced <0·15 U/ml IFN-γ (data not shown). Data are shown as mean (± SEM) from one of two subjects examined.

† Fresh PBMC were cultured alone or with grass pollen antigen for 4–5 days in the presence of chloroquine at 0, 20 and 100 μM. Supernatants were evaluated for IFN-γ production as described above. Data shown, mean (± SEM), are from four of five subjects examined.

In the second approach, the ability of anti-CD4 mAb to inhibit cytokine synthesis was evaluated by the addition of fourfold concentrated OKT4 tissue culture supernatant, or irrelevant antibody (purified normal mouse IgG), to human PBMC cultures set up as described above. As seen in Table 1 for IFN-γ synthesis, addition of this mAb at the initiation of culture largely abolished grass pollen-stimulated cytokine production in both atopic and non-atopic groups, indicating the CD4 T-cell dependence of grass pollen-stimulated cytokine production.

Finally, antigen-stimulated cultures were established in the presence and absence of chloroquine directly to examine the requirement for antigen processing by the exogenous pathway in this system. Most applications of chloroquine in studies of antigen processing have evaluated the capacity of antigen ± chloroquine-pulsed antigen-presenting cells to stimulate T hybridomas or clones in 18–24-hr assays. However, the neutralizing effects of this weak base on the acidic endosomal/lysosomal system utilized in processing of exogenous antigens were transient, potentially complicating analysis of cytokine production in 5-day cultures. We therefore evaluated the capacity of (1) primary PBMC cultures pulsed with antigen ± chloroquine, as well as (2) cultures carried out in the continuous presence of antigen and chloroquine, to generate cytokine responses.

As shown in Table 2, a 3-hr pulse of cultures with grass pollen antigens, followed by extensive washing and 5-day culture in antigen-free medium, led to IFN-γ production. This cytokine production was strongly inhibited by the presence of chloroquine during antigen pulsing, demonstrating the chloroquine sensitivity of antigen processing for these grass pollen antigens. Subsequent addition of fresh antigen to parallel cultures stimulated vigorous cytokine synthesis, demonstrating that the capacity of these PBMC to respond to antigen was not permanently inhibited (i.e. due to a non-specific decrease in viability) by chloroquine treatment.

In the second approach used in these experiments, chloroquine was added to PBMC cultures along with antigen for the duration of culture. In each of the five subjects studied, chloroquine abrogated the capacity of PBMC to respond to grass pollen stimulation. Collectively, the data are consistent with the cytokine production observed in this study being attributable to conventional antigen-specific, MHC class II-dependent T-cell activation.

### Altered ratios of IFN-γ : IL-4 synthesis in normal and atopic subjects

The relative balance of IL-4 and IFN-γ production is likely to be more important than the absolute amounts of either cytokine in determining hypersensitivity versus clinical unresponsiveness.[3,4] The ratio of grass pollen-stimulated IFN-γ : IL-4 synthesis was determined individually for each subject. Comparison of these ratios revealed a pronounced shift towards increased Th2-like activity in the circulating immune repertoire of grass pollen-sensitive individuals (Fig. 4, $P = 0{\cdot}000002$). The IFN-γ response to grass pollen was dominant in most normal subjects studied, whereas a reciprocal situation existed among the atopic subjects. As such, these ratios reflected the fact that production of both IL-4 and IFN-γ differed between normal and atopic subjects.

## DISCUSSION

Ultrasensitive assays of IL-4 and IFN-γ production were used to examine antigen-stimulated cytokine production by fresh unselected cell populations directly *ex vivo*. Markedly more intense IL-4, and weaker IFN-γ, production was found in the

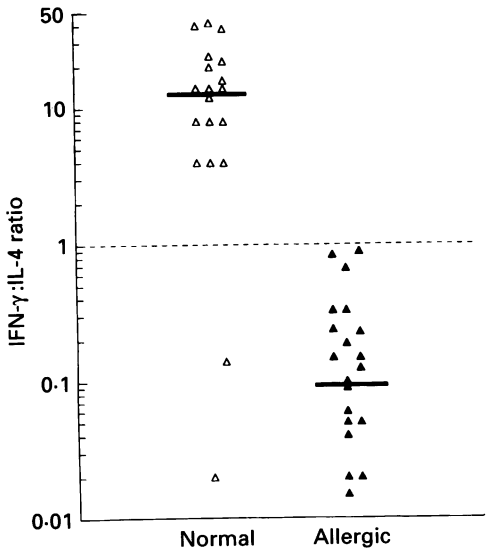


**Figure 4.** Fresh PBMC of allergic individuals exhibited markedly decreased ratios of IFN-γ : IL-4 production in response to grass pollen stimulation. For each subject, the ratio of IFN-γ : IL-4 production was calculated based on the maximum production of each cytokine observed in culture. Individuals for whom both IL-4 and IFN-γ cytokine production was undetectable were not included. A ratio of 1·0 (dashed lined) reflects balanced IFN-γ : IL-4 production. The median IFN-γ : IL-4 ratio for non-atopic subjects was 13·0, and for atopics 0·096. This difference has a *P*-value of 0·000002 by the Mann–Whitney *U*-test.

response of allergic rhinitis subjects, as measured both by the frequency of individuals making IL-4 or IFN-γ, and by the intensity of their cytokine synthesis. Notably, approximately three-quarters (19 of 26) normal non-atopic subjects also demonstrated cytokine production to these environmental antigens.

Studies using T-cell clones derived from normal and allergic subjects provided the first substantive evidence for an association between immediate hypersensitivity[5–7] or hyper IgE syndrome[8] and increases in IL-4 and/or decreases in IFN-γ synthesis. However, a recognized limitation of T-cell cloning as a tool to quantitatively infer cytokine production *in vivo* is that cloning is subject to numerous extrinsic factors that strongly influence the frequency and function of the clones obtained.[32–36] Exogenously added—or endogenously produced—cytokines represent an uncontrolled variable that can profoundly skew the composition of the panel of clones obtained. In addition, even large panels of clones (i.e. almost invariably one to five per subject, in exceptional studies[28] up to 20 allergen-specific clones from one individual) represent an extremely small proportion of the total allergen-specific T-cell repertoire *in vivo*. Taken with the growing appreciation that the Th1/Th2 paradigm reflects the extremes of the spectrum of helper T-cell activation, best observed in long-term T-cell clones, there is considerable interest in evaluation of cytokine synthesis by fresh unselected cell populations.

Consequently, many investigators have examined cytokine (protein) production by unselected, fresh mononuclear cell populations directly *ex vivo*. However, the low cytokine concentrations observed following antigen-specific restimulation and the poor sensitivity of most hIL-4 assays has led to widespread adoption of polyclonal activation as a means of T-cell activation.

Use of polyclonal activators as surrogate antigens in analysis of cytokine gene expression is problematic. The data obtained must be interpreted very cautiously, because such stimulation (1) triggers virtually all T cells rather than the allergen-specific population, which is likely to comprise a very small percentage of total T cells, and (2) represents an extremely forceful activation signal. Mitogen-driven patterns of cytokine gene expression may differ from those obtained in antigen-mediated, MHC-restricted activation.[9,37–39] Thus, this strategy risks the possibility that the conclusions drawn may not reflect cytokine responses elicited *in vivo* by antigenic stimulation.

A more conservative approach has been to utilize bronchoalveolar lavage or biopsy tissue to demonstrate altered patterns of cytokine mRNA production in asthmatic individuals[10,11] or those with ongoing infectious or parasitic diseases.[4,40–42] Although numerous precedents for translational regulation of cytokine gene expression have demonstrated that mRNA synthesis cannot necessarily be taken as tantamount to production of biologically active cytokine,[43–45] this approach provides persuasive evidence of a strong association between defined patterns of cytokine gene expression by normal cells and ongoing allergic or infectious diseases.

Clearly, cytokine production needs to be evaluated by multiple approaches since each of the currently available methods for quantification is hampered by various drawbacks. Selection of an 'optimal approach' requires recognition of the confounding factors that complicate interpretation of data from each approach and the use of independent alternatives. Thus it should be recognized that the cytokine protein levels measured in allergen-stimulated bulk culture used here reflect net IL-4 or IFN-γ production. The values obtained will be influenced by consumption of cytokine and cross-regulation of cytokine gene expression among the responding cells. Use of very short-term cultures, and daily monitoring of cytokine levels in supernatants, help to minimize these considerations, but we are well aware that the values obtained reflect minimal estimates. Use of antigen-stimulation and short-term cultures minimizes the impact of selective influences on which T-cell populations survive and which are activated. However, it should be recognized that the possibility of selective T-cell differentiation or activation cannot be ruled out for any technique utilizing *in vitro* culture.

Proliferation was not examined in this study. Several previous investigators examined PBMC proliferation in response to allergen stimulation.[46–48] Most, although not all,[49] were unable to identify a difference between allergic and normal subjects in the proliferative response to allergen stimulation. Moreover, although cytokine production and proliferation may correlate and have frequently been used together as indices of T-cell activation, numerous examples exist in which strong cytokine production is observed in the absence of proliferation to a given antigen.[50,51]

Immune responses in normal non-atopic individuals chronically exposed to environmental antigens have been reported in previous studies.[46–48,52] These reports centred on antibody production or T-cell proliferation. To our knowledge, qualitative evaluation of the cytokine responses of non-atopic

subjects to environmental antigens has not been carried out previously. Although the dominant cytokine response differed qualitatively between normal and grass pollen allergic individuals in this study, the vast majority of the 52 subjects demonstrated IFN-γ and/or IL-4 production following restimulation. Consequently, an important concern was whether the cytokine production elicited using these common environmental allergens reflected polyclonal or antigen-driven T-cell activation. Given that the intensity and kinetics of these responses fell in the same range as those normally elicited by antigen, rather than of known polyclonal activators (PHA, soluble or immobilized anti-CD3, usually some 10–100 fold higher; our unpublished data), that grass pollen stimulation was chloroquine sensitive and that it was blocked by addition of anti-CD4 mAb to culture, we believe the simplest interpretation is that this represents antigen-mediated activation. Further investigation of the responses of clinically tolerant 'normal' subjects to other common environmental antigens will be required and is currently underway.

Collectively, the data argue that the most relevant parameter in determining susceptibility to immediate hypersensitivity is not if T cells reactive to a given allergen are present in any given individual, or if they respond, but how. The decision between allergy versus clinical tolerance to environmental allergens may hinge on which form of responsiveness is induced, specifically whether the dominant response is a Th1-like or a Th2-like response, rather than on a genetically defined capacity to recognize the antigen at all. We speculate that most individuals, atopic and non-allergic, actively respond to many environmental antigens, with default Th1-like responses of non-allergic individuals actively preventing induction of hypersensitivity. Data from studies in which a Th1-like pattern of cytokine synthesis is preferentially elicited *in vivo* in the murine response to the model allergen ovalbumin (OVA),[24] argue in support of this hypothesis. Limiting dilution analysis of murine CD4 T cells enriched from mice exposed to chemically modified (Th1-inducing) versus native antigen (balanced Th1/Th2-inducing) reveal similar frequencies of proliferative T cells in the two groups, but a pronounced shift in the frequencies of Th2-like (IL-4 + IL-10 producing) and Th1-like (IFN-γ producing) CD4 T cells (R. S. Gieni, X. Yang, A. Kelso & K. T. HayGlass, manuscript submitted for publication). Further studies to address this question directly are now underway.

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