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ANALYSIS OF ALLERGEN-DRIVEN CYTOKINE SYNTHESIS PATTERNS IN GRASS POLLEN ALLERGIC AND NORMAL INDIVIDUALS

BY

YAN LI

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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To My Beloved Husband and Daughter

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ABBREVIATIONS

APC	:	Antigen presenting cells
BNHS	:	D-Biotin-e-aminocaproic acid N-hydroxysuccininmide ester
BSA	:	Bovine serum albumin
CD40L	:	CD40 ligand
CO_2	:	Carbon dioxide
CTL	:	Cytotoxic T cell
d	:	day
dpm	:	disintegrations per minute
DNA	:	Deoxyribonucleic acid
DTH	:	Delayed type hypersensitivity
ELISA	:	Enzyme linked immunosorbent assay
Exp	:	Experiment
Fc	:	Constant fragment of immunoglobulin
FCS	:	Fetal calf serum
g	:	gram
GM-CSF	:	Granulocyte-macrophage colony-stimulating factor
h	:	hour
^3H	:	Tritiated
IFN	:	Interferon
Ig	:	Immunoglobulin
IL	:	Interleukin
L.	:	Leishmania

LDA	:	Limiting Dilution Analysis
Log	:	Logarithm
LPS	:	Lipopolysaccharide
mAb	:	monoclonal antibody
mg	:	milligram
MHC	:	Major histocompatibility complex
min	:	minute
ml	:	millilitre
mRNA	:	Messenger RNA
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ng	:	nanogram
NK	:	Natural Killer
NRIg	:	Normal rat immunoglobulin
OA	:	Ovalbumin
O.D.	:	Optical density
PBMC	:	Periphery blood mononuclear cell
PBS	:	Phosphate buffer saline
pg	:	picogram
PHA	:	phytohaemagglutinin
PK	:	Prausnitz-Kuestner
PMA	:	phorbol myristate acetate
PPD	:	Purified protein derivative
PSF	:	Penicillin, streptomycin and fungzone
rIL	:	Recombinant interleukin
rpm	:	rotations per minute

S.D.	:	Standard deviation
TCR	:	T cell receptor
TGF-β	:	Transforming growth factor-β
Th	:	T helper
TNF	:	Tumor-necrosis factor
U	:	Unit
μCi	:	Microcurie
μg	:	Microgram
v	:	Volume
vs	:	Versus

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SUMMARY

The balance in which IL-4 and IFNγ production are induced following exposure to common environmental allergens is believed to be instrumental in determining whether hypersensitivity or tolerance results. Our understanding to date is largely based on extrapolations of data obtained using panels of allergen-reactive human CD4 T cell clones or, alternatively, from studies of T cell responses following polyclonal activation. Therefore, characterization of cytokine production elicited in response to allergen-specific stimulation of fresh cell populations of normal and allergic individuals directly ex vivo remains an important goal.

The objective of the present study was to elucidate the relationship between clinical sensitivity to a locally common group of inhalant allergens (grass pollens) and allergen-stimulated cytokine production in subjects with seasonal allergic rhinitis and normal controls. Our hypothesis was that a difference in the balance of cytokine production characteristic of Th1 and Th2-like responses could be demonstrated using short term, antigen-specific stimulation in primary culture of fresh PBMC. Our approach was to examine the cytokine production which is either representative of Th1 or Th2-like responses, or influential in the development of Th1 or Th2 responses, and to compare these parameters in atopic and normal subjects. Specifically, through successful development of ultrasensitive methods for cytokine detection, we were able to characterize (i) allergen-stimulated IL-4 and IFNγ production by

fresh PBMC of allergic and normal individuals directly ex vivo, (ii) IL-2 requirements for the induction of IL-4 and IFNγ gene expression, (iii) the role played by IL-12 in inducing IFNγ synthesis and (iv) the relative responsiveness of atopic and normal subjects to rIL-12 stimulation.

Using short term limiting dilution analysis or primary bulk cultures of fresh human PBMC immediately ex vivo, we found that

(i) The frequency of IL-4 producing cells in peripheral blood is significantly increased, and that of IFNγ producing cells is significantly lower in patients with seasonal allergic rhinitis compared to that of normal, non-allergic controls. The decreased ratio of IFNγ:IL-4 synthesis observed in atopic compared to normal subjects argues that an imbalance of Th1 to Th2 responses occurred upon in vivo exposure to grass pollen.

(ii) Differential requirements for IL-2 in the induction of IL-4 and IFNγ gene expression were observed. Biologically active IL-4 production was demonstrated to be IL-2 independent, while IFNγ production was significantly enhanced by the addition of rIL-2 to the cultures.

(iii) IL-12 is capable of inducing intense IFNγ synthesis by otherwise unstimulated human PBMC. Most importantly, IL-12 was further shown to enhance allergen-stimulated IFNγ production which indicates a role for IL-12 in promoting Th1-like response. IL-2 is synergistic with IL-12 in its IFNγ-inducing activity.

(iv) PBMC from allergic individuals showed an impaired response to rIL-12 stimulation in terms of IFNγ production. We speculate that this defect might be instrumental in understanding the mechanism(s) by which differential effector responses develop in allergic and normal individuals in response to in vivo allergen exposure. Moreover, they suggest that strategies which aim to redirect allergen specific cytokine synthesis patterns in vivo via administration of rIL-12 to atopic individuals should be viewed with caution.

INTRODUCTION

I. Literature review

1.1 Allergy and IgE

Allergy is an exaggerated response of immune system caused by overproduction of IgE in response to stimulation by common environmental antigens, such as those present in grass pollen, foods, house dust mites, animal danders, fungal spores and insect venoms. The most common allergic diseases are asthma, allergic rhinitis (hay fever), atopic dermatitis and food allergy. In one form or another, allergy afflicts more than 20 percent of the population, and the alarming increase in its prevalence, morbidity and mortality over the past decade has led to its designation as the "number one environmental disease" (Sutton, 1994).

Allergic diseases have been documented for centuries. Hay fever was first named in 1819 by John Bostock, who described classical symptoms of seasonal allergic rhinitis in himself and in a number of his patients. The realization that the etiologic agents for this condition were environmental and that the sensitivity to them could be demonstrated by an erythematous skin reaction came in 1860 with the observations of Salter. By the early 1920s, Prausnitz and Kustner reported that skin reactivity to allergens could be transferred from patient to patient with serum, and Coca gave the name "reagin" to the agent involved (Coca, 1943). It was not until 1967, however, that the Ishizakas in Denver as well as Bennich and Johanson in Sweden isolated human IgE and provided convincing

evidence that this newly discovered class of immunoglobulin was the most prevalent type of antibody which induced the immediate hypersensitivity.

IgE constitutes a extremely small fraction of the total antibody in human serum (Sutton, 1994). The protein sequence revealed that IgE shares the overall structure of other classes of antibody, but is distinguished from them by the sequence of its (ϵ) heavy-chain constant region. Like the μ-chain of IgM, it consists of four constant domains (Cϵ1-Cϵ4), one more than in IgG, IgA or IgD. IgE protein has a high carbohydrate content and is extremely heat liable. It loses its ability to sensitize human skin for the Prausnitz/Kustner (P-K) reaction after heated at 56°C for 2-4 hours. Unlike IgG, IgE is unable to fix complement or to cross the placenta (Ishizaka, 1989).

The most important immunological property of IgE is the ability to sensitize homologous tissues for reaginic hypersensitivity (type I allergic) reactions. The minimum concentration of human IgE antibodies required for sensitizing human skin for a positive P-K reaction is in the order of 0.2 ng/ml. An optimal latent period for sensitization is 1 to 3 days, and the sensitization persists for a long period of time (Ishizaka, 1975).

Sensitization of homologous tissue with IgE is due to its binding to mast cells and basophils. Although IgE is only present in trace amount in serum, its action is powerfully amplified by the

activities of receptors to which it binds. The high affinity receptors on mast cells and basophils, FcεRI, is responsible for the immediate hypersensitivity reaction . When a multivalent allergen associates with FcεRI-bound IgE, it crosslinks receptor molecules on the cell membrane. This triggers degranulation of the cells with rapid release of a variety of preformed vasoactive mediators, notably histamine, and the synthesis and secretion of various chemotactic factors and cytokines that attract and activate inflammatory cells (Galli, 1991). The clinical effect of this process include respiratory tract constriction and increased vascular permeability and vasodilation, resulting in difficulty in breathing, edema, and skin rashes. Another consequence of allergic reaction is the production of increased amounts of secretions by cells on mucosal surface which represent the major symptom of allergic rhinitis.

IgE antibody has been found only in mammals. In human, IgE secreting B cells are abundant in the skin, lungs and guts. It has been suggested that the level of total IgE in blood is closely associated with the risk of developing allergy in particular individuals, and predeposition to allergy appears to result from an interaction between genetic and environmental factors (Tada, 1975).

1.2. Cytokine production profiles and modulation of IgE synthesis

1.2.1. CD4 and CD8 T cells

T lymphocytes are defined by the surface expression of T cell

receptor (TCR) complexes, and play a central role in immune responses by carrying out a number of effector and regulatory functions. Based on expression of CD4 and CD8 molecules on the cell surface, T cells have been divided into two groups: CD4-T cells and CD8-T cells. This distinction generally but imperfectly correlates with fundamental functional differences. CD4 T cells provide help for B cell proliferation and production of antibodies, cross-regulate T cell functions,mediate delayed type hypersensitivity (DTH) reactions, kill target cells and also suppress antibody production by B cells. They appear to exert most of their functions through secreted cytokines by acting on the T cells that produce them in a autocrine fashion as well as modulating responses of other cells through paracrine pathway. CD8 T cells mostly carry out cytolytic functions by killing antigen-bearing target cells. A number of most recent reports also suggest that CD8 T cell play a more active role in the regulation of immune responses by producing different combination of cytokines and determining the pattern of cytokine produced by CD4 T cells and the isotype of immunoglobulin produced by B cells.

Expression of CD4 and CD8 cell surface molecules correlates with the class of major histocompatibility complex (MHC) molecules that restricts antigen recognition. The responses of CD4 T cells usually are restricted by class-II MHC molecules while the responses of CD8 T cells are restricted by class-I MHC antigen.

1.2.2. Classification of functional CD4 T cell subsets based on their cytokine secretion profile

1.2.2.1. CD4 T cell subsets in mice

In recent years, it has become clear that the type of antigen-specific immune response is quite dependent on the selection or preferential activation of peculiar subsets of CD4 helper T cells able to secret defined patterns of cytokines which lead to the triggering of strikingly different effector responses. Studies using long term murine T cell clones identified two subpopulations of CD4 T cells, termed Th1 and Th2 cells, which showed distinct patterns of cytokine production. Th1, but not Th2, cells secret interleukin-2 (IL-2), interferon-γ (IFNγ) and tumour necrosis factor-β (TNF-β), while Th2, but not Th1, cells produce IL-4, IL-5, IL-6, IL-10 and IL-13. Other cytokines, such as TNF-α, IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF), are produced by both cell types (Mosmann & Coffman, 1986 & 1989). The most important concept arising from these observations is that the different cytokine profile produced by the two subsets of Th cells results in distinct effector responses (Janewy, 1988; Mosmann, 1989). In general, Th2 cells provide excellent help for Ig synthesis by B cells, especially IgG1 and IgE, whereas Th1 cells are mainly involved in the induction of DTH responses (Cher,1987). In addition to clones that clearly fit into the description of Th1 or Th2 subsets, some CD4 T cell clones exhibit combined production of most or all cytokines made by Th1 and Th2 cells, and thus are

termed Th0 cells (Gajewski, 1988; Street, 1990; Firestein, 1989).

1.2.2.2. CD4 T cell subsets in human

The establishment of Th1 and Th2 subsets and its value in the interpretation of many different effector immune responses and disease outcomes observed in mouse models encouraged extensive investigations for its possible relevance to the human immune system. Initial studies from different laboratories on large panels of alloreactive or mitogen-induced human T cell clones revealed that only a few of them fit into clear cut Th1 or Th2 phenotypes, while the majority showed unrestricted cytokine patterns, thereby resembling murine Th0 cells (Maggi E, Paliard X and Umetsu DT, 1988).

Subsequent studies on human Th1 and Th2 clones concentrated on T cells that specifically recognize antigens involved in two classically different and prototypic immune mechanisms, such as DTH to mycobacterial antigens or to nickel and IgE responses to allergens or helminth antigens. Early evidence for the existence of human Th1 and Th2 cells was provided by analyzing the cytokine profile of a large panel of T cell clones, established from the peripheral blood lymphocytes of healthy donors, that were specific for the purified protein derivative (PPD) of Mycobacterium tuberculosis or Toxocara canis excretory/secretory (TES) antigens (Del Prete, 1991; Parronchi & Romagnani, 1991). The great majority of PPD-specific T cell clones were equivalent to murine Th1 cells,

being able to produce IL-2 and IFNγ, but not IL-4 and IL-5, following stimulation with either specific antigen (PPD) or mitogen, such as phytohaemagglutinin (PHA) or phorbol myristate acetate (PMA) plus anti-CD3 monoclonal antibody. In contrast, most TES-specific T cell clones generated from the same donor secreted IL-4 and IL-5, but not IL-2 and IFNγ, thus resembling murine Th2 phenotypes (Del Prete GF,et al, 1991). Likewise, analysis of nickel-specific T cell clones derived from the PBL of individuals with contact dermatitis and nickel-induced DTH reaction reveals that majority of them also exhibited a preferential Th1 profile (Kapsenberg, 1992). Convincing evidence for the existence of human Th1 and Th2 cells was also provided by studies on T cell clones specific for environmental allergens. Dermatophagoides pteronyssinus (DP)-specific T cell clones generated from patients with atopic dermatitis (IgE or immediate hypersensitivity) produced IL-4, but no IFNγ, whereas tetanus toxoid- or Candida albicans-specific T cell clones established from the same patients secreted IFNγ, but no IL-4, thus showing clearcut Th2 and Th1 phenotypes respectively (Wierenga, 1990; Parronchi, 1991).

Taken collectively, these data strongly support the idea that T cells with stable and definite Th1 and Th2 patterns may exist not only in mice but also in human. They also suggested a dichotomy that responses induced by environmental allergens and parasites usually associate with predominant expansion of Th2 cells, whereas some bacterial and viral antigens preferentially promote the

differentiation of Th1 cells. It is worth mentioning, however, that the response to any given antigen does not simply result in expansion of Th1 or Th2 cells, but is also associated with the proliferation of a variable proportion of Th0 cells which show the combined cytokine secretion pattern of Th1 and Th2 cells.

1.2.2.3. Functional properties of human Th1 and Th2 cells

With the convincing demonstration the existence of clearcut human CD4 Th1 and Th2 clones, further studies were then focused on the investigation of their function properties. Like their murine analog, human Th1, but not Th2, cells produce TNF-β in addition to IL-2 and IFNγ, whereas Th2, but not Th1, cells secret IL-4 and IL-5. Both subsets can produce variable amounts of IL-3, IL-6, TNF-α and GM-CSF, but in general, Th1 clones produce higher amounts of TNF-α and lower amounts of IL-3, GM-CSF and IL-6, than Th2 cells (Fiorentino DF et al, 1989 and 1991). In the murine system, IL-10 is demonstrated to be a selective product of Th2 subsets and acts on Th1 cells by inhibiting IL-2 and IFNγ synthesis. However, recent studies in humans have revealed that IL-10 is produced by both CD4 T cell subsets with a higher intensity observed in Th2 clones (Yssel H, et al, 1992 and Del Prete GF, et al, 1993).

Besides their different cytokine production profiles, human Th1 and Th2 cells also differ in their responsiveness to certain cytokines. Studies of murine T clones demonstrated that cytokines produced by either subset crossregulate the function of Th1 and Th2 cells. In

humans, much as in murine system, IL-2 was found to enhance the antigen-induced proliferation and cytokine synthesis by both cell types (Fitch, et al, 1994; Demeure, 1994), whereas IL-4 preferentially potentiates and IFNγ selectively inhibits the function of Th2 clones . Interestingly, human Th1 and Th2 clones showed different responsiveness to IL-10 in comparison with mouse Th1 and Th2 cells. While murine IL-10 acts as a selective inhibitor of cytokine production by Th1 cells (Fiorentino DF, et al, 1989), human IL-10 is shown being able to inhibit the proliferation and cytokine synthesis by both Th1 and Th2 clone in response to either antigen-specific or mitogen stimulation (Del Prete GF, et al, 1993). Thus, in humans, IL-10 is likely to be a cytokine involved in controlling the ongoing immune responses rather than in selectively inhibiting Th1 functions. However, the applicability of these results, obtained in vitro with long term T cell clones, to normal T cells in vivo in response to the physiological dose of antigens remains to be directly evaluated.

Finally, in terms of cytolytic activity and helper function for Ig synthesis, human Th1 and Th2 clones have also shown significant differences. In one report, when the cytolytic potential of both cell types was assessed, it was found that the majority of Th1, but only few Th2, clones were able to lyse the P815 murine cell line in a 4-hour PHA-dependent assay (Del Prete GF, et al, 1991). Instead, all Th2 clones effectively induce IgM, IgG, IgA and IgE synthesis by autologous B cells in the presence of specific antigen and the

degree of response was positively proportional to the number of Th2 cells added to the B cell cultures. Under the same experimental condition, Th1 cells provided B cell help for IgM, IgG, and IgA, but not IgE, synthesis, but this function was only observed at a low T cell to B cell ratio with the peak responses occurring at a ratio 1:1. Therefore, these data confirmed the previous observation that Th2 cells are able to induce IgE synthesis through their production of IL-4, whereas Th1 cells lack this function.

1.2.3. Current knowledge of mechanisms in determination of differentiation of Th1 and Th2 cells

Differential production of cytokines by helper T cells during an immune response elicited by different infectious agents or environmental allergens is likely to have important regulatory effects on the nature of responses. Therefore, understanding the factors determining the differentiation of Th1 or Th2 cells would be of great significance for immunological intervention of immune responses, potentially making it possible to control the development and outcome of certain diseases.

It has been suggested that Th1 and Th2 cells are repeatedly stimulated memory T cells that have matured into different functional phenotypes from a common pool of precursor T cell population (Thp) (Rocken, 1992; Reiner, 1993; Abehsira-Amar, 1992). According to this model, naive Thp cells mainly produce IL-2 and progress into early memory Th0 effector cells after a first

activation by specific antigen (Swain SL, et al, 1990). These cells then terminally differentiate into Th1 and Th2 cells upon repeated antigen stimulations (Firestein GS, et al, 1989 and Street NE, et al, 1990). A number of factors have been demonstrated to be involved in determining the Th cell differentiation pathway, including certain cytokines, properties of antigens, dose of antigen, distinct antigen presenting cells (APC) and utilization of different TCR-associated signal transduction pathways. These factors are hypothesized to act independently or together to commit a naive Thp cell to differentiate into effector Th1 or Th2 cells upon antigen stimulation.

1.2.3.1. Regulation by cytokines

Cytokines produced by various cell types have been suggested by many studies to play the most important role in determining the differentiation of Th1 and Th2 cells.

IFNγ and IL-4 Studies by Fitch and his colleagues provided early evidence for the roles played by IFNγ. They observed that cloning CD4 T cells in the presence of IFNγ limited the outgrowth of IL-4-producing T cells, with virtually all the clones obtained producing IFNγ (Fitch, et al., 1993). Moreover, IFNγ was also shown to inhibit the clonal expansion and effector functions of Th2 cells (Gajewski, 1988; Fernandez-Botran, 1988)). In clear contrast, studies by several groups (Swain SL, et al, 1990; Betz M, et al, 1990; LeGros GG et al, 1990) demonstrated that the presence of IL-4

during primary culture resulted in a cell population that produced IL-4 upon subsequent in vitro challenge (using polyclonal T cell activation), and that the appearance of IFNγ-producing cells was totally suppressed by the presence of IL-4. The effect of IL-4 and IFNγ in priming different Th subsets was further confirmed by the reversing effect of neutralizing antibodies. The addition of anti-IFNγ or anti-IL-4 antibodies to the priming cultures, to neutralize any endogenous IFNγ or IL-4, resulted in the generation of T cells that secreted IL-4 or IFNγ and IL-2 upon stimulation, which represent a Th2 or Th1 phenotype, respectively. Thus, it is reasonable to conclude that IL-4 and IFNγ, the principle products that characterize Th2 and Th1 cells, often oppose one another's action, not only by priming differentiation and clonal expansion of distinct subtypes of effector Th cell, but also by exerting cross-regulatory effects on each other.

IL-2 The production of IL-2 by T cells is a critical event in T cell activation. First of all, IL-2 is needed for the growth and survival of cells in the course of priming cultures. Thus, if the priming culture is carried out in the absence of IL-2, as insured by the addition of neutralizing anti-IL-2 antibody, and no other T cell growth factor is added, few if any cells survive at the end of the culture (Seder & Paul, 1994). Furthermore, if IL-4 is added to such priming culture, its action as a T cell growth factor does allow cells to emerge from the culture, but interestingly, such cells fail to produce IL-4 upon restimulation, suggesting that

IL-2 is required not only for the growth of T cells during the priming culture but also for the differentiation process of precursor T cells to Th2 cells (Seder & Paul, 1994). Since IFNγ alone does not support T cell growth in the culture, it is thus difficult to assess whether IL-2 is also required for differentiation to Th1 cells.

IL-10 Since IL-4 has been demonstrated to enhance the priming of naive T cells to cells with Th2 phenotype, the possibility has to be considered that the action of IL-4 might be mediated by factors in the culture induced by IL-4. A logical candidate for this purpose was IL-10. IL-10 is a product of Th2 cells, B cells and LPS-stimulated macrophages and is known to inhibit cytokine production by Th1 clones but not by Th2 clones in mice. It might have a similar effect on the priming process. Indeed, IL-10 was reported to diminish the priming of IFNγ producing cells, although this inhibition was less dramatic than that mediated by IL-4 (Hsieh CS, et al, 1992 and Macatonia SE, et al, 1993). However, the study further showed that the addition of anti-IL-10 antibodies to the priming culture did not prevent IL-4 from blocking the development of IFNγ producing cells. Thus, it is fair to conclude that the effect of IL-4 is not mediated by the induction of IL-10, but rather, these two Th2 cell-derived cytokines seem to act synergistically in inhibiting the differentiation of Th1 cells. Furthermore, instead of directly acting on T cells like IL-4 and IFNγ, IL-10 apparently exerts its function indirectly by acting on

antigen presenting cells, preferentially macrophages, by reducing their ability to stimulate cytokine production by Th1 cells.

IL-12 IL-12 is a newly characterized cytokine that has a unique heterodimeric structure. It was initially cloned from the conditioned medium of EBV-transformed B lymphoblastoid cell lines, but the majority of IL-12 is produced by macrophages/monocytes following appropriate stimulations (Kobayashi, 1989). Extensive studies showed that IL-12 was a potent inducer of IFNγ production by both NK cells and T cells. Interest generated quickly in questioning whether IL-12 has an effect on the differentiation of effector T cells. Indeed, there is now growing evidence that IL-12 plays a major role in the induction of Th1-like subsets in both murine and human. In an in vitro model utilizing ovalbumin-specific CD4 T cells derived from $\alpha\beta$-TCR transgenic mice, Hiseh et al. have shown that antigen combined with recombinant murine IL-12 preferentially induced the generation of Th1 clones from naive T cells (Hiseh, 1993). Furthermore, it was demonstrated with neutralizing anti-IL-12 antibody that antigen-activated macrophages, which similarly induced Th1 cells in this model, mediated this activity through the production of IL-12. The regulatory effect of IL-12 on Th1 cell differentiation has also been observed in humans (Trinchieri, 1993; Romagnani, 1992; Manetti, 1993). Dermatophagoides pteronysinus specific CD4 T cell clones generated from atopic individuals usually exhibit a Th2-like phenotype, producing IL-4 but little or no IFNγ. However, cell

lines generated from the same patients in the presence of human IL-12 showed a Th0 or Th1-like cytokine secreting profile (Manetti R, et al., 1993). Conversely, PPD-specific T cell clones which exhibit Th1-like phenotype develop into Th0 cells if generated in the presence of anti-IL-12 antibody. These results with both murine and human studies indicate that IL-12 may play a critical role in mediating the induction of Th1 cells.

Other cytokines Several other cytokines have also been shown to influence, to various extents, the differentiation of Th1 or Th2 cells. Using an in vivo system that stimulates IL-4 and subsequent IgE production, Finkelman and Gause demonstrated that injection of mice with IFNα, or with an agent known to induce IFNα in vivo, caused a decrease in the amount of IL-4 mRNA and an increase in IFNγ mRNA (Finkelman, 1991), which suggested a role of IFNα in induction of Th1 cells, possibly through enhancing the production of IFNγ. Similarly, TGFβ was also reported to suppress the development of IL-4 producing T cells while stimulating the generation of IFNγ-producing cells (Swain SL, at al., 1991). However, controversy still remains in terms of this effect of TGFβ.

1.2.3.2. Effect of APC on induction of T cell subsets

APC may have a major influence on the type of T cell response that is generated, and it is widely appreciated that certain types of APC are better at stimulating T cell responses than others. In a murine study carried out by Fitch and his colleagues, a panel of

Th1 and Th2 clones specific for ovalbumin were shown to produce cytokines and proliferate equally well in the presence of whole spleen cells. However, using purified splenic B cells as a source of APC preferentially induced the proliferation of Th2 clones while adherent spleen cell, most notably macrophages, stimulated optimal proliferation of Th1 clones (Gajewski & Fitch, 1991). In converse, proliferation of Th2 clones stimulated with whole spleen cells that were irradiated with 3000 rad, a way known to abolish the antigen presenting function of B cells while not affecting macrophages, is dramatically decreased, thus further suggesting the role of B cell or macrophage as APC in selectively induction of Th2 or Th1 response, respectively. Moreover, hepatic nonparenchymal cell, probably Kupffer cell, was reported to stimulated the proliferation of Th1 but not Th2 clones (Magilavy & Gajewski, 1989). However, controversy still remains concerning both this selective effect of different APC populations and the mechanisms by which different types of APC act to stimulate distinct T cell subsets. Cytokines produced by APCs are certainly believed to participate the regulating process and thus make it difficult to interpret the effect exerted by any single factor. In addition, cell surface receptors and ligands are suggested to be involved in regulating the differentiation pathway by APC, which however, need further indepth investigation.

1.2.3.3. Influences by antigen

It has been noted for years that the type, dose and route of

antigen exposure could profoundly alter the nature of the response that ensued (Parish, 1972). But only recently has this phenomenon been investigated in detail following the understanding of the presence of distinct subpopulations of T cells. Various groups have reported that suppression of delayed type hypersensitivity (DTH) is accompanied by increased level of antibody production. It was observed that very low concentrations of antigen often yield responses in which DTH reaction is dominant. As the antigen dose is increased, antibody responses increase while DTH responses diminish. Finally, at very high antigen concentrations, DTH reaction become dominant again (Parish & Liew, 1972). Recently, these observations were further tested by Bretscher and his colleagues in a series of in vivo experiments in which mice were infected with L. major (Bretscher, 1992). The normal susceptibility of BALB/c mice could be reversed by initially injecting the mice with a small number of parasites. Not only were the mice resistant to the initial small inoculum of L. major, they were still resistant when being rechallenged 120 days later with a normally lethal dose of parasites. These results indicate that these normally susceptible BALB/c mice developed a protective Th1 response marked by IFNγ production rather than the IL-4-dominant Th2 response they normally mount because of the initially smaller antigen dose used. Based on this observation, that antigen dose can influence the quality of the response, Bretscher has proposed that immune deviation may be a useful vaccine strategy for certain diseases. However, this is not always the case. Data presented by

Seder and Paul argue a somewhat different result (Seder & Paul, 1994). In the course of their in vitro study using cytochrome C peptide as antigen, they observed that no matter what the concentration of antigen, the determining factor for induction of Th2 clone is always the presence of IL-4. They did find that high concentration of peptide reduced priming for IFNγ production, however, this result was not observed in all experiments. Thus, they suggested that antigen dose may be one of the several factors that are important in determining the outcome of Th1 or Th2 differentiation event, however, it does not seem to be a dominant controller of this outcome.

Different types of antigen also seem to be able to influence the generation of dominant Th1- or Th2-like responses, as does the route of invasion. Most apparently, bacterial and viral antigens preferentially induce Th1 responses with the manifestation of DTH reaction, whereas environmental allergens often generate IL-4-dominant Th2 responses with consequent immediate hypersensitivity. However, understanding the mechanisms by which different antigens trigger different responses in the same person and the same antigen induces distinct responses in different individuals remains a yet-to-be-achieved goal.

1.2.3.4. Th1 and Th2 clones utilize different TCR-associated pathways

Stimulation of TCR has been shown to result in hydrolysis of PIP_2

to yield diacylglycerol and IP_3 (Imboden, 1985), an enzymatic reaction believed to be mediated by phospholipase C. Diacylglycerol presumably activates protein kinase C, while the IP_3 generates the elevation of $[Ca^{2+}]$ (Imboden, 1985). However, these events appear to occur only in some but not all T cell subsets. Fitch and his co-workers, as well as Abbas and his colleagues, reported that second messengers after stimulation with concanavalin A (Con A) or anti-TCR mAb were only observed in Th1 clones but not in Th2 clones (Gajewski & Fitch, 1990; Williams & Abbas, 1991). Furthermore, treatment with ionomycin alone induces IL-4 production in Th2 clones (Abbas A.K. et al., 1991), while such treatment induces anergy in Th1 clones (Mueller D.L. et al., 1989). These data suggest that Th1 and Th2 cells utilize a different TCR-associated signal transduction pathway, thus might explain the phenomenon that different type of APC as well as different antigen dose induce the generation of different subsets of CD4 T cells.

As discussed above, much progress has been made in understanding the factors that determine the process through which naive CD4 T cells differentiate into distinctive effector cells. Obviously, these factors act together to regulate as well as to cross-regulate in achieving a final T cell phenotype upon antigenic stimulation, and it should be emphasized that the regulation by cytokines, most notably IFNγ, IL-12, IL-4 and IL-10, may play the dominant role in determining the terminal response induced.

1.2.4. Study of CD4 T cell subsets in parasitic model

Not only extensive studies have demonstrated the existence of distinctive CD4 T cell subsets with different cytokine production profiles, further evidence has been collected on the differential effects that Th1 and Th2 cells may exert on determining the type of immune effector response a given infectious agent may elicit. In this regard, most knowledge was obtained from studies of murine responses to parasitic stimulations. Indeed, parasitic infection models provide several important, and in some cases unique, advantages for these studies. First of all, the persistent antigenic stimulation resulting from chronic infection, which is probably the most distinguishing feature of parasitic infection, often leads to polarization of T cell subsets and extreme immunoregulatory states. Furthermore, the biological diversity of the different parasites and their in vivo habitats usually results in distinct forms of antigen presentation and T cell activation , which provides an important comparative tool for understanding the cellular basis of differential T cell activation. Finally, the genetic control of many parasitic infections, which appears to reflect polymorphism that controls host processing of the organisms and their antigens, has been well-documented, and thus providing insight into the understanding of both the regulation and function of T cell responses.

The dual regulatory and effector roles of CD4 T cells are best understood in murine Leishmania major infection. Cutaneous

infection of most inbred strains with L. major leads to a localized lesion that heals spontaneously and confers resistance to reinfection. However, in a few strains, of which BALB/c is the prototype, the infection progresses to a disseminated visceral disease that is usually fatal. Extensive studies of the cellular basis of these differences reveals that the differential stimulation of CD4 T cells subsets could account for resistance or susceptibility (Reviewed by Sher & Coffman, 1987; Scott, 1989). In resistant strains of mice, such as C57BL/6 or C3H/HeN, control of infection is associated with expansion of IFNγ producing-Th1 cells in the draining lymph nodes, while progressive infection in susceptible BALB/c strain is associated with the appearance of IL-4 secreting Th2 cells (Heinzel, 1989, 1991; Kemp, 1994). The types of immune responses prominent in healing and non-healing mice further support this conclusion. Healing disease is accompanied by strong DTH reactivity, but poor antibody responses, whereas nonhealing animals have strong antibody responses and very high IgE levels, but no DTH reaction (Scott, 1989). Furthermore, Leishmania-specific Th1 lines can transfer protection to BALB/c mice, and conversely, transfer of Th2 cells exacerbates the nonhealing disease pattern (Scott, 1988).IFNγ appears to be required for the development of healing Th1 response to L. major. In vivo depletion of IFNγ in the resistant strain (C3H/HeN) with a single injection of anti-IFNγ monoclonal antibody or by disruption of IFNγ gene in vivo induced susceptibility to infection (Belosevic et al., 1989; Wang, 1994), but the antibody must be given at the time of

challenge to be effective. Repeated treatments with the antibody during the phase of development and healing of the lesion, but not during the initiation of the response, are ineffective. A logical interpretation of this result is that the presence of IFNγ at the time of antigen priming can augment protection by enhancing Th1, and decreasing Th2, development, rather than that the single injection of anti-IFNγ antibody depleted total IFNγ during the entire course of infection. Analysis of the earliest measurable T cell responses to L. major further support the protective role of IFNγ (Scott, 1991). T cells taken from draining lymph node of resistant mice (C3H) three days after infection were shown to produce IFNγ but not IL-4, whereas T cells taken from BALB/c mice produce IL-4 and much less IFNγ upon restimulation in vitro. Treatment of C3H and BALB/c mice at the time of infection with anti-IFNγ or IFNγ, respectively, reversed the pattern of this early response. Rather than directly acting on T cells, IFNγ is suggested to exert its effects through acting on antigen-presenting cell function or homing behaviour to promote preferential Th1 response (Belosevic, 1989).

Observation from similar experiments suggested that IL-4 is required for the development of Th2 responses. Treatment of BALB/c mice with anti-IL-4 conferred these mice resistance to infection with occurrence of prominent Th1 response, whereas injection of IL-4 into C3H mice induced IL-4 production and inhibited IFNγ production (Sadick, 1990). Again, the alteration in cytokine

expression induced by anti-IL-4 was only observed when applied during early phase of response (Sadick, 1990, Sher, 1992). These results are consistent with in vitro evidence that IL-4 promotes the generation of Th2 cells.

In contrast to the permanent changes in Th subset response caused by single injections of antibodies to IL-4 or IFNγ, neither short-term or long-term administration of either IL-4 or IFNγ caused reversal of the long-term response or the disease outcome (Sadick, 1990, 1991; Scott, 1991; Sher, 1992). This may either reflect the fact that cytokines have very short half-lives in vivo (typically minutes) or suggest that IFNγ and IL-4 are necessary but not sufficient for the development of Th1 and Th2 responses (Sadick, 1990, 1991).

The correlation of Th1 responses with healing and Th2 responses with exacerbation of disease are understandable in terms of the regulation of specific effector functions by cytokines. Leishmania exist in the host only within the phagolysosome of macrophages and activation of macrophages is required for elimination of the intracellular amastigotes. IFNγ has been identified as the most potent macrophage-activating factor for the killing of intracellular parasites, both in vivo and in vitro (Murray, 1983), and TNF-α has been shown to be synergistic with IFNγ in this activity (Bogdan, 1990; Liew, 1990). In contrast, IL-4, also IL-3, inhibit macrophage killing of Leishmania (Scott, 1989; Lehn, 1989; Liew, 1989), possibly by counteracting the activities of IFNγ and

TNFα at several levels, including inhibition of IFNγ release, inhibition of subsequent activation of macrophages to leishmanicidal state by IFNγ, inhibition of both IFNγ- and TNFα-induced activation of adhesion molecules and inhibition of TNFα transcription. Other Th2-derived cytokines, such as IL-10, have also been suggested to interfere with the function of macrophage by inhibiting the production of IFNγ in an accessory-cell-dependent manner (Fiorentino, 1989). Thus, the cytokines produced by Th1 or Th2 cells, in addition to cross-regulating the development of the opposing CD4 subsets, determining the fate of the parasite inside the macrophages that Leishmania obligately infects (Liew, 1989; Fiorentino, 1989).

Similar patterns of protective Th1 responses have also been observed in some other mouse parasitic models such as Trichinosis (Pond L. et al., 1989) and Schistosomiasis (Pearce EJ. et al., 1991 and Sher A. et al., 1990). This pattern, however, is not absolute. In rodents, host resistance to reinfection with Heligmosomoides polygyrus or Strongyloides venezuelensis has been attributed to Th2-mediated responses (Finkelman FD, et al., 1991 and Korenaga M. et al., 1991). So far, the role of Th cells in human immune responses to parasitic infection is still under active investigation.

1.2.5 Regulation of IgE synthesis by cytokines

IL-4 is required for induction of IgE synthesis The IgE antibody

response is highly T cell dependent, and this suggests that T cell-derived cytokines are involved in the differentiation of B cells to IgE-forming cells (Saryan, 1983; Coffman, 1986). Indeed, it has been well documented that IL-4 is a critical switch factor for IgE synthesis. Stimulation of mouse B cells with lipopolysaccharide (LPS) results in differentiation of B cells and formation of a variety of Ig isotypes except IgE (Parkhouse, 1977). However, culture of the same B cells for 5 days with LPS in the presence of IL-4 resulted in the formation of IgE and selective enhancement of IgG1 (Coffman & Paul, 1986). It was also found that culture of pure B cells with LPS together with IL-4 promoted the generation of $sIgE^+$ B cells (Coffman & Carty, 1986) and in vivo injection of anti-IL-4 antibody significantly inhibited the IgE responses in experimental animals (Finkelman, 1986, 1988). In humans, IL-4 was demonstrated to be able to induce IgE synthesis by normal unfractionated PBMC (Pene, 1988; Jaraba, 1988). The actual mechanism by which IL-4 contributes to the induction of IgE synthesis has been actively investigated but remains incompletely understood. Studies to date indicate that IL-4 acts as a crucial signal for isotype-switch to IgE. This was shown by examination of single B cells stimulated by murine (Lebman DA. et al., 1988) and human T cell clones (Gascan H. et al., 1991) as well as limiting dilution experiments with LPS-stimulated B cells in mice (Rothman P. et al., 1990). More recently, Mandler et al observed at cellular and molecular level that IL-4 induction of IgE switch by LPS-activated murine B cells occurred predominantly through sequential

switching, i.e. IgM to IgG1 to IgE (Mandler, 1993). A step in the process by which IL-4 switches antibody responses from the production of IgM to IgE antibody is by inducing the transcription of germline ϵ-chain DNA in B cells. This induction of ϵ-chain switching was shown to occur through a recombination deletion event and not through differential RNA processing (Jaraba, 1990; Gauchat, 1990; Thyphronitis, 1991). In one murine models, it was demonstrated that Ig heavy chain switching is proceeded by expression of the corresponding germline transcript (Rothman, 1990). Thus, these observations are consistent with the hypothesis that IL-4 controls IgE isotype-switching by modulating the accessibility of the ϵ switch region to a putative common switch recombinase.

IL-4 is necessary but not sufficient for the induction of IgE synthesis by highly purified B cells. Addition of IL-4 alone or in combination with a variety of other cytokines, including IL-5 and IL-6, is not effective in inducing IgE synthesis in highly purified B cell suspensions (Vercelli, 1989). Additional signals are required for IL-4 induced IgE synthesis to occur. These second signals can be provided by T cells or a variety of B cell activators as well as anti-CD40 mAb and hydrocortisone.

Second signals for IgE synthesis In addition to be an important source of IL-4, T cells further support IgE synthesis by providing physical contact to B cells. Experiments carried out by Geha and his colleagues showed that mixtures of T and B cells produce IgE

upon incubation with IL-4 only when the T and B cells are cultured in the same well, but not when they are separated by a semipermeable membrane (Vercelli, 1988). Furthermore, IL-4 induced IgE synthesis is strongly inhibited by mAb specific for cell adhesion molecules. Besides the cognate interaction possibly through recognition by the TCR/CD3 complex on CD4 T cells of MHC class II antigen plus peptides on B cells, noncognate T/B interaction was also suggested to support IL-4-dependent IgE synthesis (Parronchi, 1990). The mechanisms involved in the latter interaction are still poorly understood. One speculation is that an inducible membrane-associated molecule is involved in the B cell differentiation pathway and this molecule may represent the ligand for a B cell activation antigen.

In addition to T cells, there is a growing list of direct B cell activators that can synergize with IL-4 to induce IgE synthesis. In this regard, Finkelman and Jabara groups have reported that stimulation with IL-4 and EBV induces T-cell-independent IgE synthesis in human B cells (Thyphronitis, 1989; Jabara, 1990). More recently, it has been shown that highly purified B cells costimulated with IL-4 and various mAb to CD40 synthesize high level of IgE antibody (Jabara HH. et al., 1990 and Ke Z. et al., 1991, Renz, 1994). Stimulation of B cells from nonatopic donors with anti-CD40 mAb, in the absence of IL-4, results in a small increase in IgG synthesis but no IgE or IgM. When both anti-CD40 and IL-4 are added, IgG production increases slightly, however,

large amount of IgE synthesis is observed. Furthermore, when B cells from atopic patients were stimulated by anti-CD40 alone, in contrast to the result described above, increased amount of IgE production was observed and IL-4 was found to up-regulate CD40 expression on B cells (Renz, 1994). The different results obtained from different testing groups may reflect in vivo pre-exposure to IL-4 of B cells from atopic patients. CD 40 is a 50 kd surface glycoprotein expressed on B cells, dendritic cells, epithelial cells, and carcinomas. Recent studies indicate that the natural ligands of CD40 is expressed on activated T cells, mostly CD4 but also some CD8 as well as basophiles/mast cells. An increasing body of evidence suggests that CD40 is involved in the regulation of B cell growth and activation events, in that crosslinking of CD40 with immobilized anti-CD40 or cells expressing CD40-ligand induces B cells to proliferate strongly, and addition of IL-4 allows the secretion of IgE following isotype switch (Reviewed by Banchereau, 1994).

Hydrocortisone has also been found to enhance IL-4-induced IgE synthesis both in vivo (Zieg, 1994) and in vitro by normal unfractionated mononuclear cells in human (Wu CY. et al., 1991) and $sIgE^{+}B$ cells isolated by cell sorting are also inducible by hydrocortisone and IL-4 (Jabara HH. et al., 1991). The mechanism involved remains unknown, however, these observations that steroid hormone-receptor interaction can provide help for IgE synthesis dictates some caution in prescribing steroids for allergic

diseases.

Taken together, these data indicate that the second signals required for IgE production can be delivered to B cells through different activation pathways. The exact mechanisms by which these diverse signals synergize with IL-4 in stimulating IgE synthesis remain to be elucidated. It is possible, however, that these different pathways may share the ability to activate switch recombination events in B cells while IL-4 acting to render the Cε locus accessible.

Modulation of IgE synthesis by other cytokines Although IL-4 is critical for the induction of IgE, other cytokines have also been found to regulate IL-4 induced IgE synthesis.

Particularly important, IFNγ has been demonstrated to be capable of inhibit IgE synthesis both in vitro and in vivo. Romagnani et al. (1988) have observed that the capacity of human and mouse T cell clones to induce IgE production directly correlated with the ratio of secreted IL-4 to IFNγ. IFNγ was also reported to antagonize the effect of IL-4 by down-regulate the expression of CD23 expression on B cells (DeFrance T. et al., 1987). The role of IFNγ as an inhibitor of IgE production is further supported by clinical trials. Treatment with IFNγ in vivo lowered the IgE serum levels of patients with hyper-IgE syndrome (King, 1989). With similar treatment, atopic dermatitis patients showed clinical improvement and a reduction in the spontaneous in vitro synthesis of IgE

(Boguniewicz, 1990). The mechanism by which IFNγ inhibits IgE synthesis is still under active investigation. Data up to date suggest that IFNγ may exert its function by acting on the pathway of B cell activation. It is also possible that IFNγ may act indirectly on T cells to either down-regulate IL-4 production or induce the production of other cytokines that can inhibit IgE synthesis.

Another recently described cytokine, IL-13, has been demonstrated to be a strong inducer of IgE synthesis other than IL-4 (Defrance, 1994; Punnonen, 1994). It has been reported that IL-13 selectively stimulated IgE production from B cells co-cultured with activated T cells or with anti-CD40 mAb (Defrance, et al., 1994). The further observation that neutralizing anti-IL-4 antibodies failed to antagonize the IgE stimulatory effect of IL-13, in a T cell-free culture system, demonstrated that IL-13 operates through an IL-4-independent mechanism. In addition, IL-13 was found to exert its function preferentially on $sIgD^+$ B cells which suggested that the IgE-inducing activity of IL-13 is likely resulted from isotype switch rather than from differentiation of IgE-committed precursors. Thus, IL-13 appears to contribute to the B cell maturation process by its unique way which might operate in some atopic individuals.

Several other cytokines are also implicated to be involved in modulation of IgE synthesis. IL-5, a nonisotype-specific B cell growth factor, and IL-6, a B cell differentiation factor, were

reported by several investigators to up-regulate IgE synthesis by human PBMC induced by IL-4 (Pene J. et al., 1989, Vercelli D. et al., 1989). Indeed, endogenous IL-6 seems to be critical for Ig production including IL-4 induced IgE production by PBMC, inasmuch as anti-IL-6 antibody strongly inhibits the IgE level in such cultures. Furthermore, TNF-α was shown to be synergistic with IL-4 in inducing IgE synthesis by enhancing IL-4-induced germline ϵ-chain transcription in purified B cells (DeVries, 1991). In contrast, IFNα and TGFβ have been shown to counteract the activity of IL-4 in stimulating IgE production in experimental animals and humans (Pene, 1988; Finkelman, 1991). In addition, IL-12, at picomolar concentration, markedly inhibits IL-4-induced IgE synthesis by human PBMC in vitro as reported by Kiniwa et al. (Kiniwa, 1992, Morris, 1994). Since IL-12 was demonstrated as a strong inducer for IFNγ production, it was first speculated that the IgE-inhibiting effect of IL-12 was mediated by IFNγ. However, treatment of human PBMC or mice with anti-IFNγ antibody did not totally reverse the IgE synthesis suppressed by IL-12 (Morris, 1994). Thus, it suggested that IL-12 can influence IgE production by both inducing IFNγ production and by an IFNγ-independent mechanism.

II. An overview of the present study

The primary goal of this study was to elucidate the relationship between clinical sensitivity to a common group of inhalent allergens (grass pollen) and cytokine production by fresh PBMC following physiologic (allergen-mediated) short term stimulation in subjects with allergic rhinitis and normal controls . Although much work has been done in analyzing the ralationship between human hypersensitivity and putative cytokine production, most of them utilized T cell clones or polyclonal activation as experimental approaches due to the low level of cytokine production by fresh PBMC under antigen-specific stimulation and the lack of sensitive detecting methods. The data obtained up to date have been interpretated as being consistent with preferential Th2-like activation patterns in atopic individuals. However, careful examination of the primary data reveals several fiaws and/or potential problems. These include (i) extrapolation from a very limited number of T cell clones derived per subject (frequently 1-3 T cells) to the entire T cell repertoire in vivo, (ii) the selective pressures demonstrated during T cell cloning which can dramatically skew the composition of the panels of T cell clones on which the data are based (discussed above in detail on page 9-16), and (iii) the contradictory results obtained from such studies in which some groups found decreased IL-4 and no changes in IFNγ, some shown decresed IFNγ and no changes in IL-4 and some reported changes in production of both cytokines (see page 135 for detailed

discussion). The use of such experimental approaches (described above in Section 1) may yield results that do not represent the in vivo situation elicited by physiological stimuli. In the present study, through the successful establishment of sensitive cytokine detecting methods, we were able to examine the cytokine production by fresh PBMC in response to physiological (allergen) stimulation, and compare these parameters in atopic and normal subjects. Our hypothesis was that a difference in the balance of cytokine production characteristic of Th1 and Th2-like responses could be demonstrated using short term antigen specific stimulation in primary culture of fresh PBMC.

The approaches we developed for analysis of the cytokine gene expression pattern were short term antigen stimulated primary bulk cultures and limiting dilution analysis of human PBMC directly ex vivo.

Limiting dilution analysis (LDA) is quantitative approach which allows estimation of the frequencies of cells participating in an immune response. Its main advantage is that it allows determination of the frequency of one cell population, which may be of very low abundance, dispersed in another. Extensively used in animal studies, LDA has also been used to characterize human T cell responses by examining proliferative cell frequency (Teppler et al., 1993; Schauf et al., 1993), CTL precursor frequencies (Sharrock et al., 1990; Carmichael et al., 1993) or the frequency

of cells producing IL-2 (Moretti et al., 1992; Waldman et al., 1992; Schulick et al., 1993) in a number of clinical conditions. These studies examined the intensity of human T cell activation rather than the nature of the response induced. Traditional LDA approaches often utilize up to 10-18 day culture in the presence of antigen, alloantigen or polyclonal activators, extensive washing and then antigen or polyclonal restimulation for 24-48 hours followed by evaluation of the parameters examined (ie. IL-2 production) as means of determining precursor frequencies. Our choice, however, of using short term culture directly ex vivo was driven by two considerations: (i) our interest was in those cells currently, as opposed to precursors which are potentially, capable of cytokine synthesis, and (ii) Extensive evidence has demonstrated that the in vitro conditions under which human or murine T cells are clonally expanded can dramatically skew the data obtained. By evaluating cytokine production elicited via short term, antigen-restimulation of fresh PBMC, our intent was to minimize the selective effects associated with extended culture and unphysiologic T cell activation protocols. Antigen was chosen as the means of activation instead of polyclonal stimulator because it more closely represents the physiological signals encountered in vivo. Furthermore, IL-4 and IFNγ were selected as readouts in preference to IL-2 (as used to evaluate T cell function in other systems) as they provide a more detailed information of the characteristics of the response (ie. Th1 vs. Th2) induced. As such,

this method represent a useful approach for evaluating ongoing cytokine production in the circulating immune repertoire in response to physiologic activation signals.

The present study demonstrated that (i) The frequency of IL-4 producing cells in peripheral blood is significantly increased and the frequency of IFNγ producing cells is significantly lower in patients with seasonal allergic rhinitis compared to that of normal subjects. The decreased ratio of IFNγ to IL-4 synthesis indicates an imbalanced Th1 to Th2 response upon in vivo exposure to grass pollen; (ii) IL-12 is capable of inducing intense IFNγ synthesis by otherwise unstimulated human PBMC as well as substantially enhancing antigen-stimulated IFNγ synthesis. IL-2 is synergistic with IL-12 in its IFNγ-inducing activity. (iii) PBMC from allergic individuals showed an impaired response to rIL-12 in terms of IFNγ production. We speculate that this defect might be instrumental in understanding the mechanism by which differential effector responses develop in allergic and normal individuals upon in vivo allergen challenge.

MATERIAL AND METHODS

I. MATERIALS

1.1 Patients and clinical evaluation

The study groups consisted of 26 grass pollen allergic individuals (9 females) and an equivalent number of normal non-atopic volunteers (13 females) as controls.

Allergic individuals were recruited on the basis of (i) a minimum of a two year documented history of seasonal allergic rhinitis severe enough to have regular daily treatment with a topical corticosteroid spray and/or H1 receptor antagonists. (ii) positive epicutaneous tests (wheal diameter at least 4mm greater than negative control) to the grass pollen mix used for in vivo and in vitro testing (Grass pollen mix 1649, consisting of June/Kentucky Blue grass, Timothy grass, Orchard grass, Brome grass and Red-top grass, Hollister-Stier/Miles, Canada, Toronto, Ontario). Normal volunteers had no history of asthma or other allergic disorders including allergic rhinitis. They exhibited negative epicutaneous tests to the grass pollen mix used in this study. All participants were between 18-35 years of age. This study was carried out following review by the University of Manitoba Ethics Committee on Human Subjects in Research.

1.2 Chemicals and reagents

Hydrochloric acid, ethyl alcohol, sodium azide, sodium phosphate monobasic, sodium carbonate, sodium bicarbonate and

paraformaldehyde were purchased from CanLab (Winnipeg,MB). Bovine serum albumin, P-nitrophenyl phosphate (PNPP), concanavalin A(Con A) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, Tween 20 and Sodium hydroxide from Mallinckrodt Canada Inc. (Mississauga, Ontario). Glutaraldehyde and 2-Mercaptoethanol from Kodak (Rochester, N.Y.). Trypan Blue from Matheson Coleman and Bell (Ohio, USA). HBSS (Hank's balanced salt solution), L-glutamine, penicillin, streptomycin and fungizone from Flow Laboratories (Mclean, VI). RPMI 1640 from Gibco Laboratories Life Technologies, Inc. (Grand Island, NY). ^{3}H-thymidine from ICN Radiochemical (Montreal). BNHS (D-Biotin-e-aminocaproic acid N-hydroxysuccinimide ester) from Boehringer Mannheim (Montreal, PQ). DMSO (Dimethyl sulfoxide) and 2-propanol from Fisher Scientific (Ottawa, Ontario). Streptavidian-alkaline phosphatase from Jackson Immuno Research Laboratories (West Grove, PA). Readysafe scintillation fluid from Beckman (Mississauga, Ontario). Histopaque-1077 and EDTA from Sigma Chemical Co.(St.Louis. MO). Grass Pollen Mix from Hollister-Stier/Miles, Canada). PHA from Difco (Detroit, MI). Recombinant human IL-2 and anti-human-IL-2 Ab were gift from Chiron Corp (Emeryville, CA). Recombinant human IL-4 and anti-IL-4 Ab were kindly provided by Sterling Winthrop Inc. (Collegerville, PA). Recombinant human and mouse IL-12 were gifts from Hollmen-LaRoche. Purified polyclonal rabbit anti-human IFNγ and purified monoclonal anti-human IFNγ cocktail (MIF 3009 and MIF 3125 recognizing

spatially distinct epitopes) were kindly provided by Dr. F. Jay, University of Manitoba, (Winnipeg, Canada). Standard IFNγ was provided by Dr. C. Laughlin (NIAID, NIH) and Hybridoma CT.h4S cell line was from Dr. W. Paul (NIAID, NIH). Hybridoma HT-2 cell line was obtained from Dr. K. Rock (Harvard Medical School, Boston, USA). Hybridoma OKT4 cell line (anti-CD4 Ab) and 2A3 (anti-IL-2 receptor Ab) were provided by ATCC (American Type Culture Collection, Rockville, MD).

1.3. EQUIPMENT

Laminar flow biological safety cabinets (model Nu-408EM-400 and NU-408FM-600) were purchased from Nuair Inc. (Plymouth, MN). Water-jacketed incubator model 3158 was purchased from Forma Scientific, Division of Mallinckrodt Inc. (Marietta, OH) and model WJ501-TAUA was from Revco Scientific Inc.(Asheville, U.S.A.). Sorvall RT 6000 B refrigerated centrifuge was a product of Dupont Canada Inc. (Burnaby, B.C.) and TJ-6 centrifuge was made by Beckman (Mississauga, ON). UV max kinetic microtitre reader with Softmax software was a product of Molecular Devices Corporation (Menlo Park, CA). Spectrophotometer was a product of Carl Zeiss (Germany). PHD cell harvester (model 200A) was a product of Cambridge Technology Inc. (Cambridge, MA). Liquid scintillation analyzer (Packard Tri-Carb 2200CA) was purchased from Packard Instrument Company (Downer Grove, IL). Vortex-Genie was produced by Fisher Scientific (Ottawa, ON). One microscope was purchased from Carsen Medical Scientific Co. (Winnipeg. MB). Centriprep-30 concentrators

were products of Amicon (Oakville, ON). 96 well ELISA plates, polystyrene flat-bottom tissue culture plates, U-bottom plates and 24 well tissue culture plates were purchased from Corning Science Products (Missisauga. ON). 96 well V-bottom plates were purchased from Nunc, Denmark. 96 well ELISA plate washer was a product of Skatron Instruments As, Norway. Cell irradiation was done at Manitoba Cancer Foundation using a Cobalt 60 source. Software used for frequency calculation in limiting dilution analysis was generously provided by Dr. C. Orosz, Ohio State University, USA.

II. METHODS

2.1. Purification of PBMC (peripheral blood mononuclear cells) from freshly collected human blood

20 ml whole blood was collected into 1 ml of 2.7% EDTA, diluted with equal volume of saline solution and then gently layered as approximately 10 ml volume onto 3.5 ml Histopaque-1077. PBMC enrichment was typically carried out within 30 minutes of blood collection and always within 2 hours. After 30 minutes centrifugation at 1600 rpm, PBMC were collected from the interface of the red blood cells and the serum. PBMC were then washed twice in serum-free saline solution. The number and the percentage of viable cells were determined by staining cell preparation with trypan blue exclusion and counted using a haemocytometer. The yield of cells from 20 ml blood was normally 30-40 x 10^6 and the cell viability was more than 99%.

2.2. Determination of protein concentration of grass pollen mix

2.2.1. Lowry protein assay

Serially diluted protein standard solution (ovalbumin 2 mg/ml) and grass pollen samples were supplemented with 0.15 N NaCl to 1 ml. Equal volumes (1.5 ml) of Lowry solution A (1% $CuSO_4.5H_2O$) and solution B (2% Sodium Tartrate) were mixed first and the final volume was raised to 150 ml by addition of solution C (2% Na_2CO_3). The final solution was well mixed and freshly prepared for each assay. 5 ml of this solution was added to each standard and sample tube and vortexed vigorously. After 15 min incubation at room temperature, 0.5 ml 1 N phenol reagent was added and mixed well. Absorbency was determined at 720 nm in a spectrophotometer after the tubes were allowed to sit another 30 min and vortex again. Protein concentrations of the samples were calculated by reference to the standard curve.

2.2.2 BCA protein assay

The modified Bicinchoninic acid (BCA) protein assay system was used for quantitation of protein concentration in grass pollen mix. Serially diluted protein standard and samples were added to a 96 well ELISA plate followed by addition of working reagent (mixture of reagent A and B provided by the manufacturer). After 30 seconds shaking on a microtiter plate shaker, the plate was covered and incubated at 37°C for 30 minutes. Absorbency at 570 nm was read using a UV max microtiter plate reader and the protein

concentration in the sample was determined according to the standard curve.

2.3. Determination of optimal Grass Pollen concentration for in vitro stimulation of PBMC

2.3.1. By examining proliferation of PBMC in bulk culture

Initially, proliferation assays were carried out to determine the optimal concentration and kinetics of grass pollen mediated stimulation for use in later assays. Essentially, cells were purified and cultured at 6 x 10^5/well in 100 ul complete tissue culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 50 uM 2-mercaptoethanol, 100 units/ml penicillin, 100 ug/ml streptomycin, 100 units/ml fungizone, and 10% heat-inactivated fetal calf serum) in the absence and presence of different concentration of Grass pollen mix (5%, 2.5% 1% and 0.5% dilution of grass pollen, corresponding to approximately 2000, 1000, 400 and 200 ug/ml). After 5 day incubation at 37°C in 5% CO_2, each microwell received 1 uCi of [^{3}H] thymidine. The culture was incubated for another 6-8 hours and the contents of each well were harvested onto glass fibres. [^{3}H]- thymidine uptake was measured by liquid scintillation counting.

2.3.2 By determining frequencies of cytokine producing cells in LDA

To directly examine the impact of different antigen concentration

on cytokine production, parallel LDA was set up by utilizing the same experimental conditions with the antigen concentration as the only variable factor. Specifically, PBMC from 3 independent subjects were cultured from 3 x 10^3 to 1 x 10^5/well in the presence of 40 or 200 ug/ml grass pollen allergen. 48 replicate cultures were set up for each cell concentrations. After 3-day incubation at 37°C, culture supernatant was harvested and analyzed for IFNγ and IL-4 production. The frequency of cytokine producing cells was calculated using the maximum likelihood method (As described in detail in a later section).

2.4. Irradiation of cells

Cells (autologous fresh PBMC) to be used as APC (antigen presenting cell) in vitro were isolated, washed, counted and then suspended in complete culture medium. They were irradiated at 3000 rads, 6000 rads or 8000 rads in a ^{60}Co irradiator, washed again, recounted and used for culture at 2 x 10^4 cells/well.

2.5. Preparation and purification of mAb

2.5.1. Hybridoma growth

Hybridoma OKT4(anti-human-CD4) and 2A3 (anti-IL-2 receptor) were grown in 500 ml tissue culture flask at 1 x 10^5/ml in complete culture medium. Cells were left in a 37°C incubator until exhaustion (< 50% viability) and the supernatants were harvested by centrifugation for later mAb purification.

2.5.2. Purification of mAb

Tissue culture supernatants were purified by ion exchange chromatograghy and gel filtration using the Pharmacia Biopilot system. Briefly, supernatants were extensively dialysed against low ionic strength citrate buffer (0.01M, pH 5.3) and concentrated via ion exchange chromatography using S-Sepharose 35/100. The mAb containing fraction was eluted by a gradient of increasing ionic strength (NaCl 0-0.8, Na citrate 0.01 M, pH 5.3), and then applied directly to a gel filtration column (Superdex 200, 60/600 in PBS) with the mAb collected in that fraction corresponding to 150-200 kD.

2.5.3. Determination of mAb concentration

The mAb (2A3: mouse IgG1) concentration was determined by ELISA as follows. ELISA plate was coated overnight with goat anti-mouse IgG (H+L) at 5 ug/ml in coating buffer. After blocking and washing, dilutions of concentrated tissue culture supernatants or fractions eluted from the ion exchange purification process were added to the plate and incubated at 37°C for 3 hours. Serially diluted normal mouse IgG was used as standard starting from 17 ug/ml with 16 dilutions. After another wash, biotinylated goat anti-mouse IgG1 was added for 2 hour incubation at 37°C. The plate was washed again followed by addition of SA-alkaline phosphatase. After 1 hour incubation and washing, the ELISA was completed by adding substrate tablets (p-nitrophenyl phosphate in $MgCl_2$, 10% diethanolamine) and the absorbency at 405 nm was determined by an UV max kinetic

microtitre reader.

2.6. Short term limiting dilution assay

A short term limiting dilution assay system was developed to evaluate the frequency of grass pollen reactive, IL-2, IL-4 and IFNγ-producing cells in PBMC directly ex vivo (see results). The procedure was carried out as follows. PBMC were serially diluted and seeded in V bottom 96 well plates at concentrations from 1 x 10^5 to 3 x 10^3/well. 24, 36 or 48 replicates were used for each responder cell dilution depending on the experiment. Cultures were set up in the presence of 1000 ug and/or 400 ug/ml grass pollen mix with or without recombinant human IL-2 at 10 WHO IU/ml. As controls, 10 or 20 wells/ cell concentration (i) containing no responder cells or irradiated cell only, (ii) containing responder cell but no antigen in the presence or absence of IL-2, (iii)containing double diluted responder cells plus constant number of irradiated PBMC used as APC, or (iv) containing responder cells in the presence of anti-CD4 mAb, were included for different assays. In order to evaluate the antigenic specificity of cytokine production in this LDA, identical cultures were set up using purified (HayGlass and Stefura, 1991) normal rat IgG at 50 and 500 ug/ml as nominal antigen instead of grass pollen extract. After 3-4 day incubation at 37°C in 5% CO_2, supernatants were harvested and analyzed immediately or stored at -20°C.

2.7. Limiting dilution analysis calculation

Minimal estimates of the frequency of PBMC from a given patient producing IL-2,IL-4 or IFNγ in response to allergen stimulation were calculated by analysis of the Poisson distribution relationship between the number of responder cells added to the limiting dilution microwell and the percentage of cultures which failed to produce that particular cytokine. For each assay, a given well was considered positive if the reading was greater than three standard deviations above the mean reading obtained from negative control wells which lacked responding cells. Frequency was calculated using the maximum likelihood method.

2.8. Cytokine determination

2.8.1. IL-2

HT-2 cells are highly responsive to IL-2 and were used for IL-2 quantitation. After being washed 3 times with cytokine free culture medium , 7 x 10^3 HT-2 cells in 50 ul fresh medium were added to each well which contained 50 ul of tissue culture supernatant in sterile 96 well, flat bottom plate. Cultures were incubated at 37°C in 5% CO_2 for 24 hours, and a total of 50 ug MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)in 10 ul RPMI 1640 was then added to each well. After another 6 hour incubation at 37°C in 5% CO_2, 190 ul of stop solution (2:1 2-propanol:H_2O 0.04 N in HCl) was added to each well. Each well was then mixed 20 times with a multichannel pipet in order to

completely solubilise the formazan dye. The plate was left at 37°C for 10 more minutes, and A_{560} was determined by an UV max kinetic microtitre reader. Each assay was calibrated using human rIL-2 standard titred from 5.2-666 pg/ml (specific activity of standard was 1.8×10^7 WHO IU/mg) with IL-2 deficient medium used as negative controls. The sensitivity of this assay was typically 26 pg/ml and this HT-2 cell line does not respond to IL-4 below a concentration of 500 pg/ml.

2.8.1. Maintenance of HT-2 cell

The murine IL-2-dependent cell line, HT-2, was maintained in RPMI complete culture medium ($1-2 \times 10^4$ cells/ml) supplemented with recombinant IL-2 at 20 U/ml (1332 pg/ml) in 25 cm^2 flasks. Every 2-3 days, as required, the cells were subcultured and/or used for detection of IL-2. While not required, the cells were stored in liquid nitrogen for extended periods.

8.2.3. IL-4

The CT.h4S cell line, which is highly responsive to human IL-4, was used in an MTT based assay. Briefly, log phase cells were washed 3 times with cytokine free culture medium immediately prior to culture and counted. For optimal sensitivity, the viability of the cell has to be more than 95%. Using 96 well flat bottom plates, 5×10^3 cells in 50 ul fresh complete culture medium were then added to each well which contained 50 ul tissue culture supernatants. Optimal sensitivity was obtained when cells were

cultured at 37°C for 40 hours before 50 ug MTT in 10 ul RPMI 1640 were added to each well. After another 6 hour incubation, 190 ul stop solution (2:1 2-propanol: H_2O made 0.04N in HCl) was added to each well. Each well was vigorously pipetted, the plate was incubated for 10 more minutes, and A_{560} was determined by an UV max kinetic microtitre reader. IL-4 deficient medium was used as negative control in each assay. In order to rule out the possibility that the CT.h4S cells proliferated in response to IL-2 which is produced by the PBMC, either anti-human IL-2 Ab was used in assays at a concentration enough to block 250 pg/ml IL-2 or, as an additional approach, CT.h4S cells hyposensitivity to IL-2 were routinely monitored by regularly determining the IL-2 responsiveness of CT.h4S (see below). If the responsiveness to IL-2 developed, the cells were discarded and fresh, IL-2 nonresponsive stock was started from liquid nitrogen. The specificity of this bioassay was determined by parallel analysis of replicate supernatants in the presence of purified polyclonal anti-IL-4 Ab to neutralize the activity of IL-4. This assay detected 0.5-1.0 pg/ml IL-4, quantitating amounts above 1 pg/ml. As an internal control and to monitor assay sensitivity, a standard curve (0.625- 80pg/ml) generated with human rhIL-4 (Specific activity was 1.8×10^7 U/mg) was carried out in quadruplicate for each assay. Standard deviation was usually <5%, frequently 2-3%.

2.8.4. Maintenance of CT.h4S cells

The CT.h4S cells were maintained in RPMI complete culture medium

(0.5-1 x 10^5 cells/ml) supplemented with 2000 pg/ml recombinant human rIL-4 in 25 cm^2 flask. Every 3 days the cells were subcultured and/or used for detection of IL-4.

2.8.5. IFNγ

2.8.5.1. Biotinylation of anti-IFNγ mAb

Anti-human-IFNγ mAb cocktail MIF 3009 and MIF 3125, developed by Dr. F. Jay, which recognize spatially distinct epitopes, were purified from ascites. A biotin-spacer conjugate (BNHS) was dissolved in anhydrous DMSO at 2 mg/ml and immediately added to antibody cocktail at the ratio of 1:4 (BNHS:antibody v/v). The reaction was typically allowed to proceed for 2 hours at room temperature in the dark, following which unbound biotin was removed by extensive dialysis against PBS at 4°C. The optimal concentration of mAb cocktail for use in this assay was determined by ELISA.

2.8.5.2. IFNγ ELISA

96 well ELISA plates were coated with purified polyclonal rabbit anti-human IFNγ (PIF 3004) at 1 ug/ml in coating buffer(0.05 M carbonate buffer, pH 9.6) for 2 hours at 37° C or overnight at 4°C. Each plate was then blocked with blocking buffer (1% BSA prepared with PBS, pH 7.4) for 30 min-2 hours at room temperature and washed extensively. Culture supernatants and an internal IFNγ standard were serially diluted and added to the plate. After 3 hours incubation at 37°C , plates were washed 5 times, following which

biotinylated anti-human IFNγ (MIF 3009 and MIF 3125) was added for an overnight incubation at 4°C or 2 hour incubation at 37°C. The plate was then washed before streptavidin-alkaline phosphatase was added for 30 minutes at 37°C. The plate was washed again , p-nitrophenyl phosphate in $MgCl_2$, 10% diethanolamine was added and A_{405} was determined using the UV max kinetic microtitre reader at 30 and 60 minutes. The internal standard used in this assay was an IFNγ containing PHA stimulated PBMC supernatant calibrated against Human IFNγ reference reagent Gg 23-901-530 (specific activity 7 x 10^5 U/mg, 1 U = 115 pg, provided by Dr. C. Laughlin,NIAID,NIH). The lower limit of detection was typically 0.25 U/ml, with quantitative measurement of IFNγ at 1.0 U/ml. Standard deviation ranged from 5-10% in most assays.

2.8.6. IL-12

2.8.6.1. Bulk culture for IL-12 production

Bulk culture was used for IL-12 assay. In brief, purified PBMC were cultured at 10, 6, or 2 x 10^7/ml (50ul/well) in 96 well round bottom plates using complete culture medium . Cells were cultured at various conditions including: (i) unstimulated, (ii) polyclonally stimulated with 0.0075% and 0.0019% pansorben, (iii) stimulated with 400 ug/ml and 100 ug/ml grass pollen extract. Duplicate cultures were set up for each group. Culture supernatants were harvested at multiple time intervals (20 hours - 5 days) and analyzed immediately or stored at -20°C.

2.8.6.2. Determination of IL-12

IL-12 is a recently discovered cytokine. In contrast to the bioassays for IL-1, IL-2, IL-3, IL-4 and IL-6, that use cloned cell lines as the responder population, an IL-12 responsive cell line available for routine use in a bioassay to quantitate IL-12 has not yet been identified. Although a number of MAb to human IL-12 have been derived, and an ELISA for measuring IL-12 protein has been previously described (Chizzonite et al, 1991), the poor sensitivity and the inability to detect biologically active cytokine limited its usage.

Based on the ability of IL-12 to stimulate the proliferation of PHA-activated T lymphoblasts, an antibody-capture bioassay was suggested by Dr. M. K. Gately and R. Chizzonite (Hoffmann-La Roche, New Jersey) which is basically as follows. Human IL-12 is captured from IL-12 containing culture supernatants by rat anti-human IL-12 MAb adsorbed to an ELISA plate. The test fluid is then washed from the wells and replaced with a PHA-activated human lymphoblast suspension. The lymphoblasts proliferate in response to the captured IL-12, and [^{3}H]-thymidine incorporation is measured. Although this method is very sensitive and specific to biologically active IL-12, the assay sensitivity is highly dependent on successful preparation of human PHA blasts. Besides, it is time-consuming (5-6 day/assay) and requires radioisotope involvement.

As IL-12 has been demonstrated to have the ability of stimulating

CD4 T cell and NK cell to produce large amounts of IFNγ, we therefore developed an alternative "antibody-capture bioassay" to determine IL-12 production. Essentially, 96 well flat bottom plates were coated with anti-human IL-12 antibody at 10 ug/ml (50 ul/well) in sterile coating buffer and incubated for 3 hours at 37°C. After the plate was washed 3 times with RPMI 1640, Grass pollen or polyclonally stimulated culture supernatants and an internal IL-12 standard which was serially diluted were added to the plate for 3 hour incubation at 37°C. The plate was washed 3 times with serum free RPMI to remove other cytokines in the experimental sample which might synergize with or antagonize the ability of IL-12 to activate the PBMC. PBMC which were thawed from liquid nitrogen or purified from freshly collected whole blood were added to the plate. After different culture periods, supernatants were harvested for determination of IFNγ production induced by IL-12 in the culture supernatants (see results). Since the use of fresh human PBMC saved the trouble (and variability) of preparation of PHA blasts and we have an well established ELISA for IFNγ detection, this alternate antibody-capture bioassay provided a simple, rapid as well as highly sensitive and specific means for quantitating human IL-12.

2.8.6.3 Characterization of IFNγ inducing activity of IL-12

Bulk culture was used to characterise biological activities of IL-12. Thawed or freshly purified PBMC were cultured at 1 x 10^7 and 3 x 10^6/ml (50ul/well) in various conditions (i) cells alone

(ii) with different concentrations of IL-12 (iii) with different dose of IL-2 (iv) with IL-12 in the presence or absence of IL-2. After different culture periods (20-40 hours) at 37°C, supernatants were harvested for determination of cytokine production.

2.9. Statistical analysis

Normally distributed data are presented as mean values + SD. Statistical significance was determined using Student's t test. Non-parametric data are presented as median values and statistical significance was determined with the Mann Whitney U test.

RESULTS

I. Identification of optimal culture conditions for LDA

1.1. Determination of optimal concentration of grass pollen for in vitro stimulation of PBMC

The first technical variable we investigated was the optimal concentration of grass pollen mix that was maximally effective in stimulating cytokine production in limiting dilution microcultures. An initial dose-response relationship was determined by measuring proliferative responses of PBMC to various concentrations of grass pollen mix in conventional bulk culture. Cells were cultured at 6.0×10^5/well in complete culture medium in the presence of 0-2000 ug/ml grass pollen mix. After 5 day incubation, each well received 1 uCi/ml ^{3}H-thymidine and ^{3}H-uptake was determined by scintillation counting. As shown in Table 1, the optimal antigen concentration was not sharply defined over the range from 100-2000 ug/ml, but in most cases, 400 to 1000 ug/ml antigen yielded the best proliferative response of PBMC.

To evaluate the possibility that the optimal concentration of antigen for cytokine production might be substantiallly different from the optimum for proliferation, bulk cultures were established using a range of antigen concentrations as carried out for proliferation assays (HayGlass & Stefura, unpublished data). The results indicated a similar optimum for both parameters.

Moreover, to directly examine the impact of different antigen concentration in limiting dilution analysis where the number of

responder cells varies from 3 x 10^3 to 1 x 10^5, we also established parallel LDA experiments using 400 and 2000 ug/ml grass pollen allergen in 3 independent subjects. The frequency of negative wells did not differ significantly with the use of different antigen concentrations in this system. Thus, the IL-4 frequency estimate calculated from data obtained using 200 ug/ml grass pollen is 1/64,352 (95% confidence limits, 53-83 x 10^3) while data obtained following stimulation with 40 ug/ml antigen is 1/61,818 (52-82 x 10^3, $p > 0.05$).

Table 1

Effect of concentration of grass pollen extract on bulk culture PBMC proliferation

	^{3}H-uptake (DPM)			
	Subjects			
Antigen concentration	1	2	3	4
2000ug/ml	33254	72346	26193	75488
1000ug/ml	35944	71949	50937	100680
400ug/ml	46965	72978	72688	71689
200ug/ml	29377	53017	61793	58642
0	18892	34804	41617	51904

Legend: PBMC were cultured at 6 x 10^5/well in the presence of varies concentration of grass pollen extract and [^{3}H] thymidine incorporation (dpm) was measured by liquid scintillation counting 5 days later. Results were presented as the mean value of duplicate culture at each condition.

1.2. Kinetics of antigen driven IL-4 and IFNγ production

In order to evaluate antigen stimulated cytokine production as close as possible to the in vivo situation, a characterization of the kinetics with which IL-4 and IFNγ responses were elicited following in-vitro antigen activation was necessary. Mononuclear cells from five subjects with allergic rhinitis were cultured for six daysin the presence of grass pollen extract. Concentrations used ranged from 0 to 4000 ug/ml. Each day, culture supernatants were harvested and cytokine production was measured. As can be seen from Figure 1 and Figure 2, antigen driven IL-4 and IFNγ production was undetectable prior to day 3. Although the day of peak IL-4 or IFNγ gene expression varied slightly between individuals, those who were to produce these cytokines had done so by day 3 or 4. Cytokine production reached a plateau or had decreased by day 5 and 6 in all cases.

In summary, study of the kinetics of cytokine synthesis in in vitro bulk cultures demonstrated that IL-4 and IFNγ protein were detectable in culture supernatants 2-3 days following antigen specific stimulation and reached maximum at 3-5 days. IL-4 level was decreased after 3 days whereas IFNγ production was decreased or plateaued from day 4. Based on the above results, 3 day or 4 day cultures were used in all limiting dilution assays.

A kinetic study was also done by establishing 1 day and 3 day limiting dilution assays. It was demonstrated that the cytokine

level was undetectable in day 1 culture supernatants, but at day 3, both IL-4 and IFNγ were readily detected (data not shown). Use of longer culture periods yielded lower frequencies suggesting that 3-4 day was the optimal culture period under the condition tested.

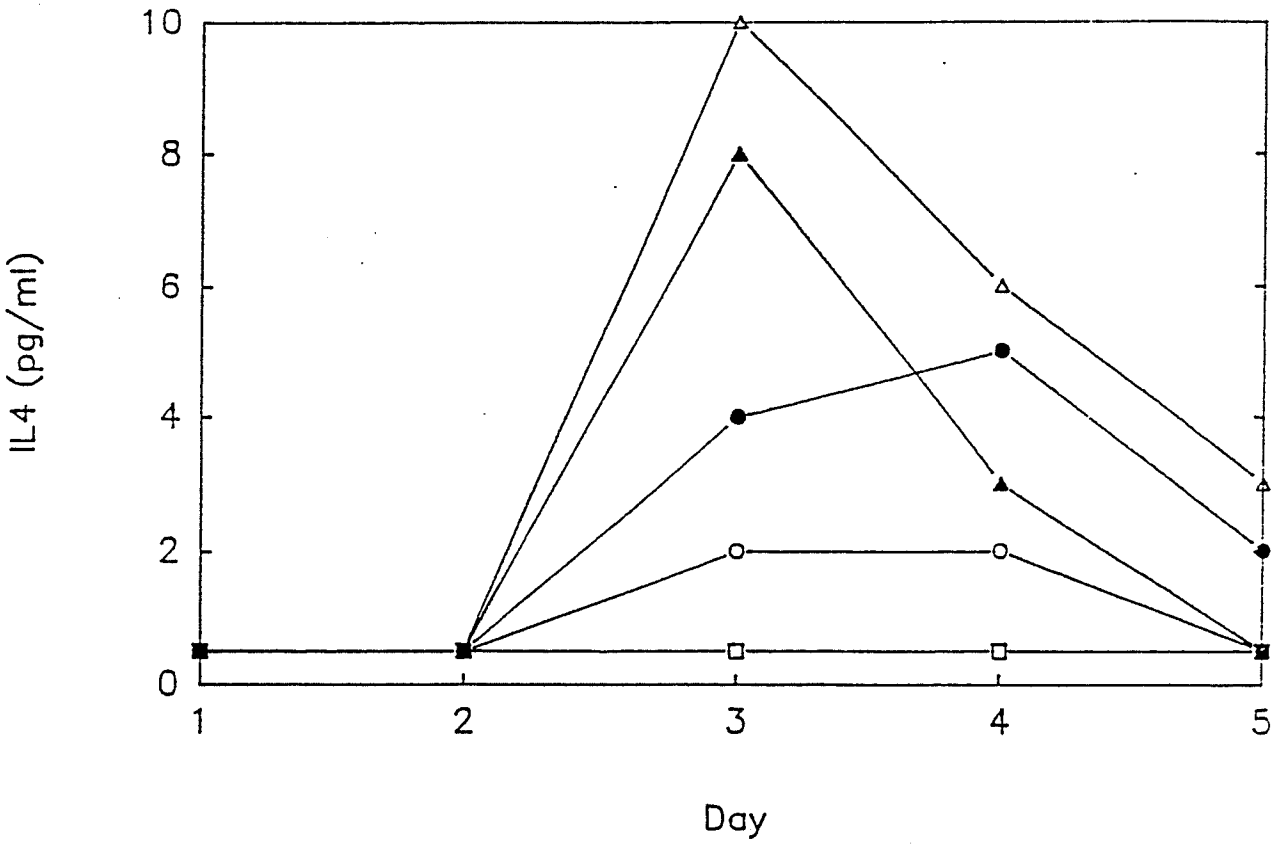

Figure 1: Kinetics of antigen-driven IL-4 production. PBMC from 5 individuals with allergic rhinitis were purified and cultured in the presence of grass pollen extract for 6 days. Each day, supernatants were harvested and IL-4 productions were tested using the MTT-CT.h4S bioassay.

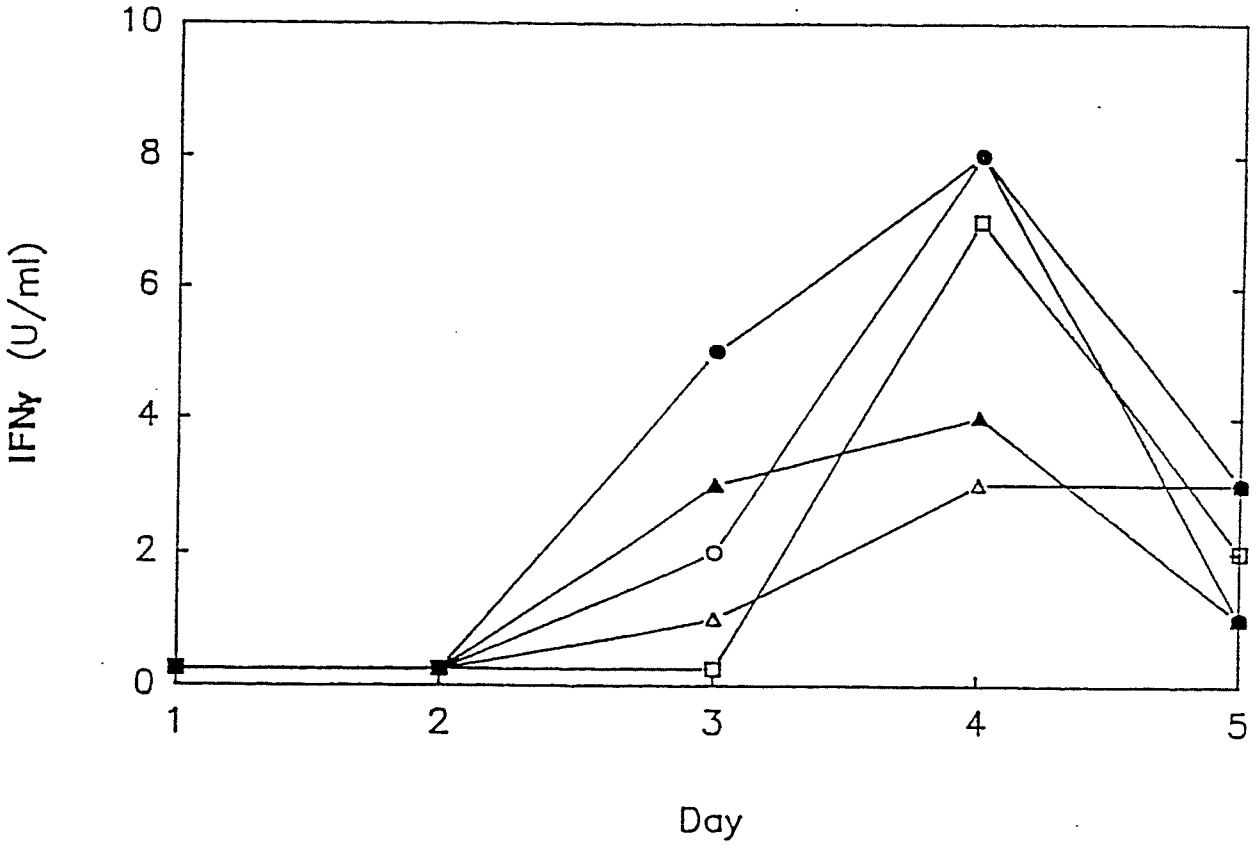

Figure 2: Kinetics of antigen-driven IFNγ production. PBMC from 5 individuals with allergic rhinitis were purified and cultured for 6 days in the presence of grass pollen extract. Each day, supernatants were harvested and tested for IFNγ production by ELISA.

II. DEVELOPMENT OF SENSITIVE CYTOKINE MEASUREMENT ASSAYS

The purpose of this study was to examine the cytokine production pattern of human individuals with immediate hypersensitivity in comparison to that of normal control individuals. Considering the limitations in interpreting the results from studies of long term cultured T cell clones, we decided to use an approach which we believed to be more reflective of the in vivo situation. Specifically, we chose to characterize antigen driven cytokine synthesis by PBMC from grass pollen allergic individuals directly ex vivo in short term (3 days) limiting dilution cultures. A major problem in previous attempts to quantitate cytokine production by fresh derived PBMC has been assay insensitivity. Most assays are capable of detecting IL-4 only at concentrations > 50-100 pg/ml, therefore requiring the use of unphysiological stimuli such as PHA, immobilized anti-CD3 or PMA and Ca^{++} ionophore in primary cultures. These stimuli may elicit cytokine production qualitatively different from that observed following conventional activation (Yang & HayGlass, 1993; Imada, et al., unpublished). Because antigen-driven cytokine production in short term cultures, especially in limiting dilution culture conditions, is of much lower intensity than that stimulated by polyclonal activator in long term T cell clones or hybridomas, a prerequisite for successful detection of cytokine synthesis in this study was the availability of highly sensitive and specific cytokine measurement techniques. We therefore initially made efforts to optimize

techniques for quantitation of very small amounts of IL-4 and IFNγ with the smallest permeable intra-assay variations.

2.1. Development of sensitive IL-4 assay

CT.h4S, a subline of CTLL, is highly responsive to human IL-4, hyporesponsive to IL-2 and does not respond to any other known cytokines. Since most in vitro culture systems, especially human samples, contain very low level of IL-2, CT.h4S cells bioassay provided a potentially valuable approach for human IL-4 detection and quantitation. In previous attempts, [^{3}H] thymidine incorporation was usually used to measure the CT.h4S cell proliferation (W. Paul, personal communication). Considering that the use of [^{3}H] thymidine was time consuming and labour intensive, we thereby developed and employed a colorimetric assay system based on that first introduced by Mosmann in 1983. This procedure based on the pale yellow salt [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) which is cleaved by active mitochondria to form a dark blue formazan product. This can be completely solubilized in acidic-isopropanol and quantified by microtitre plate reader. This method was proven to be a more sensitive (3-10 times increased in sensitivity than ^{3}H-thymidine method, Gieni, Li & HayGlass, manuscript in preparation) and rapid way to detect living and growing cells.

As shown in Figure 3, the MTT-based CT.h4S bioassay detected 0.5-1

pg/ml rIL-4, quantitating amounts above 1-2 pg/ml. The standard deviation in this assay, even at very low IL-4 concentrations, was less than 3%. This is a major improvement over the ^{3}H-thymidine assay which was usually 10-15%.

Figure 3

Sensitivity of MTT-CT.h4S assay for IL-4

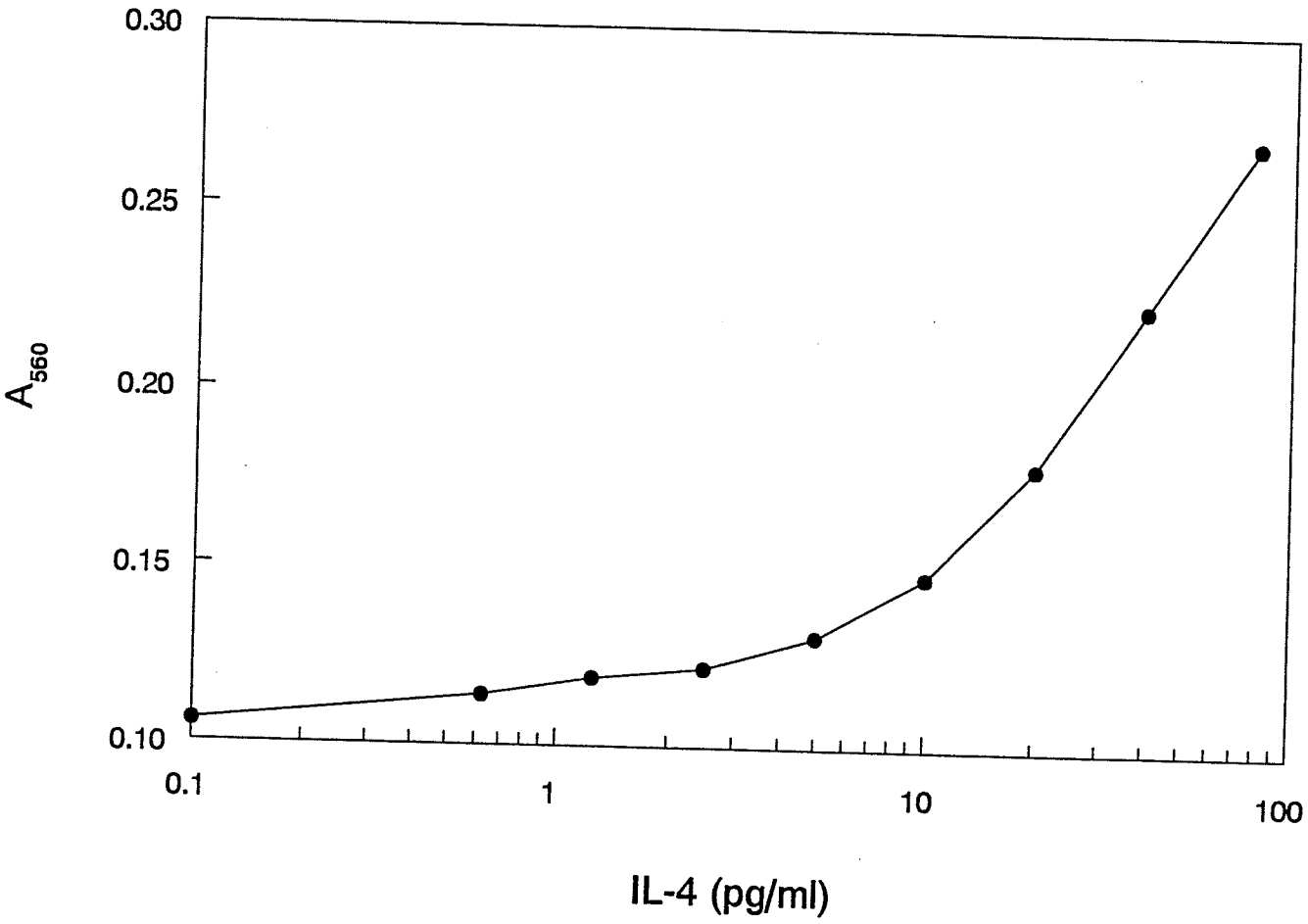

Figure 3: CT.h4S cells were cultured at 5000/well with rIL-4 from 80 to 0.625 pg/ml. Cultures were incubated for 40 hours at 37°C followed by addition of MTT for another 6 hour incubation. Data shown are mean + S.D. (S.D. are too small to be seen).

Assay specificity is critically important. Here, it was determined by parallel analysis of samples in the presence of neutralizing anti-IL-4 Ab. As also shown in Figure 4A, anti-IL-4 at 40 ng/ml totally blocked the activity of 40 pg/ml IL-4 and neutralized more than 80% activity of IL-4 at 80 pg/ml, these levels are substantially above those elicited in antigen-driven culture of PBMC (see below). Given that the parent line from which CT.h4S was initially derived is known to be highly IL-2 sensitive, we continuously monitored the IL-2 responsiveness of the CT.h4S cells used in this study. As shown in Figure 4D, CT.h4S cell did not proliferate in response to 25 U/ml rIL-2 (1700pg/ml). Moreover, deliberate addition of IL-2 at 0.25 U/ml (17 pg/ml) and 1 U/ml (66pg/ml) (levels similar and or above those seen in antigen-stimulated culture of PBMC respectively, see below) in a standard IL-4 assay (Figure 4B and 4C) did not alter the assay sensitivity.

In order to maintain assay sensitivity and specificity, regularly monitoring the CT.h4S growth condition was absolutely necessary. These cells have been reported to "spontaneously" acquire IL-2 sensitivity, thereby making them useless for IL-4 assay (ie, after several months of continue culture) (C. Kinzer, personal communication), and we observed a similar phenomenon (see below). We dealt this problem by utilizing the following procedures. The CT.h4S cell line was routinely maintained in complete tissue culture medium supplemented with a high level of recombinant human IL-4 (2000 pg/ml). Every several weeks, the

response of cells to IL-4, or to IL-2 was measured to confirm the specificity of the assay. One phenomenon we found was that after being maintained in regular culture condition for a long time , the CT.h4S cells lost their sensitivity to and/or specificity for IL-4. As shown in Figure 5A, the cells which had been maintained for about 1 year can only respond to IL-4 at above 10 pg/ml, and they also showed a very high responsiveness to IL-2 even at 0.625 U/ml (Figure 5B). At this stage,the approaches we took to ensure sensitivity of this assay was either to propagate new cells from the original source (ie, stored in liquid nitrogen) or to monoclonally select for cells which had the best sensitivity and specificity as was initially carried out in Paul's laboratory. Figure 6 shows the response of new CT.h4S cells to IL-4 and IL-2. The detection level of IL-4 was 1.25-2.5 pg/ml and the cells did not respond to IL-2 even at 25 U/ml (1700 pg/ml).

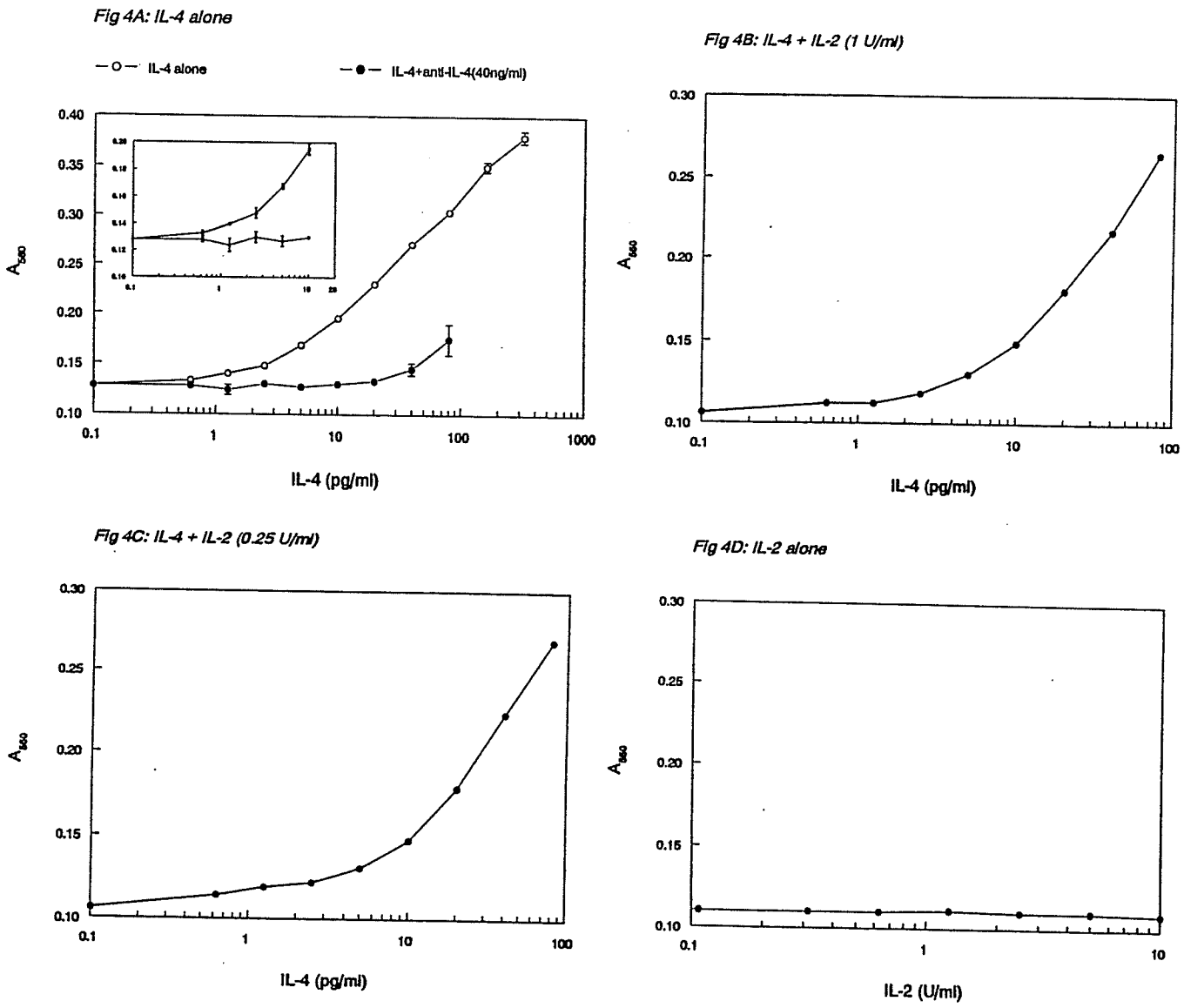

Figure 4. Specificity of MTT-based CT.h4S bioassay for IL-4. A neutralizing anti-IL-4 Ab at 40 ng/ml(A), rIL-2 at 0.25 U/ml (B) and 1 U/ml (C) were added to standard MTT-CT.h4S assays for IL-4. Standard rIL-4 ranged from 80 to 0.625 pg/ml. CT.h4S cell response to rIL-2 (10-0.16 U/ml) were also tested (D). Data were shown as mean + S.D. Note that SD are too small to be seen except in expanded graph (inset Figure 4A).

Figure 5

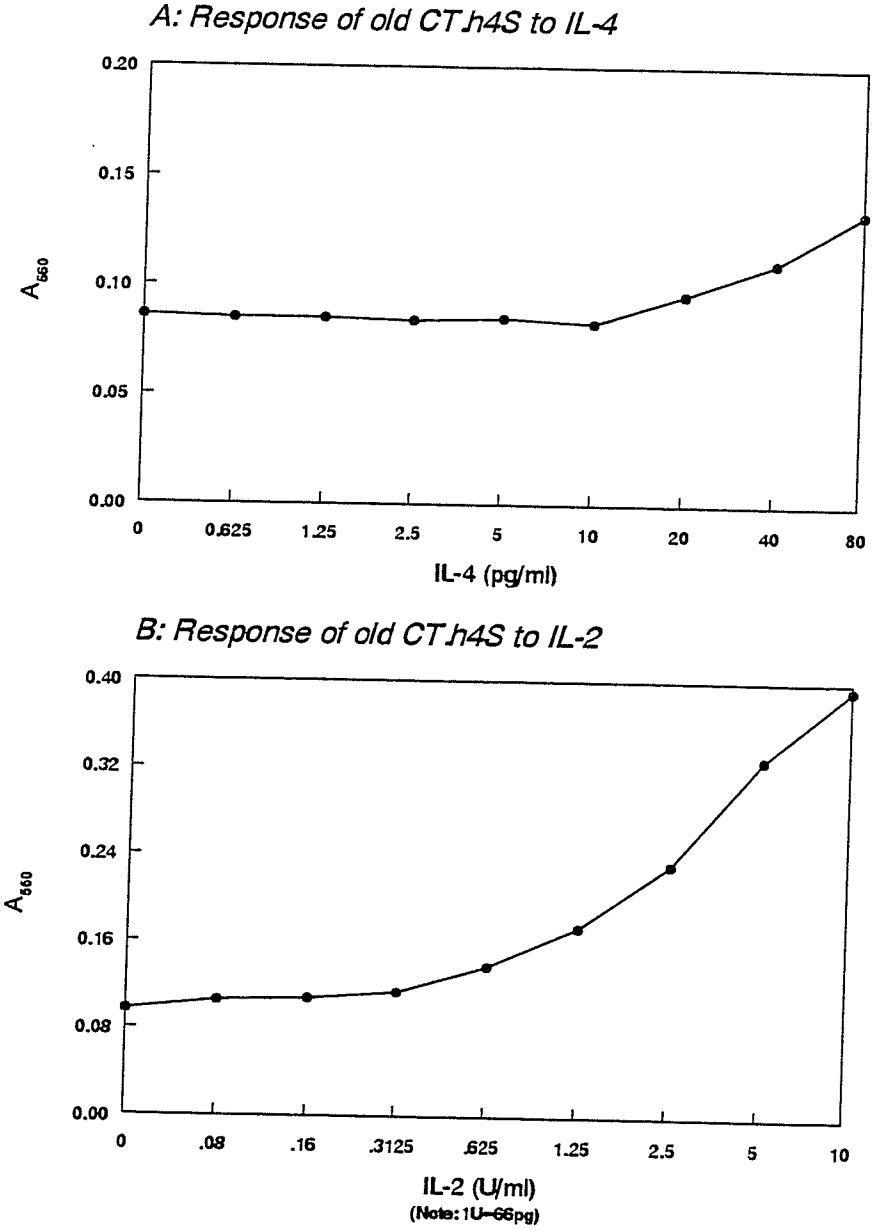

Figure 5: Responses of old CT.h4S to IL-4 (A) and IL-2 (B). CT.h4S cells, which had been maintained for about 1 year, were washed and cultured in the presence of IL-4 (80-0.625 pg/ml) or IL-2 (20-0.16 U/ml). After 40 hours, MTT was added to each well followed by 6 hour incubation. Absorbance were determined at 560nm. Data are shown as mean of triplicate cultures.

Figure 6

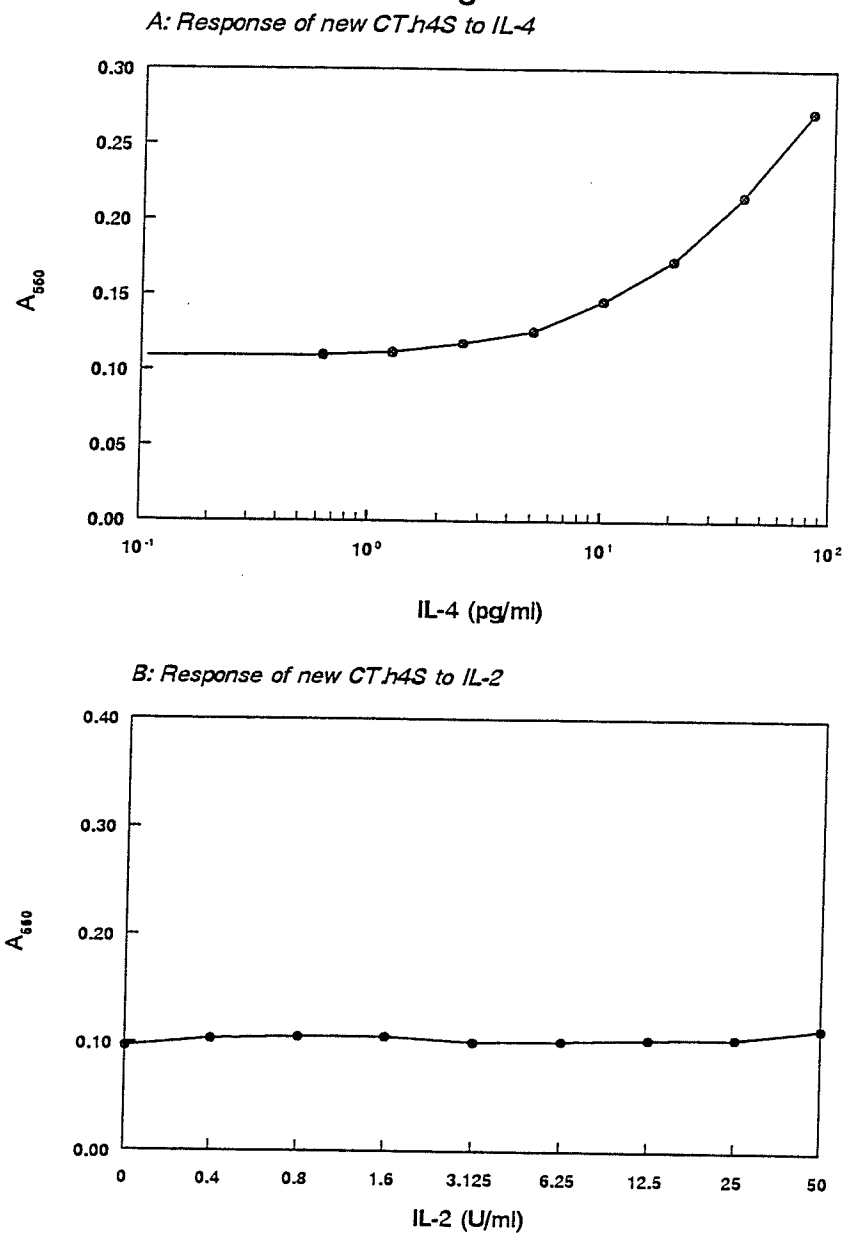

Figure 6: Responses of new CT.h4S to IL-4 (A) and IL-2 (B)
New CT.h4S cells were washed and cultured in the presence of rIL-4 (80-0.625 pg/ml) and rIL-2 (20-0.16 U/ml, equivalent to 1332-10.6 U/ml) for 40 hours followed by addition of MTT. Absorbency at 560-690 nm was determined after 6 hours. Data are shown as mean of the triplicate cultures.

Another important technical detail that we found also contributed to the sensitivity of the assay was the way to manipulate the cells before each assay. First, the CT.h4S cells had to be subcultured one day before being used because the assay sensitivity is highly dependent on the cells being in log phase growth. Second, the washing style, for removing the high level of IL-4 existing in the culture medium, had an important effect on CT.h4S cell response to IL-4. As demonstrated in Figure 7, cells being washed one time for 40 or 60 seconds at 1600 rpm did not differ in their response to IL-4 (1-2.5 pg/ml), but cells being washed 2 times for 60 seconds each at 1600 rpm detected IL-4 at 5 pg/ml (2-5 fold poorer sensitivity). Moreover, cells which were washed 3 times for 5 minutes each time at 1600 rpm exhibited even worse response to IL-4 (sensitivity: 10-20 pg/ml). Optimal assay sensitivity was obtained by washing cells at 1200 rpm just one time for 40 seconds. Under these conditions, 1.25 pg/ml IL-4 was the detection level. This was the method we adopted for washing CT.h4S cells in all remaining assays.

Thus, as demonstrated above, this MTT-based IL-4 assay was highly sensitive and specific. It detected IL-4 at about 1-2.5 pg/ml and did not respond to IL-2 below 25 U/ml (1700 pg/ml). Since the substrate does not interfere with the measurement of the product, there is no need for removal and washing steps, thus helping to increase the speed of the assay and minimize variability between

samples which was typically 2-3%. Moreover, it avoided the use of potentially hazardous radioisotopes. The optimal sensitivity and specificity were maintained by monitoring the cell condition and using the best washing style.

Figure 7

Effect of washing CT.h4S on IL-4 assay

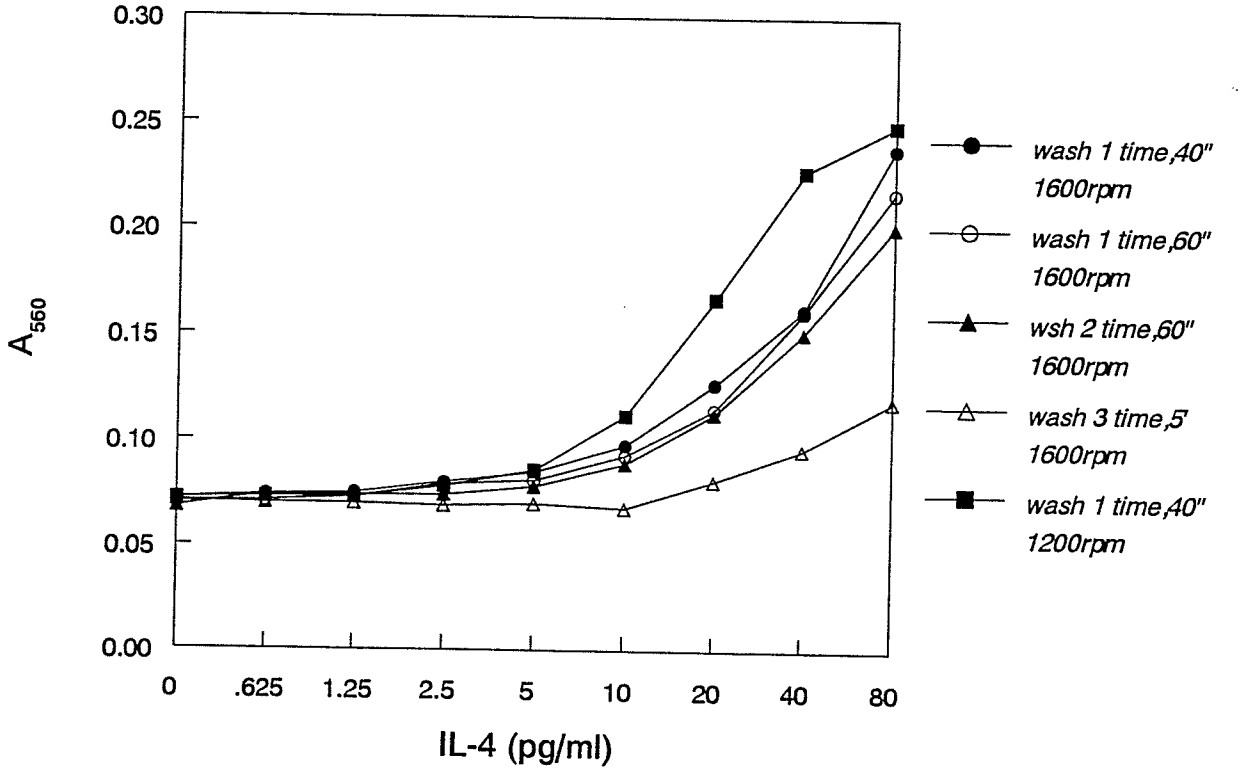

Figure 7: Effect of washing on response of CT.h4S to IL-4. CT.h4S cells were washed differently,

A: 1 time for 40 seconds at 1600 rpm,
B: 1 time for 60 seconds at 1600 rpm,
C: 2 times for 60 seconds each at 1600 rpm,
D: 3 times for 5 minutes each at 1600 rpm,
E: 1 time for 40 seconds at 1200 rpm .

The cells were then used in a standard MTT-based IL-4 assay with the concentration of rIL-4 ranging from 80 to 0.625 pg/ml. Data were shown as mean of duplicate cultures.

1.2. Establishing sensitive IFNγ ELISA

Until recently, many IFNγ assays were based on its anti-viral activity. The assays currently available include viral inhibition assays inhibition of WEHI-279 cell proliferation (for murine IFNγ), induction of MHC class II antigen and several others. Most of these assays offer a high degree of sensitivity. However, as all bioassays, they share the potential disadvantage that other cytokines existing in the culture supernatant may synergize with or antagonize the effects of IFNγ. Moreover, particularly in the case of inhibition assays (as opposed to the proliferation assay), non-specific inhibitory effects of metabolites present in exhausted tissue culture supernatants may interfere with the results. Although the use of neutralizing antibodies to other cytokines has been helpful in dealing with these non-specific effects, the high variation observed when detecting of low amounts of IFNγ limited their application for antigen-driven IFNγ quantitation and detection in short term cultures. Therefore, we developed a simple ELISA for human IFNγ determination which was highly sensitive, specific and with a very low intra-assay variation. Although this ELISA did not have the sensitivity of some bioassays, the inherently higher level of IFNγ (compared to IL-4) produced in antigen-stimulated cultures did not require use of a bioassay.

Polyclonal anti-human IFNγ Ab PIF 3004 and a monoclonal anti-human IFNγ Ab cocktail MIF 3009 and 3125 which recognize spatially

distinct epitopes on natural IFNγ (provided by Dr. F. Jay) were used as capture agents. As shown in Figure 8, the lower limit of detection was typically o.125 U/ml, with quantitative measurement of IFNγ at 1.0 U/ml. Standard deviation ranged from 5-10% in most assays.

In order to optimize the assay sensitivity, other capture antibodies were also tested. Comparison of the assay sensitivities obtained by combining use of these Abs is shown in Figure 9. Using PIF 3003 instead of PIF 3004 as the capture antibody detected IFNγ concentrations at and above 1 U/ml. This was the worst in the several assays tested. Since further layers on the sandwich have been suggested to increase assay sensitivity, mouse anti-rabbit IgG antibodies were either used as coating reagents before applying first capturing antibody PIF 3003 or conjugated with biotin to detect second capturing antibody. These two approaches yielded sensitivities of 0.25 and 1 U/ml which did not improve the standard sandwich assay we initially developed.

Based on the sensitivity of the assay and the availability of the antibodies, we chose to use the standard sandwich ELISA with PIF 3004 and MIF 3125/3009 as capturing antibodies. It was highly specific to IFNγ and exhibits a very small intra-assay variation. Moreover, it has the advantage of being very rapid and simple in operation. The sole limitation of this assay, in common with all ELISA, is that it does not directly assess biological activity of IFNγ.

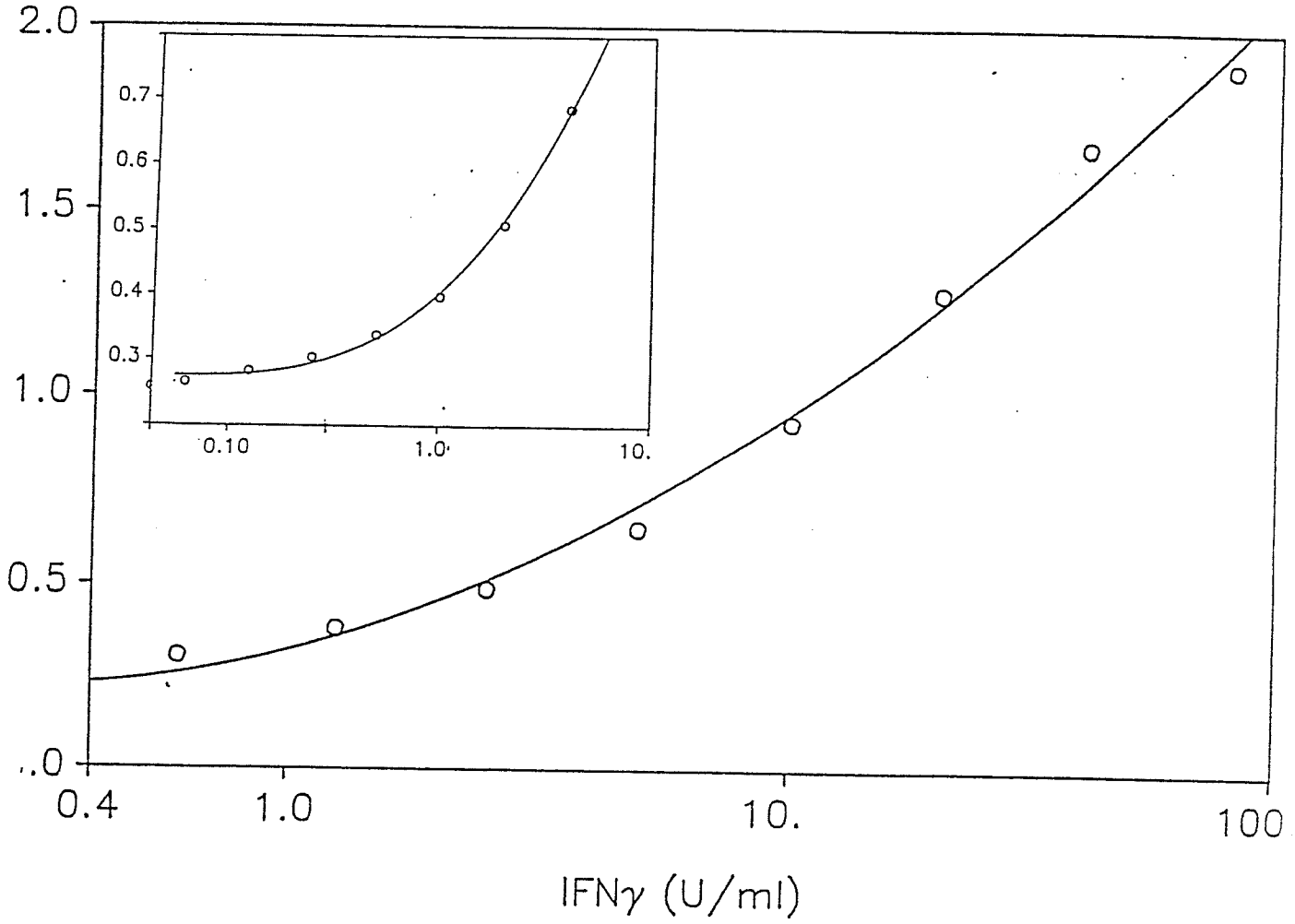

Figure 8: Sensitivity of IFNγ ELISA. Purified polyclonal anti-human IFNγ (PIF 3004) was used as capturing antibody in combination with a biotinylated mAb cocktail (MIF 3125/3009) in a streptavidin-alkaline phosphatase based ELISA. Data shown are means + S.D. of triplicate cultures.

Figure 9

Comparision of sensitivity of IFNγ ELISA

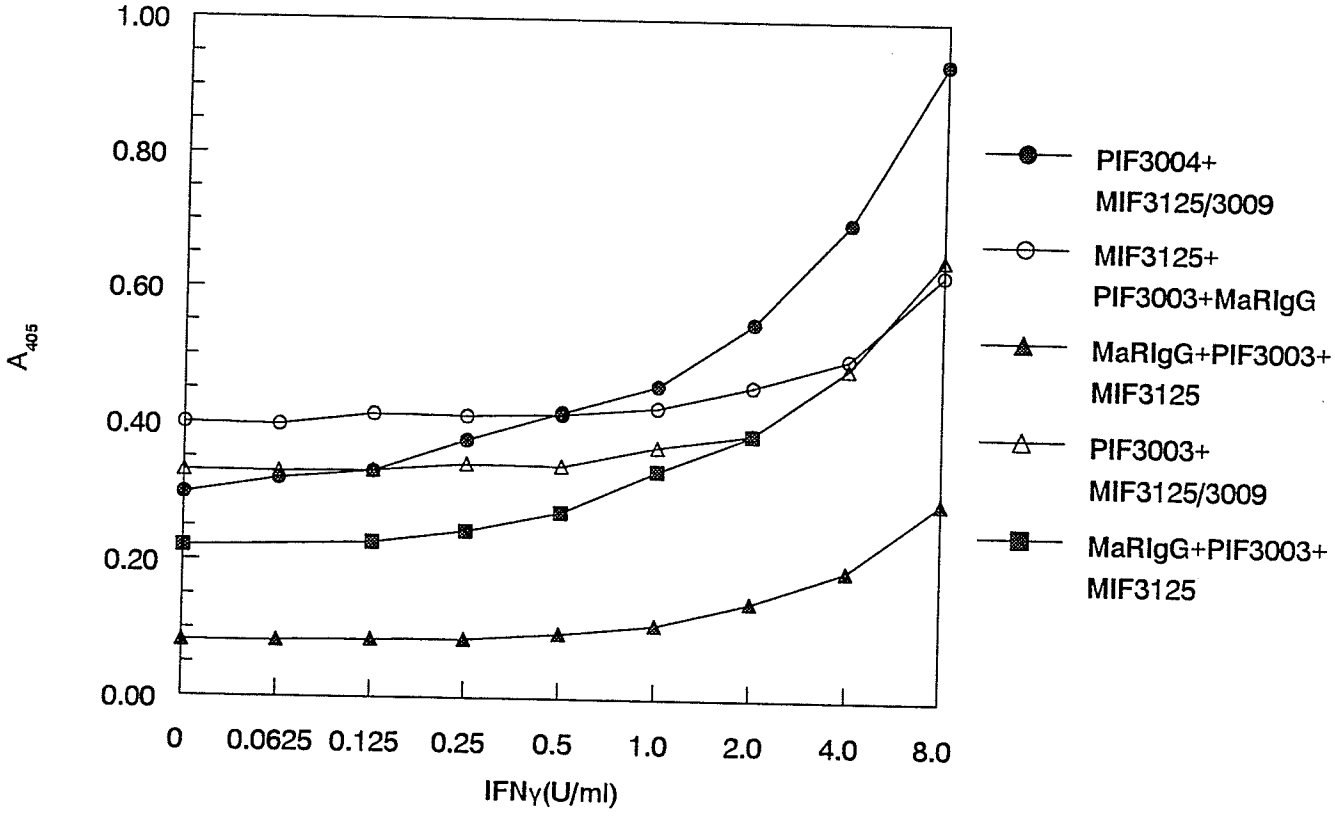

Figure 9: Determination of the IFNγ ELISA configuration which yields maximum sensitivity. Different combinations of antibodies were used for IFNγ ELISA. The first Ab listed is the capture reagent.

A: PIF3004 + IFNγ + biotinylated MIF3125/3009,
B: MIF3125 + IFNγ + PIF3003 + biotinylated mouse anti-rabbit IgG,
C: mouse anti-rabbit IgG + PIF3003 + IFNγ + biotinylated MIF3125/3009,
D: PIF3003 + IFNγ + biotinylated MIF3125/3009,
E: mouse anti-rabbit IgG + PIF3003 + IFNγ + biotinylated MIF3125/3009.

2.3. MTT-based IL-2 assay

HT-2 cells, which are highly responsive to murine and human IL-2, were used to determine IL-2 production in tissue culture supernatants from antigen stimulated primary cultures. Basically carried out as for the IL-4 assay , this method quantified IL-2 at and above 0.3125 U/ml (21 pg/ml) (Figure 10). Compared with using ^{3}H-thymidine as seen in Figure 10, MTT-assay, as was found in the CT.h4S assay, provided a more sensitive lower limit of detection (0.3125 vs.1 U/ml), a wider linear portion of the standard curve to be used for IL-2 quantitation, and smaller intra-assay standard deviations (3% vs.10-15%). Moreover, this assay is highly specific to IL-2. As demonstrated in Figure 11, neutralizing anti-IL-2 Ab at 1: 1000 totally blocked the proliferation of HT-2 cells stimulated by IL-2 at 75 pg/ml and neutralized 50% activity of IL-2 at 250 pg/ml. IL-4 below a concentration of 500 pg/ml, which is much higher than the levels observed even in polyclonally stimulated cultures, and other cytokines did not significantly interfere with the HT-2 cell proliferation (Our unpublished data and Bottomly, 1991).

These assays we established provide exquisitely sensitive and specific detection systems, allowing analysis of cytokine gene expression by normal PBMC in response to short term antigen stimulation. Particularly relevant to limiting dilution analyses are the small intra-assay standard deviations observed (typically

2-3%), making it possible to distinguish positive from negative wells at extremely low levels of cytokine synthesis and leading to a clear separation between negative and positive cultures.

Figure 10

Sensitivity of IL-2 assay

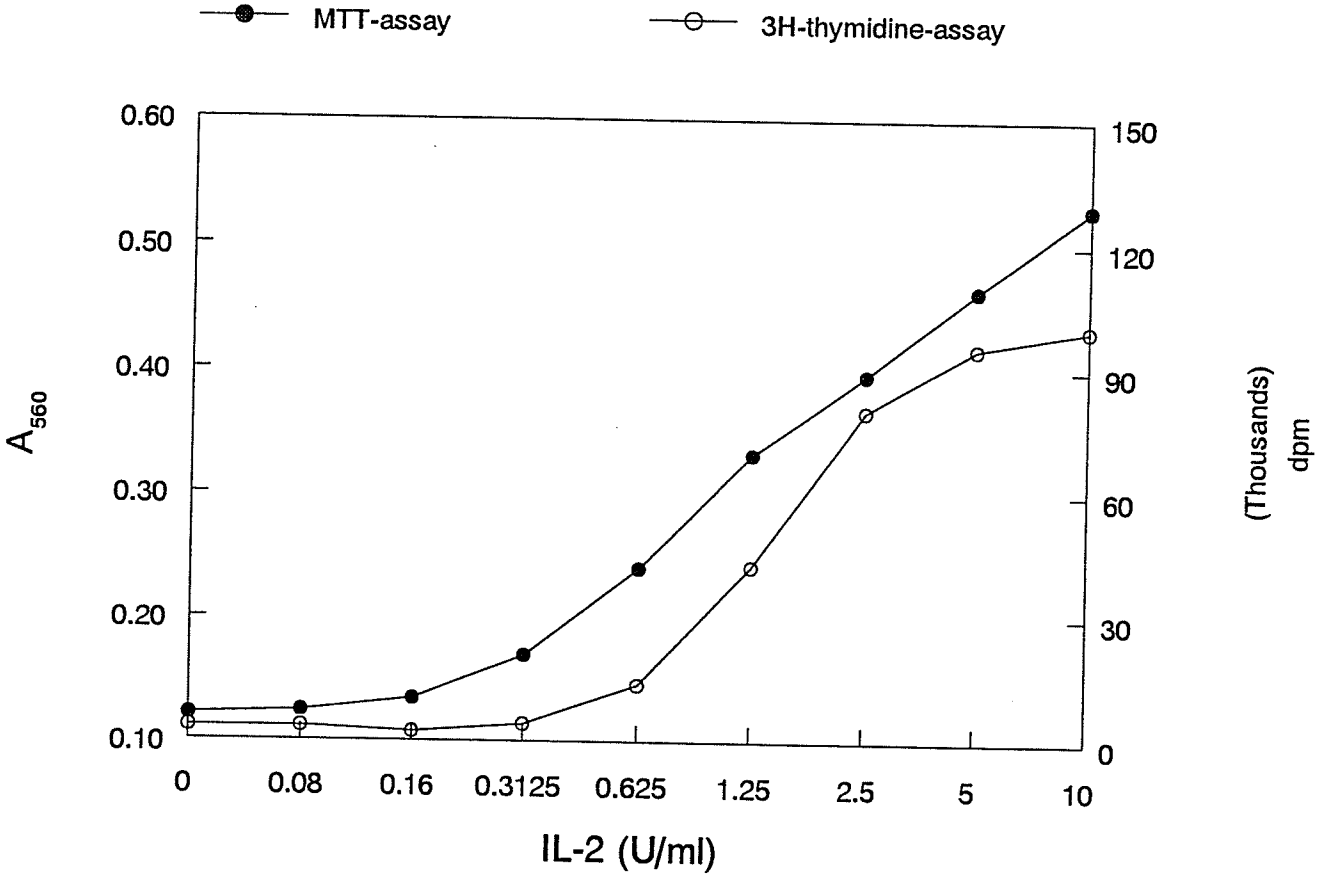

Figure 10: Sensitivity of MTT-based HT-2 bioassay for IL-2. The HT-2 cells were washed and cultured in the presence of rIL-2 from 20 to 0.16 U/ml. MTT was added at 24 hours followed by 6 hour more incubation. Absorbency was determined at 560-690 nm. Data shown are means of triplicate cultures.

Figure 11

Specificity of IL-2 assay

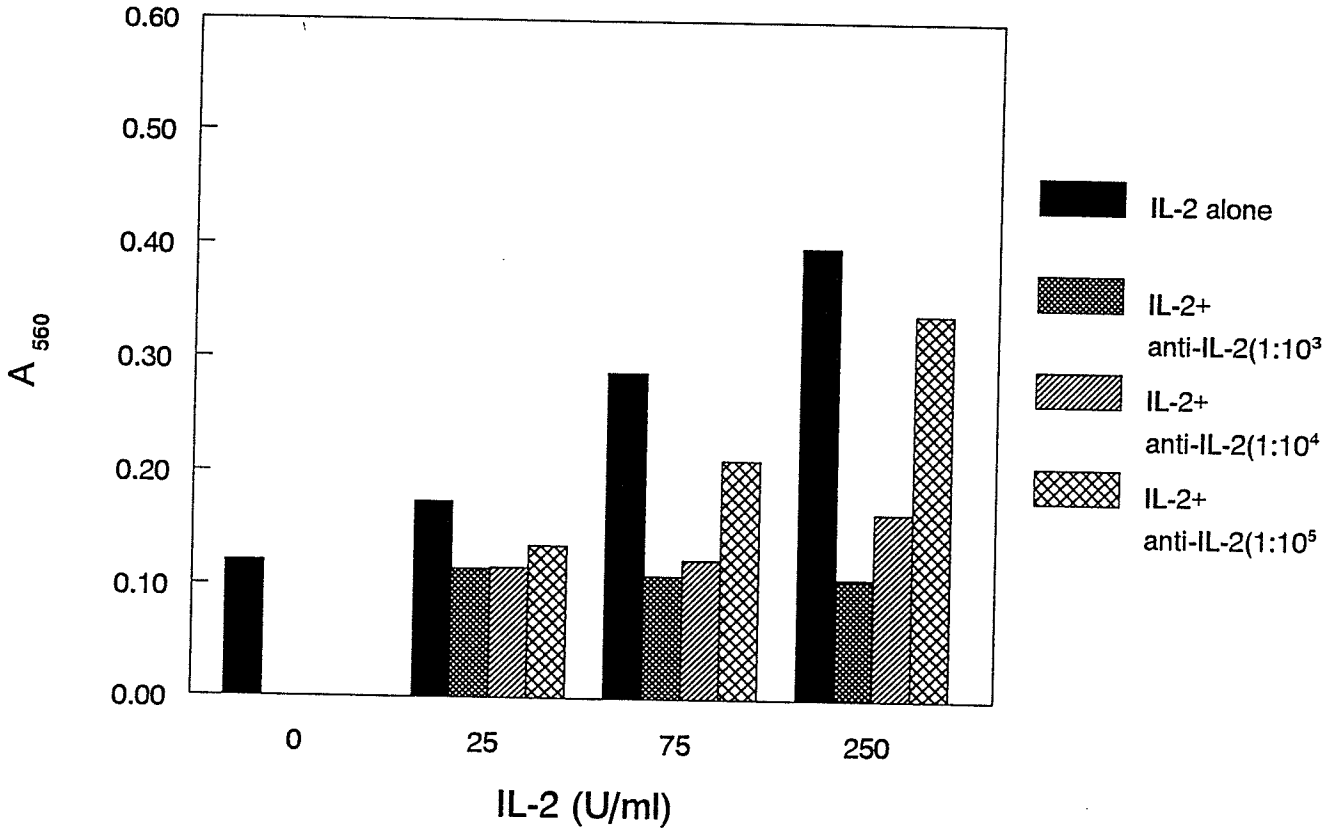

Figure 11: Specificity of MTT-HT.2 bioassay for IL-2. Anti-IL-2 at 1:1 x 10^3, 1:1 x 10^4 and 1:1 x 10^5 were added to cultures concurrently with 25, 75 and 250 U/ml human rIL-2 in a standard MTT-HT.2 bioassay. Data shown are means of duplicate cultures at each condition.

III. Allergen-driven cytokine synthesis

3.1. Limiting dilution analysis of the frequencies of allergen-driven cytokine synthesis

Limiting dilution analysis (LDA) is a quantitative approach which allows estimation of the frequencies of cells participating in an immune response (reviews, Lefkovits and waldman, 1984; Sharrock et al., 1990; Kelso et al., 1991). Its main advantage is that it allows determination of the frequency of one cell population, which may be of very low concentration, dispersed in another. Extensively used in animal studies, LDA has also been used to characterize human T cell responses by examining proliferative cell frequencies (Teppler et al., 1993; Schauf et al., 1993), CTL precursor frequencies (Sharrock et al., 1990; Carmichael et al., 1993) and the frequencies of cells producing IL-2 (Moretti et al., 1992; Waldman et al., 1992; Schulick et al., 1993) in a number of clinical conditions. These studies examined the intensity of human T cell activation rather than the nature of the response induced.

Much attention currently centres on the hypothesis that the relative commitment of the immune response to Th-1 vs. Th-2 like cytokine production following vaccination or nature exposure to antigen strongly influences the consequent effector responses and disease outcome. Meaningful characterization of such ongoing responses requires explicit examination of cytokine production

patterns. Such studies have not been carried out to date (Medline, Nov., 1994). Traditional approaches for LDA in mice or humans frequently utilize up to 10-18 day of culture in the presence of antigen, alloantigen or polyclonal activators, extensive washing and by restimulation for 24-48 hours with antigen or mitogen (Groves, 1994; Sharrock, 1990). These approaches provided substantial information for the association between hypersensitivity and increased IL-4 production. However, there are several important reservations to them. Using T cell clones is subject to a number of extrinsic factors which strongly influence the numbers and functions of the clones obtained. Moreover, even large panels of clones represent a very small proportion of the total allergen-specific T cell repertoire in vivo. It should also be recognized that use of polyclonal activators as surrogate antigens risks the possibility that the conclusions drawn are not representative of those elicited in vivo by antigenic stimulation.

In an effort to circumvent these difficulties, we therefore developed a novel strategy of LDA allowing us to evaluate the frequency of existing, grass pollen reactive IL-4 or IFNγ producing cells in peripheral blood of allergic and non-atopic individuals. Short term, antigen driven limiting dilution analysis as used in this study provided a minimal estimate not of the precursor frequency but of the frequency of mature, previously differentiated, circulating, allergen reactive cells in vivo.

As such it enables us to obtain a minimal estimate of the size of the existing, circulating peripheral repertoire responsive to grass pollen.

As shown in Figure 12, the frequency of IL-4 and IFNγ producing cells was calculated from the negative log of the number of nonresponding wells at each cell concentration. In order to be valid, data must fit the zero order term of the Poisson distribution equation, a condition met only if the presence or absence of a secreting cell is the single limiting factor determining if that cytokine will be detected in the assay. Satisfying this condition results in a linear relationship between the number of cells per well and the number of negative wells. The number of cells plated per well for one non-atopic and one allergic individual is plotted against the log of the percentage of negative wells for IFNγ (Figure 12a) and IL-4 (Figure 12b). The relationship was consistently linear, confirming that the number of cytokine producing cell plated was the single variable limiting IL-4 or IFNγ production under the experimental condition used. From these plots, the frequency of PBMC producing particular cytokines could be estimated as the input cell number yielding 37% negative replicates. Evaluation of this data by the maximum likelihood method yields a frequency estimate and a frequency range corresponding to the 95% confidence limits as shown.

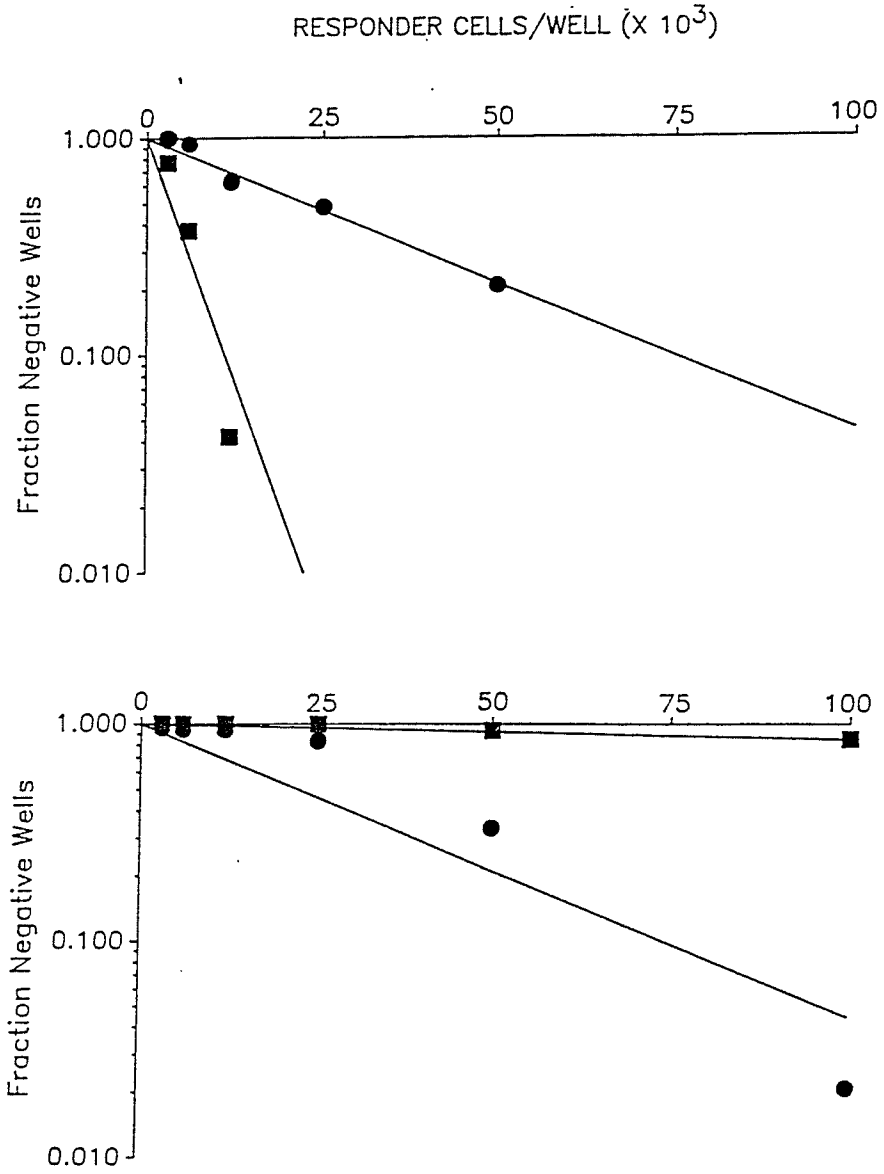

Figure 12: Limiting dilution analysis of IFNγ (top panel) and IL-4 (lower panel) producing PBMC of normal and grass pollen allergic individuals. 3,000 - 100,000 PBMC were cultured (48 replicates per cell concentration) for 3-4 days in the presence of 200 ug/ml grass pollen mix. Culture supernatants were harvested and examined for the presence of IL-4 by CT.h4S bioassay or IFNγ by ELISA as described. Results shown are from one experiment typical of more than 10 performed. ■: Normal ●: Allergic

3.2. Determination of appropriate cutoff for positive and negative cultures in limiting dilution analysis

Use of limiting dilution analysis to obtain frequency estimates requires one to distinguish positive from negative wells. The accepted standard used as a detection limit is the mean value of the background, or unresponsive, wells plus three standard deviations. Thus, if the A_{560} obtained in analysis of the 48 wells of cells cultured without antigen gave a mean value of 0.300 $\pm$ 0.004, then the cutoff for "positivity" in that assay would be 0.312. Supernatants scoring above 0.312 would be counted as positive (ie. containing $\geq$ 1 cell producing cytokine in response to grass pollen) while those $\leq$ 0.312 would be counted as negative. This approach is extensively used in limiting dilution analysis (Sharrock, 1990) in animal and human systems.

Despite the fact that this is the accepted method of carrying out LDA calculations, we wished to determined the impact of arbitrarily changing the threshold for positivity. Our intent was to determine if changing the threshold to "mean background $\pm$ 2SD", or $\pm$ 4SD, would substantially alter our results and conclusions. We therefore examined the impact of using different thresholds for "positive" wells on the calculated frequency obtained. The data presented in Figure 13 make use of the conventional threshold of three SD above background. Re-analysis of the data using thresholds of four and five SD respectively yields similar frequencies, based on their

falling within the 95% confidence limits of the frequencies obtained using three SD. In the example considered in Table 2 (one subject in Figure 13), this corresponds to frequency estimates (95% confidence limits) of 1/257,701 (190-402 x 10^3) , 1/280,560 (204-446 x 10^3) and 1/309,520 (260-635 x 10^3) for IL-4 producing cells using 3, 4 and 5 SD as thresholds, respectively. None of these values are significantly different from one another ($p < 0.05$).

The significance of this finding is that it demonstrates that minor variations in the intensity of cytokine production in any given well, which may act to push that individual value slightly above or below the cutoff selected, has minimal impact on the final calculated frequency estimated. At the same time, it must be understood that the value obtained reflect <u>minimal</u> estimates of the frequency of cells producing these cytokines in response to grass pollen stimulation. This is a characteristic of all limiting dilution analyses.

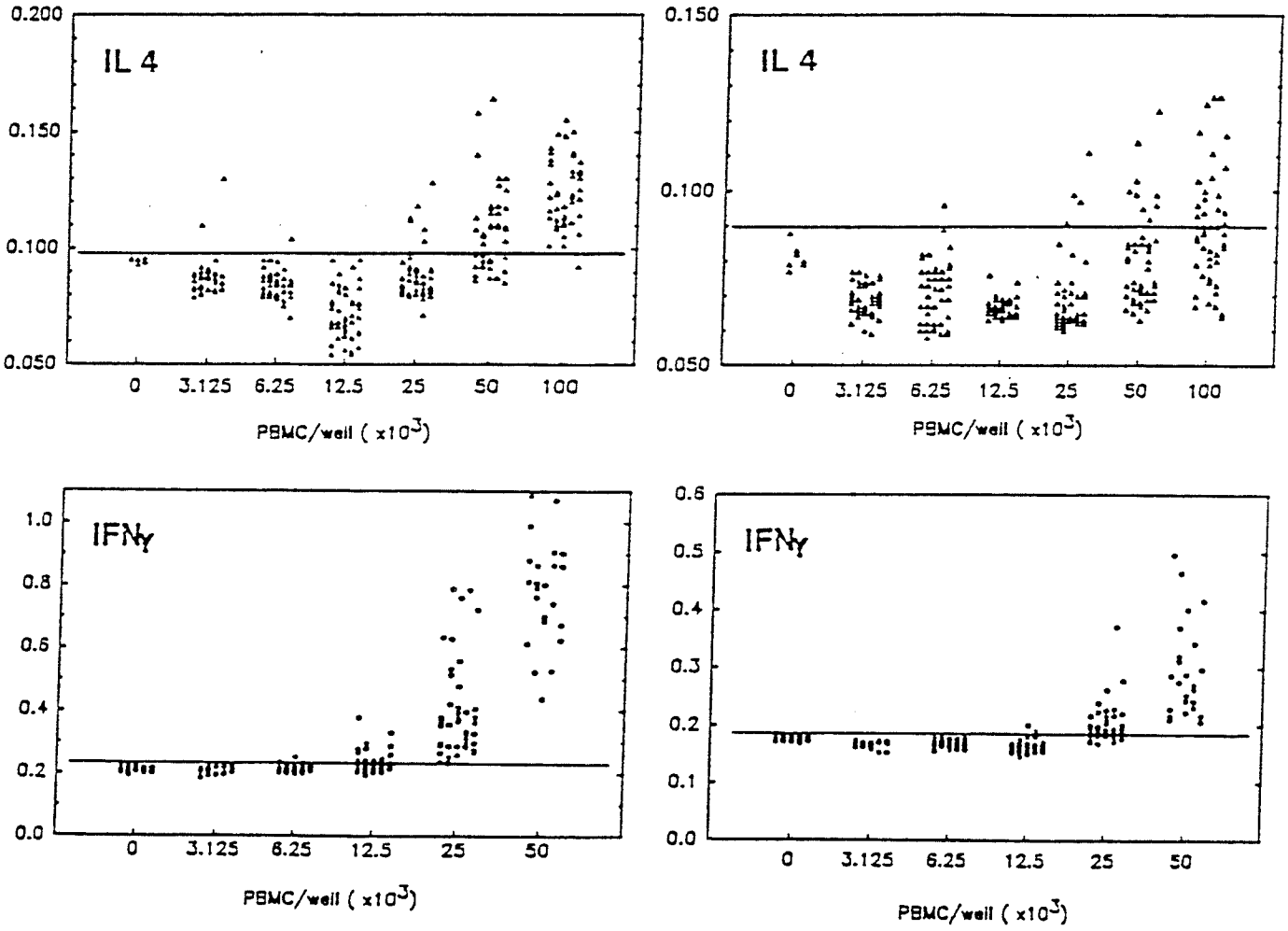

Figure 13: Levels of IFNγ or IL-4 production by PBMC in each limiting dilution cultures. Cells from two subjects were cultured from 100,000 to 3,125 cells per well for 3 days. 48 replicates were set up for each cell concentration. Culture supernatants were tested for IFNγ production by ELISA and IL-4 level by CT.h4S bioassay.

Table 2

Comparison of cytokine producing frequencies obtained by using different thresholds

Subject	Threshold used	Calculated frequency of cytokine producing cells	
		IL-4	IFNγ
1	3 SD	1/257,701 (190-402 x 10^3)	1/33,140 (27,620-41420)
	4 SD	1/280,560 (204-446 x 10^3)	1/37,570 (31,200-47,230)
	5 SD	1/309,520 (260-635 x 10^3)	1/41,460 (34,480-52,550)
2	3 SD	1/27,571 (23-34 x 10^3)	1/63,988 (52,774-81,252)
	4 SD	1/28,490 (24-35 x 10^3)	1/63,440 (52,310-82,580)
	5 SD	1/30,720 (26-38 x 10^3)	1/82,950 (67,460-107,670)

Legend: Comparison of frequencies of cytokine producing cells obtained by using different thresholds. Frequency estimates and 95% confidence limits for IL-4 and IFNγ producing cells from 2 subjects (raw data of Figure 15) were calculated using 3, 4 and 5 SD as thresholds to determine the impact of arbitrarily changing the threshold for positivity.

3.3. Different cytokine synthesis patterns are exhibited by allergic rhinitis subjects and non-allergic individuals

3.3.1. Imbalanced frequency of IFNγ or IL-4 producing cells in allergic individuals

In an attempt to characterize the cytokine synthesis patterns elicited by grass pollen, 3-4 day limiting dilution analysis was used to determine the frequency of fresh PBMC producing IL-4 or IFNγ from normal and allergic individuals. As can be seen from Table 3, in the 19 individuals available for detailed analysis of IL-4 and IFNγ frequencies, a median frequency of 1/196,000 PBMC capable of detectable IL-4 production was observed amongst allergic individuals in response to grass pollen stimulation. Normal controls exhibited approximately 5-fold lower frequency with 1/834,000 capable of producing IL-4 (Mann Whitney U test $p=0.023$, $n=19$).

In marked contrast to the elevated frequency of IL-4 producing cells observed in allergic rhinitis patients, the frequencies of grass pollen reactive IFNγ producing cells was approximately three-fold lower than normal individuals (1/11,200 in normals vs. 1/35,800 in atopics. Mann Whitney U test $p=0.016$)

Put differently, a median of 51 of 10^7 PBMC from allergic donors made IL-4 in response to allergen, whereas 11 of 10^7 cells from non-allergic volunteers did so. The median frequency of IFNγ

producing cells among allergic individuals was $279/10^7$ PBMC, significantly lower than the $893/10^7$ observed in the normal population.

Table 3

Limiting dilution analysis of PBMC producing IFNγ or IL-4 in response to allergen specific restimulation

Patient	Observed Frequency IFNγ	Observed Frequency IL-4	cells/10^7 PBMC producing: IFNγ	cells/10^7 PBMC producing: IL-4
Normal:				
N5	1/29,000	1/4,700,000	335	2
7	1/11,000	1/490,000	893	20
11	1/13,000	1/920,000	746	11
15	1/7,700	1/180,000	1290	57
16	1/85,000	<1/9,000,000	117	<1
20	1/5,700	1/830,000	1754	12
21	1/6,900	1/4,800,000	1449	2
23	1/7,300	<1/9,000,000	1370	<1
32	1/21,000	1/740,000	481	13
Median	1/11,208	1/834,000	893	11
Allergic:				
A2	1/36,000	1/71,000	279	141
9	1/46,000	1/190,000	218	51
11	1/48,000	1/29,000	209	348
13	1/45,000	1/76,000	222	131
17	1/17,000	1/2,300,000	575	4
19	1/30,000	1/390,000	337	25
20	1/28,000	1/370,000	352	27
26	1/25,000	1/48,000	408	209
27	1/11,000	1/200,000	893	50
28	1/15,000	NA	649	NA
Median	1/35,000	1/196,000	279	51

Legend: Minimal estimates of the frequency of PBMC producing IFNγ or IL-4 in response to grass pollen stimulation are presented for each patient directly and, by calculation, as the number of cells producing that cytokine per 10^7 fresh PBMC. NA: not available

Figure 14

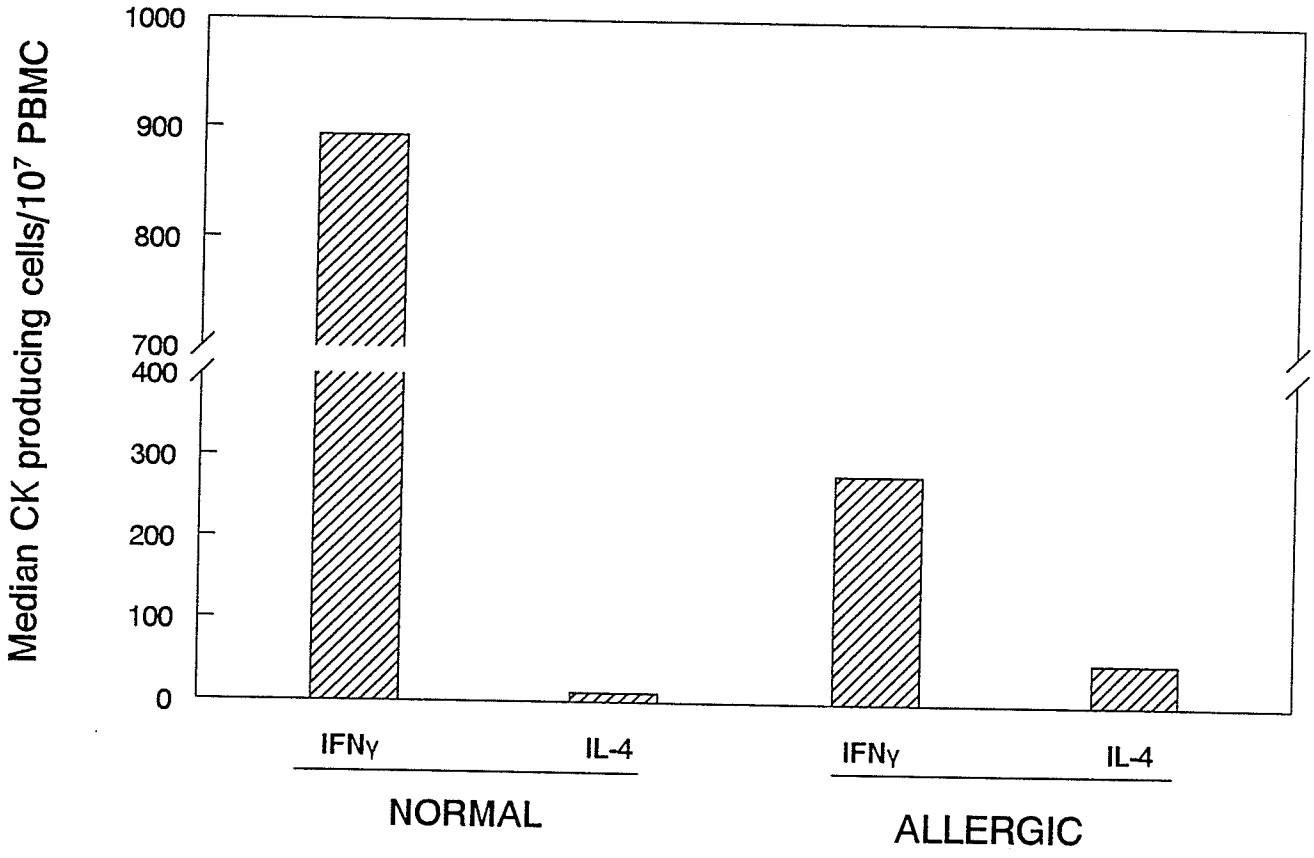

Figure 14: Median number of cells producing IFNγ or IL-4 per 10^7 fresh PBMC from allergic and normal populations.

3.3.2. Altered ratio of IFNγ:IL-4 synthesis

Evidence suggests that the relative balance of IL-4 and IFNγ production is more important than the absolute amounts of either cytokine in determining hypersensitivity versus clinical unresponsiveness. The ratio of the median frequencies of IFNγ to IL-4 producing cells in normal and allergic individuals was calculated based on the data in Table 4 and shown in Figure 15. The frequencies of cells producing IFNγ compared to those making IL-4 in response to grass pollen stimulation yields a ratio of 81:1 in normal individuals. In the grass pollen allergic population, it was approximately 5:1, some 15 fold lower. Therefore, it indicates a profound shift towards increased Th-2 like activity in the circulating immune repertoire of grass pollen sensitive individuals.

Similarly, work done by other members of the lab revealed that the magnitude of cytokine production (measured in bulk cultures) reflects a mean 13 fold decrease in the ratio of IFNγ: IL-4 production between the two study groups ($p<0.007$). Thus, although the IFNγ response to grass pollen remains dominant in most allergic and in all normal individuals studied, comparison of either the frequency of responding cells or the intensity of cytokine production in vitro reveals a marked reduction in the IFNγ:IL-4 ratio amongst allergic individuals which may be more relevant to the clinical consequences.

Figure 15

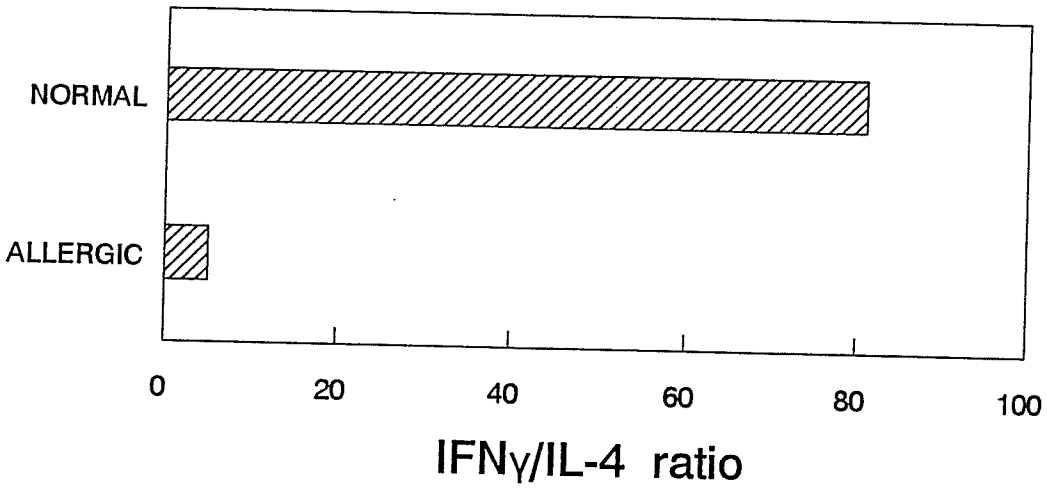

Figure 15: Allergic individuals exhibit a decreased ratio of IFNγ:IL-4 production by PBMC in response to grass pollen stimulation. The median ratio of the frequencies of PBMC producing IFNγ and IL-4 was calculated from the data in Table 2 for normal volunteers (median IFNγ:IL-4 ratio = 81, range 23-1370, n = 9) and allergic individuals (median IFNγ:IL-4 ratio = 5.5, range 0.6-144, n = 8)

3.4. The increased IL-4 : IFNγ ratio observed in atopic individuals following grass pollen stimulation is antigen specific

To examine specificity in this antigen-driven assay system, mononuclear cells were stimulated under the same conditions used above but (i) in the absence of any antigen stimulation, or (ii) substituting irrelevant antigens in place of grass pollen. Cells from three subjects (two normal individuals and one grass pollen allergic donor) were cultured (i) with purified normal rat IgG at 500 and 50 ug/ml, (ii) with ovalbumin at 500 and 50 ug/ml and (iii) without antigen stimulation. 24 replicate cultures were set up for each cell concentration. As demonstrated in Table 4, the frequency of IL-4 producing PBMC was below the limit of detection ($<1/2 \times 10^6$) for all subjects examined following ovalbumin or normal rat-IgG stimulation. Similarly, the frequency of normal rat-IgG specific IFNγ producing cells is below the limit of detection in the subjects examined. Interestingly, IFNγ production was readily detected for all 3 subjects in response to OVA stimulation. The frequency ranged from 1/11,207 to 1/674,692, similar to that observed in normal grass pollen non-allergic donors. Since all subjects do not have a record of allergy to ovalbumin and to normal rat IgG, this explains the predominant IFNγ response observed. Also noticed here is that the frequency of IFNγ producing cells induced by ovalbumin is much higher than that observed following rat IgG stimulation. As ovalbumin is a common antigen to which most individuals are chronically exposed whereas normal rat

IgG is rarely encountered, the response induced by ovalbumin after in vitro restimulation likely represents a boosted secondary response which is much stronger than the primary (undetectable) immune response elicited in vitro by normal rat IgG. Also illustrated in Table 4, in the absence of antigen stimulation (but in the presence of rIL-2 at 10 U/ml and fetal calf serum), a very low to undetectable frequency of IL-4 or IFNγ synthesis was observed. These results indicated that the increased IL-4 synthesis with the consequent high IL-4 : IFNγ ratio observed in allergic rhinitis donors following grass pollen stimulation is antigen specific. It is worth mentioning that PBMC cultured in the presence of fetal calf serum, even in the presence of IL-2 (10 U/ml), did not produce detectable level of cytokines (IL-4 and IFNγ) (Table 4 and some other data not shown here), thus eliminate the concern that FCS in the culture medium would activate PBMC in a non-specific way.

Table 4

Antigenic specificity of grass pollen stimulated IL-4 and IFNγ production in limiting dilution analysis

Subject	Antigen	Frequency of cytokine producing cells IL-4	IFNγ
Allergic 1	Grass pollen	1/27,571 (23,105-34 179	1/63,988 (52,774-81,253)
	Ovalbumin (500 ug/ml)	$<1/2 \times 10^6$	1/11,207 (8,350-17,036)
	Ovalbumin (50 ug/ml)	$<1/2 \times 10^6$	1/12,623 (9,666-18,939)
	N. Rat-IgG (500 ug/ml)	$<1/2 \times 10^6$	1/131,195 (85,988-276,623)
	N. Rat-IgG (50 ug/ml)	$<1/2 \times 10^6$	1/70,702 (49,998-120,666)
	None	$<1/2 \times 10^6$	$<1/2 \times 10^6$
Normal 1	Grass pollen	$<1/2 \times 10^6$	1/8,500 (6,340-12,890)
	Ovalbumin (500 ug/ml)	$<1/2 \times 10^6$	1/17,207 (12,962-25,482)
	Ovalbumin (50 ug/ml)	$<1/2 \times 10^6$	1/32,145 (23,889-49,120)
	N. Rat-IgG (500 ug/ml)	$<1/2 \times 10^6$	1/674,692 (316,480-511,6510)
	N. Rat-IgG (50 ug/ml)	$<1/2 \times 10^6$	1/674,692 (316,480-511,6510)
	None	$<1/9 \times 10^6$	1/277,982 (159,613-107,5764)

continue on next page

Subject	antigen	Frequency of cytokine producing cells IL-4	IFNγ
Normal 2	Grass pollen	$<1/2 \times 10^6$	1/27,048 (22,479-33,946)
	ovalbumin (500 ug/ml)	$<1/2 \times 10^6$	1/15,893 (11,976-23,617)
	ovalbumin (50 ug/ml)	$<1/2 \times 10^6$	1/32,970 (24,545-50,205)
	N. Rat-IgG (500 ug/ml)	$<1/2 \times 10^6$	1/103,064 (69,709-197,624)
	N. Rat-IgG (50 ug/ml)	$<1/2 \times 10^6$	$<1/9 \times 10^6$
	None	$<1/2 \times 10^6$	$<1/9 \times 10^6$

Legend: Specificity of grass pollen stimulated IL-4 and IFNγ production in limiting dilution analysis. PBMC from 1 grass pollen allergic donor and 2 normal individuals were cultured in the absence of antigen or in the presence of ovalbumin or normal rat IgG at 500 and 50 ug/ml. Culture supernatants were harvested after 3-4 days and examined for IL-4 or IFNγ production. Frequencies were calculated using maximum likelihood method with 95% confidence limit (shown in bracelets). Note: $1/2 \times 10^6$ is the detection limit for the cell concentrations and number of replicates examined.
N. Rat-IgR: purified normal rat-IgG

3.5. Addition of irradiated autologous PBMC as supplemental APC does not alter the frequency of IL-4 and IFNγ producing cells in this LDA system

Pragmatic considerations such as the limited amount of fresh blood obtainable from each subject, our belief that the frequency of cells reactive to allergen was likely to be very low and our desire to evaluate the response of the intact circulating repertoire to antigen rather than polyclonal stimulation, led us to examine unfractionated mononuclear cell populations rather than purified CD4 T cells co-cultured with irradiated, T cell depleted PBMC as a source of APC. Two earlier reports (Adams et al., 1991; Waldman et al., 1992) indicated that the presence of antigen presenting cells in the unseparated mononuclear cell preparations, approximately 6-10% of the total, was not a limiting factor at the PBMC concentrations tested in their studies. To verify this ourselves, we examined the impact of adding irradiated autologous PBMC as a supplemental source of APC in our LDA cultures. We hypothesized that a decrease in the frequency of the negative wells (ie. an increased number of positive wells) following addition of these irradiated APC would suggest that a shortage of antigen presenting cells was artificially decreasing our frequency estimates for IL-4 and IFNγ producing cells. However, as shown in Table 5, we found that at the responder cell concentrations used for frequency calculations, the presence of 5,000 irradiated PBMC

as supplemental APC did not alter the frequency of wells in which cytokine production was observed. This confirmed that a shortage of APC was not a concern in influencing the results obtained under the assay condition used.

Table 5

Impact of additional APC on the calculated frequency of IL-4 and IFNγ producing cells

		# negative wells/total wells					
		IL-4			IFNγ		
Subject	PBMC/well:	12	6	3	12	6	3 ($x10^3$)
1	no APC	22/24	24/24	24/24	9/24	15/24	22/24
	+ APC	22/24	22/24	24/24	10/24	16/24	23/24
2	no APC	24/24	24/24	24/24	0/24	2/24	11/24
	+ APC	24/24	24/24	24/24	0/24	1/24	12/24
3	no APC	23/24	24/24	24/24	19/24	23/24	23/24
	+ APC	22/24	23/24	24/24	18/24	24/24	24/24
4	no APC		N.A.		13/24	23/24	24/24
	+ APC		N.A.		15/24	23/24	24/24
5	no APC		N.A.		6/24	16/24	24/24
	+ APC		N.A.		10/24	17/24	20/24
6	no APC	20/24	21/24	22/24	16/24	19/24	23/24
	+ APC	18/24	23/24	24/24	16/24	20/24	24/24

Legend: Addition of irradiated autologous APC does not decrease the frequency of wells which fail to produce detectable level of IL-4 or IFNγ. PBMC were cultured at 12,500, 6,250 and 3,125 per well in the absence or presence of irradiated autologous PBMC as a supplemental source of APC. Cytokine production in any given well was deemed positive when IL-4 or IFNγ assay values were greater than the mean plus three SD of values obtained from wells cultured with all components except responder cells. N.A. not available

3.6. Effect of IL-2 on IFNγ and IL-4 synthesis

The demonstration of increased frequencies of IL-4 producing cells in individuals with allergic rhinitis suggests a pivotal role for IL-4 in induction and maintenance of immediate hypersensitivity. To therapeutically modulate the patterns of cytokine production, and consequently block the IgE secretion elicited in allergic individuals after exposure to allergens, it is necessary to characterize the signals involved in regulation of Th1 or Th2 responses. Most animal studies carried out after in vivo antigen-specific or polyclonal stimulation indicate that IL-2 is required for the induction of IL-4 response (Powers, 1988; Weinberg, 1990; Seder, 1991). To verify this in our human allergy study, we examined the requirement of IL-2 in antigen-driven IL-4 and IFNγ synthesis.

In our preliminary experiments, addition of IL-2 at low concentrations (10 U/ml) was found to increase the observed frequency of pollen reactive IFNγ producing cells in most patients, both allergic and normal individuals (Table 6). Consistent with observations of other investigators (Sharrock, 1990), levels of IL-2 $\leq$ 25 U/ml fail to elicit (non-specific) IFNγ production in cultures set up without antigen stimulation (Data not shown). In contrast, addition of exogenous IL-2 (10 U/ml) failed to increase the intensity or the frequency of IL-4 production by antigen stimulated PBMC (data not shown). This observation is consistent

with reports (Yang and HayGlass, 1993) of the dependence of IFNγ gene expression, and relative independence of IL-4 gene expression, with respect to IL-2 production.

Table 6

Effect of rIL-2 on IFNγ production

	Frequency of IFNγ producing cells	
	no rIL-2	+rIL-2 (10 U/ml)
Allergic		
1	1/26,423 (19,761-39,862)	1/11,998 (9,312-16,860)
2	1/33,467 (25,925-47,195)	1/16,079 (12,486-22,574)
3	1/30,510 (22,713-46,458)	1/28,286 (21,529-41,223)
4	1/28,111 (21,014-42,444)	1/34,231 (26,416-48,611)
5	1/32,911 (24,333-50,654)	1/15,896 (12,275-22,547)
6	$<1/9 \times 10^6$	1/96,708 (66,499-177,212)
7	1/34,206 (26,547-48,077)	1/12,852 (9,965-18,094)
8	1/42,292 (31,080-66,155)	1/18,907 (14,751-26,322)
9	1/34,688 (25,636-53,623)	1/35,355 (26,646-52,522)
10	$< 1/9 \times 10^6$	1/41,837 (31,076-63,999)
Normal		
1	1/21,518 (16,268-31,772)	1/7,059 (5,422-10,113)
2	1/27,427 (20,470-41,546)	1/10,015 (7,736-14,219)
3	1/32,911 (24,373-50,654)	1/10,417 (8,047-14,764)
4	1/13,539 (10,147-20,334)	1/6,904 (5,323-9,833)
5	1/30,278 (23,539-42,423)	1/15,892 (12,361-22,246)
6	1/36,826 (27,312-56,513)	1/11,778 (9,111-16,650)
7	1/16,541 (12,463-24,585)	1/16,005 (12,368-22,674)

Legend: Effect of IL-2 on IFNγ production. PBMC from 10 allergic and 7 normal individuals were cultured in the absence or presence of IL-2 (10 U/ml) for 4 days. IFNγ production was determined in the culture supernatants and the frequency of IFNγ producing cells was calculated by maximum likelihood method.

IV. Characterization of the effects of IL-12 on IFNγ synthesis by human PBMC: differential responses of grass pollen allergic and non-allergic individuals

The type and amount of cytokine production by allergen-reactive CD4 T cells during an immune response plays an important role in regulating the nature of the response. Th1 cells which produce IL-2 and IFNγ mediate delayed-type hypersensitivity response and activate macrophages. In contrast, responses induced by Th2 cells which predominantly produce IL-4 and IL-5 result in generation of IgE secreting cells with the consequence of immediate hypersensitivity. Th1 and Th2 cells appear likely to differentiate from a common pool of precursors (Rocken, 1992; Reiner, 1993; Abehsira-A, 1992) and the signals controlling the differentiation pathway as well as the activation of differentiated T cells are still poorly defined. Cytokines produced by T cells and accessary cells have been demonstrated, at least in part, to be involved in the differentiation of Th subsets, most prominently IL-12 and IL-10. IFNγ has been reported to promote differentiation of precursor T cells into Th1 cells whereas IL-4 has the opposite activity, inducing differentiation into Th2 cells. Recently, a novel cytokine termed natural killer cell stimulatory factor (NKSF) or IL-12 was shown to have an inhibitory effect on the development of IL-4 producing cells and to play an inducing role in promoting Th1-like responses (Roberto et al., 1993 and Germann et al., 1993).

IL-12 is a heterodimeric cytokine produced by monocytes /macrophages, B cells and possibly other accessory cell types. Many studies have shown that IL-12 is active on T and NK cells in vitro (and in vivo in animal models), affecting cytotoxicity, cellular proliferation and production of lymphokines. (Kobayashi et al., 1989, Wolf et al., 1991, Gubler et al., 1991, Perussia et al., 1992, Susan et al., 1991, Roberto et al., 1993, Cnang-you Wu et al., 1993, Annalisa et al., 1993, and Germann et al., 1993). Of particular relevance to our study, IL-12 is reported to be highly efficient in inducing IFNγ production by T and NK cells. It acts synergistically with other IFNγ inducers such as IL-2 and mitogens. As we demonstrated in our limiting dilution analysis experiments, patients with allergic rhinitis developed an increased frequency of IL-4 producing cells while the frequency of IFNγ secreting cells was substantially decreased compared to that of normal individuals after in vitro grass pollen restimulation. This suggests an allergen-induced shift towards Th2-like response. Thus, factors which influence the commitment of Th1 or Th2 cell and the activation of IFNγ or IL-4 producing cells are of central importance in determining the effector response induced by grass pollen.

Although much work has been done to characterize the biological activities of human IL-12, all human studies that examined the effects of IL-12 to date had been in systems stimulated by polyclonal activators such as anti-CD3, PHA and phorbol diester. In

our study, besides confirming the IFNγ inducing activity of IL-12 in our antigen specific system, we also aimed to analyze the responses to IL-12 of the grass pollen allergic and non-allergic individuals. We hypothesized that such information may be very important for the understanding of the mechanism(s) underlying the different effector responses induced and the maintenance of hypersensitivity in atopic individuals.

4.1. IL-12 induces the synthesis of IFNγ by unstimulated human PBMC

To determine the role played by IL-12 in the synthesis of IFNγ, unfractionated human PBMC were cultured at a concentration of 5 x 10^5/well (50 ul/well) for 3 days, in the absence and presence of recombinant human IL-12 at different concentrations. No grass pollen was present. As demonstrated in Figure 16, in the absence of exogenous IL-12, IFNγ production by unstimulated PBMC was undetectable. Approximately 1 U/ml of IFNγ production was seen when 5 pg/ml of IL-12 was added to the culture, and 20 pg/ml IL-12 induced a significantly increased IFNγ release (50 U/ml). The response of cells to IL-12 was dose-dependent (Fig 16), and it was also influenced by the duration of the culture. Kinetic studies revealed that the level of IFNγ is about 2-5 fold higher in 5-day culture supernatants than that of 3 day supernatants. Therefore, consistent with other reports, our results indicates that IL-12 is capable of stimulating human PBMC to produce IFNγ in a dose-dependent way.

Figure 16

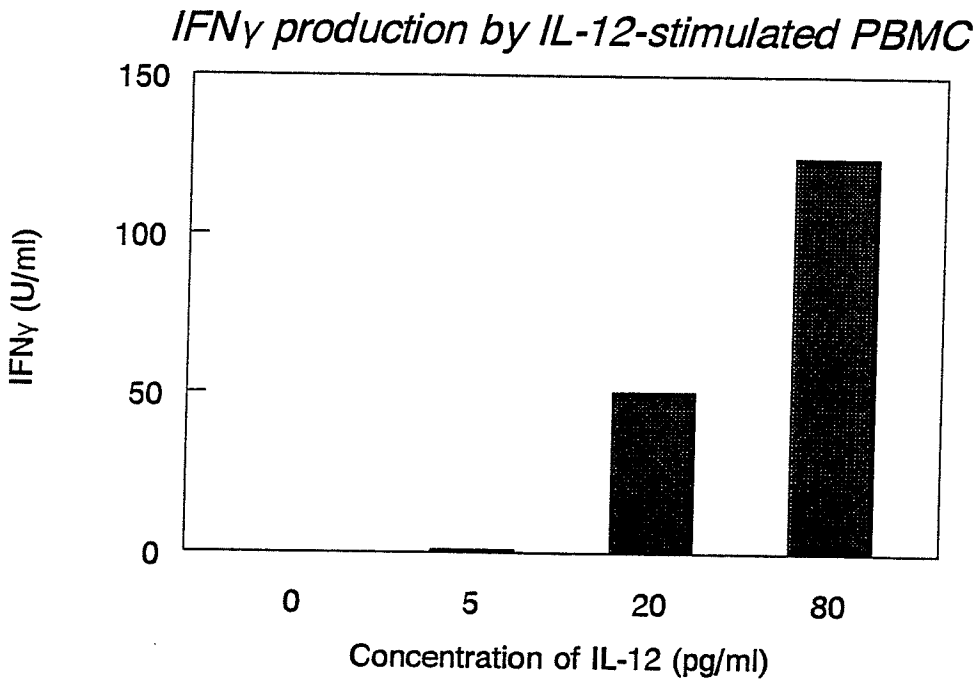

Figure 16: IL-12 induced IFNγ production by Human PBMC. Purified PBMC (5 x 10^5 / well) were cultured for 3 days in the absence and presence of human rIL-12 at 5, 20 and 80 pg/ml. Culture supernatants were tested for IFNγ production by ELISA. Data shown is from one representative experiment of three.

4.2. IL-2 synergizes with IL-12 in inducing IFNγ production

We next examined the effect of IL-2, which has been reported to synergize with IL-12 for the induction of IFNγ production (Kobayashi, 1989; Wolf, 1991; Chang, 1993; Roberto, 1993). In the example considered (one of the 6 subjects tested), both IL-12 (25 and 100 pg/ml) and IL-2 (50 U/ml) were not very efficient in stimulating PBMC to secrete IFNγ when used alone (Figure 17). However, the production of IFNγ after 20 hours of culture with IL-12 (100 and 25 pg/ml) was increased 18- and 67-fold in the presence of 50 U/ml IL-2 (30.9 and 6.6 U/ml of IFNγ as compared to 570.9 and 430.2 U/ml). Table 7 summarizes the results of all 6 subjects examined. As anticipated based on previously published reports and our data in Figure 17, IFNγ production was significantly enhanced upon stimulation with both IL-12 and IL-2.

Figure 17

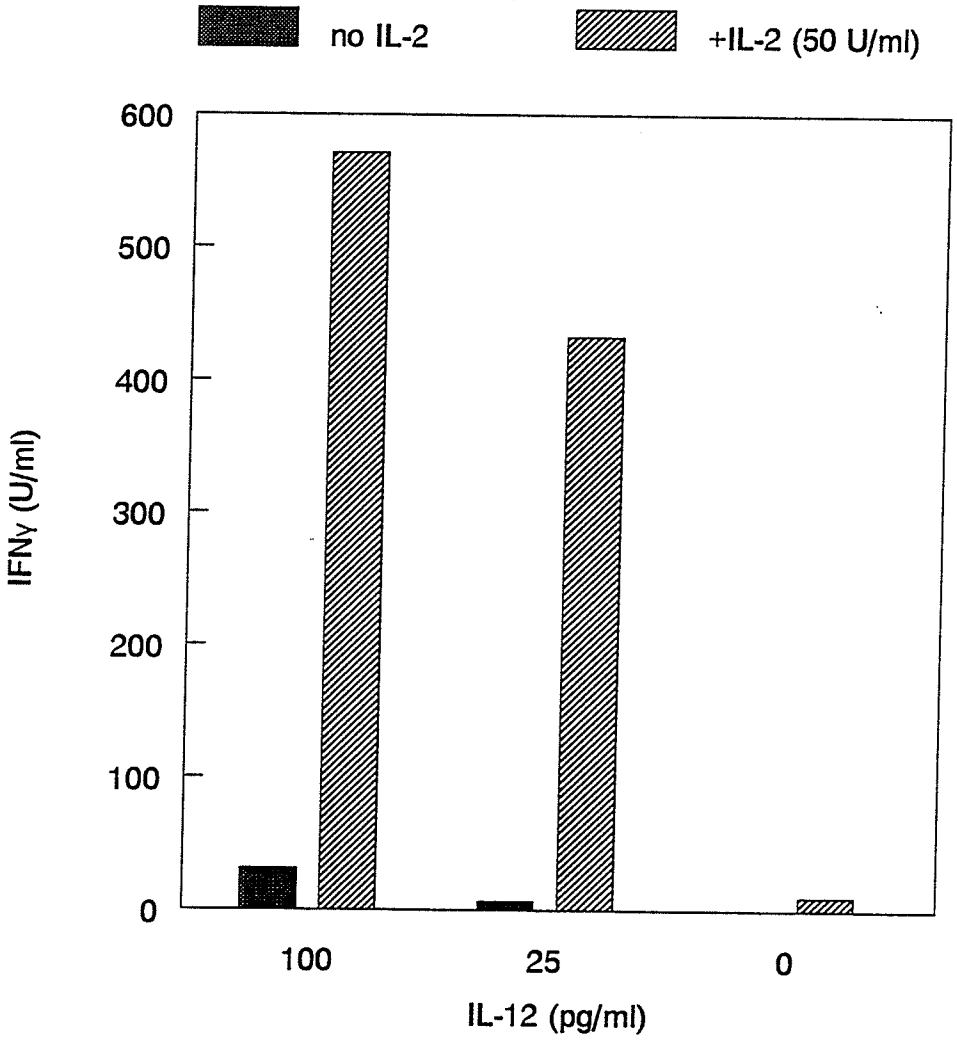

Figure 17: IL-2 synergizes with IL-12 in inducing IFNγ production by PBMC from a normal healthy subject. Cells were cultured with IL-12 for 20 hours in the absence and presence of 50 U/ml IL-2. Culture supernatants were tested for IFNγ production by ELISA. Data shown is from one experiment of six.

Table 7

IL-2 synergizes with IL-12
in inducing IFNγ production by human PBMC

	IFNγ (U/ml)				
IL-12 (pg/ml)	100	25	100	25	0
IL-2 (u/ml)	0	0	50	50	50
1	30.9	6.4	570.9	432.6	9.9
2	3.3	1.3	593.0	143.7	1.5
3	62.7	7.0	1364	557.8	21.1
4	65.0	29.2	1029	456.9	121.2
5	1.6	1.3	1584	979.5	<1
6	603.5	393.4	>>>>	2004	857.0

Legend: IL-2 synergizes with IL-12 in inducing IFNγ production by human PBMC. Cells from 6 normal individuals were cultured with IL-12 for 20 hours in the absence and presence of 50 U/ml IL-2. Supernatants were collected and tested for IFNγ production by ELISA. Data shown are means of duplicate cultures.

4.3. Differential responsiveness to IL-12 observed in allergic and normal individuals

Our demonstration that grass pollen allergic individuals exhibited a higher frequency of IL-4 producing cells and a lower frequency of IFNγ secreting cells than that of normal donors, and the observation that IL-12 is capable of inducing IFNγ production (Figure 17, Table 7) prompted us to examine whether there is a difference in responsiveness to IL-12 between the allergic and non-allergic individuals. Purified PBMC from 6 normal and 7 atopic donors were cultured, alone, or with different combinations of IL-12 and IL-2. After 20 hours, the supernatant fluids were analyzed for their content of IFNγ. As can be seen, the median IFNγ response of IL-12 stimulated PBMC obtained from allergic individuals was substantially below that of healthy, non-atopic subjects cultured under the same conditions (Table 8, columns 1, 2).

IFNγ production is strongly dependent on the presence of IL-2 (Yang and HayGlass, 1993; Vilcek, 1985; Kelly, 1987). Thus, we next determined the IL-12 driven IFNγ response in the presence of exogenous rIL-2. As shown in Table 8, IL-2 used at 50 U/ml strongly enhanced the capacity of IL-12 to stimulate IFNγ synthesis (Table 8, column 3, 4).

However, the striking finging in these preliminary experimnets was that the median responses of allergic individuals were substantially (10-20 fold) lower than those of mormal subjects.

This difference in the responsiveness of the twogroups is similar in the presence or absence of exogenous IL-2. At the same time, we recognized that a concern in the interpretation of these experiments was the "spontaneous" induction of IFNγ synthesis frequently observed following culture with high concentrations of IL-2 to "non-specifically" activate proliferation and/or cytokine gene expression is well recognized (Sharrock, 1990; Table 8, column 5). We therefore carried out pilot experiments examining the relative IL-12 responsiveness of healthy, non-atopic and grass pollen allergic subjects using substantially lower levels of exogenous IL-2. As demonstrated in Table 9, use of different concentration of IL-12 and IL-2 generated similar results, indicating that potential influence by the dosage of stimulation which may result in the difference of response is not a concern. At the same time, further experiments with more subjects, and a more complete titration of IL-2 and IL-12 will be required.

Thus, our initial data indicates that the response to IL-12 shows a profound difference between normal and atopic individuals. This suggests that decreased responsiveness to IL-12 may possibly contribute to the decreased ratio of IFNγ:IL-4 production induced by grass pollen observed in allergic patients .

Table 8

Differential responses of allergic and non-allergic individuals to IL-12 and/ or IL-2 (Exp 1)

	IFNγ (U/ml)				
IL-12(pg/ml)	100	25	100	25	0
IL-2 (U/ml)	0	0	50	50	50
Normal:					
1	30.9	6.4	570.9	432.6	9.9
2	3.3	1.3	593.0	140.7	1.5
3	62.7	7.0	1364	557.8	21.1
4	65.0	29.2	1029	456.9	121.4
5	1.3	1.6	1584	979.5	<1
6	603.5	393.4	>>>>	2004	857.0
median	46.8	6.7	1197	507.4	15.5
Allergic:					
1	<1	<1	253.6	32.8	<1
2	2.0	<1	38.9	3.8	<1
3	<1	<1	81.9	24.2	<1
4	92.8	37.5	1084	852.1	12.4
5	29.9	22.4	987.1	784.4	48.1
6	40.6	<1	9.1	73.1	<1
7	0.3	0.3	93.6	39.9	<1
median	2.0	<1	93.6	39.9	<1

Legend: Differential responses of grass pollen allergic and non-allergic individuals to IL-12 and /or IL-2. PBMC (5 x 10^5/well) from 7 atopic and 6 normal donors were cultured in the absence or presence of different combination of IL-12 and IL-2. Supernatants were collected after 20 hours and IFNγ production was measured by ELISA. Data shown are mean of duplicate cultures.

Table 9

Differential responses of grass pollen allergic and non-allergic individuals to IL-12 and/or IL-2 (Exp 2)

	IFNγ (U/ml)					
IL-12(pg/ml)	100	20	0	100	20	0
IL-2 (U/ml)	10	10	10	50	50	50
Normal:						
1	772.5	490.2	22.5	2481	1867	368.6
2	402.6	168.6	0.5	N.A	1262	20.3
Atopic:						
1	127.7	57.9	0.4	349.3	185.2	1.2
2	43.3	13.7	0.3	140.6	58.9	1.1

Legend: PBMC from 2 normal and 2 atopic individuals were cultured with (100 and 20 pg/ml) or without IL-12 in the presence of 10 or 50 U/ml IL-2. After 40 hours of culture, supernatants were collected and measured for IFNγ production by ELISA. Data shown are means of duplicate cultures at each condition.

4.4. IL-12 significantly augments the antigen-induced IFNγ production by human PBMC

In the course of our study on the effect of IL-12 on IFNγ synthesis by unfractionated human PBMC, we have begun to determine the effects od IL-12 in aantigen specific system. In a pilot experiment, cells from 2 normal individuals were stimulated with IL-12, alone, or in combination with grass pollen (400 ug/ml), in the absence and presence of 5 U/ml IL-2. As can be seen in Table 10, allergen triggered IFNγ production was increased 2-10 fold upon addition of IL-12 (at 5, 20, 80 pg/ml). Supplementation of cultures with low levels of IL-2 (5 U/ml) further augmented the secretion of IFNγ. We are currently in the process of evaluating more subjects to determine if there is a difference between normal and allergic individuals in IFNγ synthesis in response to Ag stimulation.

Taken collectively, this component of our study confirms that IL-12 is capable of inducing IFNγ synthesis by human PBMC and that IL-2 required for full expression of this activity. In addition to enhancing mitogen-induced IFNγ production, which was previously reported extensively, we demonstrated that IL-12 is very efficient in augmenting IFNγ synthesis induced by grass pollen. A significant difference of response to IL-12 was also observed between grass pollen allergic and non-allergic individuals. This differential sensitivity to rIL-2 (in combination with antigen stimulation) by allergic and non-allergic individuals has obvious implication for the therapeutic administration of rIL-12 in vivo.

Table 10

rIL-12 enhances antigen-stimulated IFNγ production

		IFNγ (U/ml)							
IL-12(pg/ml)		0	5	20	80	0	5	20	80
IL-2 (U/ml)		0	0	0	0	5	5	5	5
1	no Ag	20.5	36.8	93.2	242.3	<1	75.6	202.7	322.5
	+ Ag	177.6	352.4	416.8	541.4	138.9	470.4	1080	1094
2	no Ag	4.0	148.1	280.1	303.0	15.3	254.4	452.1	774.3
	+ Ag	125.5	493.3	883.2	1189	168.8	871.7	1630	2351

Legend: IL-12 enhances the antigen induced IFNγ production. PBMC from 2 individuals were stimulated with IL-12, alone, or in combination with grass pollen (400 ug/ml), in the absence and presence of 5 U/ml IL-2. Culture supernatants were harvested after 5 days and IFNγ was tested by ELISA.

DISCUSSION

From the initial demonstration that IL-4 and IFNγ reciprocally regulate polyclonally stimulated murine IgE production, intense effort has been devoted to determine if human hypersensitivity results from an imbalance in the production of these antagonistic cytokines. However, direct analysis of allergen-specific cytokine synthesis by fresh PBMC has been problematic because of the extremely low level of IL-4 and IFNγ production and the lack of sensitive detecting methods. Research has then been focused on the derivation and characterization of T cell lines and clones, or alternatively , polyclonally activated cytokine production by fresh PBMC. In agreement with results obtained from animal models, such studies suggested an increased IL-4 production in allergic individuals (Wierenga JI., et al., 1990). However, both of these experimental approaches have well recognized limitations due to their unphysiological conditions used, and thus reservations have to be made in interpreting the results obtained . Characterization of cytokine production elicited following antigen-specific stimulation of fresh cell populations from normal and allergic individuals, and furthermore the mechanisms that control the induction and expression of these cytokine genes remains an important goal.

In the present study, through the successful development of ultrasensitive cytokine detection methods, we examined (i) allergen-specific cytokine synthesis by fresh PBMC of allergic and

normal individuals directly ex vivo, (ii) IL-2 requirements for the induction of IL-4 and IFNγ gene expression, and (iii) role of IL-12 in inducing IFNγ production and furthermore, IL-12 responsiveness of normal and atopic individuals.

I Technical considerations for in vitro culture and cytokine determination

1.1 Limiting dilution analysis (LDA) vs. other methods

Many investigators utilize bulk culture for studying cytokine synthesis because it is simple to carry out and the cytokine level in such cultures are relatively high thus making it easier to be for them to be detected. However, bulk culture is associated with several important disadvantages: (i) cytokine consumption occurs simultaneous with production, thus the cytokine level detected is the net production rather than total production, and (ii) potential cross-regulatory activity of cytokines secreted by different cell types in the culture can not be avoided. In other words, secretion of certain cytokines in the culture system may inhibit or facilitate the synthesis of other cytokines even with the usage of short term culture period. In this regard, the application of limiting dilution analysis (LDA) techniques can certainly provide a better approach.

The main advantage of LDA is that it allows quantitative estimation of the frequency of antigen-specific cells which exhibit a

particular function. With the minimization of cytokine consumption and cross-regulatory effects seen in bulk culture condition, it is particularly well suited to the identification of rare cells, the activity of which may be undetectable even in primary bulk cultures. Spurred by an increased appreciation of the pivotal role played by Th1 and Th2-like responses in different disease states (Modlin and Nutman, 1993), we developed an LDA method which enable us to evaluate the IL-4 and IFNγ production by allergen-reactive fresh human mononuclear cell populations following short term culture directly ex vivo.

This approach can be readily extended to the evaluation of other cytokines and used to characterize the relative dominance of Th1 or Th2-like responses in vivo. Application of this technique should facilitate efforts to establish clear cut correlations between different patterns of cytokine production in different subjects and disease exabertion vs. resolution in vivo. Moreover, the limited number of cells required, the ease with which they can be obtained and the relatively short time period required for analysis, facilitates longitudinal analysis of low frequency antigen-reactive cells in individuals.

It should be noted that although the LDA system used in the present study has several advantages, there also are certain inherent shortcomings, the major one is that the need of multiple cell concentrations and replicate cultures (ie. 24 or 48 replicates per cell concentration in the present study) is inevitably associated

with intensive work for carrying out cell cultures and later cytokine assays. Furthermore, substantially more cells may be needed for each assay than that for bulk culture. It should also be remembered that the frequency estimates obtained via LDA are always minimal estimates and the "true" frequency in vivo may be higher.

An alternative approach to detailed analysis of in vivo antigen-driven responses which demonstrates great promise utilizes in situ hybridization (Kay et al., 1991) or RNA-PCR analysis (Yamamura et al., 1991; Karp et al., 1993; Pirmez et al., 1993). These strategies have one major advantage over the method we used in that no culture period is required. This entirely dispenses with concerns about skewing the expansion of different T cell subsets in vivo. However, there are several disadvantages associated with these mRNA based methods, (i) there are noted effects caused by the inherent invasiveness of the biopsy procedures used on patient recruitment and their willingness to undergo longitudinal analysis, (ii) mRNA synthesis is not necessarily the same as production of biologically active cytokine (Beutler et al., 1986; Lagoo et al., 1994), and moreover (iii) detection of cytokine mRNA-positive cells by in situ hybridization can not distinguish between antigen-specific cells and those specific for other antigens which have also been activated. In terms of studies with allergic subjects, in the absence of in vivo provocation tests (Durham et al., 1992) which is

a risky and unpleasant procedure, in situ hybridization provided little information to the comparisons between groups of normal and allergic subjects following whatever level of nature booster immunization is provided by the environment at the time of study. In contrast, our short term LDA method provides antigen-specific restimulation of cytokine gene expression which targets those cells reactive only to the antigen in question.

Yet an additional approach which has been successfully used to quantitatively evaluate human cytokine gene expression by normal cells is ELISPOT analysis of PBMC or CD4 T cell enriched populations (Mahanty et al., 1992, 1993). These studies provided instrumental evidence for preferential expansion of Th2-like response by normal cell population directly ex vivo. Although individuals used in these studies with active nematode or helminth infections are likely to exhibit a much higher frequency of IL-4 producing cell than individuals with less intensive polarized response, such as immediate hypersensitivity, ELISPOT may prove to be very useful in this application as well.

1.2. Using fresh whole PBMC vs. long term T cell clones or fractionated CD4 T cell populations

A number of investigators have used long term T cell clones to characterize allergen-stimulated cytokine production by normal and allergic individuals. This experimental approach provided the first substantial evidence for an association between hypersensitivity or

hyper-IgE syndrome and increased IL-4 synthesis. However, a recognized limitation of T cell cloning as a tool to quantitatively infer cytokine production in vivo is that it is subjected to a number of extrinsic factors which strongly influence the number and function of the clones obtained (Gajewski, TF, 1989; Romagnani, S. 1992, 1993; Scott, P. 1991). Exogenously added or endogenously produced cytokines represent an uncontrolled variable which can profoundly skew the composition of the panel of clones obtained. Thus, results derived from such studies may not reflect the real in vivo situations. Moreover, even large panels of clones (ie. in exceptional cases, up to 20-30 clones from one individual) represent only a very small proportion of the total allergen-specific T cell repertoire in vivo. Considering these limitations inherent with using of T cell clones, we choose to study the cytokine synthesis by fresh PBMC following allergen stimulation directly ex vivo, a strategy which would provide information much closer to the physiological signals in vivo. However, one problem associated with studying cytokine synthesis by fresh cells, as discussed above, is the extremely low level of cytokines produced, thus making the development of sensitive detecting methods a prerequisite for such study.

In addition, in the present study, unfractionated mononuclear cell population was used for examination of cytokine production rather than purified CD4 T cells supplemented with irradiated PBMC as a source of APC. This consideration was based on (i) the limited

amount of fresh blood obtainable from each donor, (ii) our belief that the frequency of cells reactive to allergen was likely to be very low, and (iii) our desire to evaluate the response of the intact circulating repertoire to antigen stimulation. Two earlier reports (Adams et al., 1991; Waldman et al., 1992) indicated that the existence of antigen presenting cells in unseparated mononuclear cell preparations, approximately 6-10% of the total, was not a limiting factor at the PBMC concentrations tested in their studies. To verify this, we examined the impact of adding irradiated autologous PBMC as a supplemental source of APC. A decrease in the frequency of negative wells following addition of these irradiated cells would suggest that a shortage of antigen presenting cells was artificially decrease the observed frequency of IL-4 or IFNγ producing cells. However, we found that at the responder cell concentrations used for frequency calculations, the presence of different concentrations of irradiated PBMC as supplemental APC did not alter the frequency of wells in which cytokine production was observed (Table 6), confirming that a potential shortage of APC while examining whole PBMC was not a concern.

1.3. Antigen-specific stimulation vs. polyclonal activation

In an effort to circumvent the limitations associated with utilization of T cell clones, some investigators have examined fresh mononuclear cells directly ex vivo. However, the low

concentrations of cytokine production following short term antigen-specific in vitro restimulation, the limited sensitivity of most human-IL-4 assays, and the difficulties in detecting differences in Northern analysis of IFNγ and IL-4 gene expression upon allergen re-stimulation (Gauchat, J-F. et al., 1991) led many groups to adopt polyclonal activation to evaluate cytokine synthesis patterns.

Use of polyclonal activators as surrogate antigens in analysis of cytokine gene expression in response to allergen exposure is problematic. The data obtained must be interpreted very cautiously, because such stimulation (i) triggers virtually all T cells rather than the allergen-specific population which is likely to make up a very small percentage of total T cells, and (ii) represents an extremely strong activation signal which may stimulate cytokine gene expression qualitatively different from that evoked by antigen-specific signals. Use of such approach risks the possibility that the conclusions drawn may not be the same as those elicited in vivo by antigenic stimulation.

Successful development of sensitive measurement techniques enable us to studies the allergen-induced cytokine synthesis pattern of allergic and normal individuals. A major advantage of antigen specific stimulation in comparison to polyclonal activation is that they specifically activate the cells of interest. Cells specific for antigens other than the one(s) being examined are not targeted,

thereby dramatically increasing the signal to noise ratio and allowing characterization of cytokine producing cells present at very low frequency.

In contrast to purified, recombinant allergens, commercial pollen extracts typically represent complex mixtures of multiple allergens. The latter were deliberately selected for use in this study as the vast majority of allergic individuals exhibit hypersensitivity to multiple entities. While use of such heterogeneous antigen collections can be counterproductive for some objectives, such as mapping T cell epitopes and identifying immunodominant determinants, evaluation of IL-4 and IFNγ responses to a panel of related allergens has the inherent advantage that it reflects the broader picture of cytokine production elicited in vivo by allergens to which that individual exhibits clinical sensitivity and to which that individual is customarily exposed. It should be noted that the possibility that the cytokine production induced by component(s) of the heterogenous grass pollen mixture reflects a polyclonal T cell activation can not be totally excluded. However, given that both the intensity and the kinetics of these responses fall into the same range as that elicited by antigen rather than that induced by known polyclonal activators such as PHA and anti-CD3, and given that different cytokines are undetectable for different individuals, we believe that the simplest interpretation is that this represents antigen-mediated activation.

1.4. Parameters examined

The optimal approach for characterizing the in vivo T cell response varies considerably, depending on the intended objective. The success of therapeutic efforts aimed at amplification or reconstitution of general T cell responsiveness (ie. in HIV infected individuals) can be effectively studied by examining the frequency of IL-2 producing cells (Teppler et al., 1993; Richard DS., et al.,1993). However, our interest is in individuals with allergic diseases which represent more restricted or antigen-specific immunological dysfunctions, thus analysis of Th1 vs. Th2-like pattern of cytokine gene expression, ie. IFNγ vs. IL-4 production respectively, will be more informative than determining the capacity to generate cytokine responses in general by using IL-2 synthesis as a readout.

Proliferation was not examined in this study. Several previous investigators examined PBMC proliferation in response to allergen stimulation in bulk culture or LDA (Cavaillon et al., 1988; Ownby et al., 1979; Phillips et al., 1987). Most of them were unable to identify a difference in the frequency of proliferating cells between allergic and normal individuals (Gauchat 1991). The frequent observance of antibody responses and T cell proliferation in normal non-atopic individuals chronically exposed to environmental allergens are well established (Romagnani, 1990; Sallusto, 1993; Parronchi, 1992). These and other studies in human

and murine systems suggest that the most relevant parameter should not only reveal if an allergen-reactive T cell responds but how. Evidence supporting this hypothesis was provided by some murine studies. In one report by Gieni and HayGlass, limiting dilution analysis of mouse CD4 T cells treated with chemically modified or native antigen revealed similar frequencies of proliferative T cells in the two groups, however, a pronounced Th1- or Th2-like response was observed in mice treated with chemically modified antigen or native antigen, respectively, indicating the differential cytokine synthesis pattern, but not the general proliferative response, is the instrumental parameter in this study system (Gieni & HayGlass, 1994, submitted).

II. Allergen-induced cytokine synthesis pattern observed in allergic and normal individuals

2.1. Allergic individuals exhibit markedly stronger IL-4 and weaker IFNγ responses to grass pollen

In the present study, by examining cytokine synthesis by fresh human PBMC following allergen specific stimulation , we demonstrated that patients with seasonal allergic rhinitis exhibited an imbalanced cytokine synthesis profile compared to that of normal individuals upon exposure to common environmental allergen. Specifically, the average frequency of IL-4 producing cells was found to be approximately 5-fold higher, while the frequency of IFNγ secreting cells was 3-fold lower in PBMC of

atopic patients than that observed in normal subjects (Table 3). To our knowledge, this is the first direct evidence of an imbalance of cytokine production among fresh, antigen-stimulated PBMC in atopic individuals, supporting our hypothesis that the allergen elicited immune response in such patients is skewed toward Th2-like patterns of cytokine gene expression which give rise to the overproduction of IgE antibody.

Over the recent years, many groups focused their efforts on investigating the mechanisms underlying the development of allergic diseases. Due to the low concentration of cytokine production by fresh allergen-stimulated PBMC and the lack of sensitive cytokine-measuring techniques, most studies utilized long term T cell clones or polyclonal-activated fresh cell populations to characterize the cytokine synthesis pattern in atopic individuals. These studies indeed provided plentiful information about the association between hypersensitivity or hyper-IgE syndrome and elevated IL-4 production. However, as discussed above, there are potential limitations associated with these approaches (as discussed above), thus, the interpretation of the results obtained need to be very cautious.

Furthermore, the current consensus that imbalanced IL-4 and IFNγ production may account for the generation of allergic diseases such as immediate hypersensitivity and broadly related disorders like atopic dermatitis and hyper-IgE syndrome has been general accepted

based on evidence provided by individual studies. However, careful examination of the literature reveals conflicting conclusions as to the nature of the specific defect in cytokine synthesis (Imada & HayGlass, in press). In many studies, IFNγ production was reported to be essentially normal in atopic subjects (Romagnani, 1989; Paganelli, 1992; Tang, 1993; Quint, 1989), whereas in others it was markedly reduced (Byron, 1992; Chan, 1993; Jujo, 1992; Rousset, 1991) or even elevated (Grewe, 1994; Tang, 1994). IL-4 production has usually been reported as substantially higher (Romagnani, 1989 and 1994; Jujo, 1992; Pene, 1994), but there also some exceptions (Van der Pouw Kraan, 1994). In some studies, neither IL-4 nor IFNγ production was different (Quint, 1989) whereas in others syntheses of both cytokines were affected, with decreased IFNγ and increased IL-4 secretion (Jujo, 1992; Rousset, 1991) compared to the responses elicited in normal controls. Some of these discrepancies may reflect different etiologies for related but distinct hypersensitivity states (ie. atopic dermatitis vs. allergic rhinitis). However, the variation frequently observed by different groups studying the same disease may 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 subject may be extrapolated to the entire allergen specific T cell repertoire, or that potentially unphysiological stimuli such as polyclonal activators were used as surrogate antigens (Imada & HayGlass, in press). Thus, we believe that our

data obtained by examining fresh PBMC following allergen-specific stimulation directly ex vivo should represent the real situation in vivo much closer than that obtained by studies using T cells clones or polyclonal activations. It should be noted, however, further confirmation of the present observation is also necessary by examining more subjects.

2.2. Altered ratio of IL-4:IFNγ production observed in atopic patients

The ratio of Th1:Th2 cytokine synthesis elicited following allergen challenge is demonstrated to be critical in a number of systems (Del prete, 1988; Swain, 1988; Bass, 1989) in determining the class/subclass of antibody or type of immune response induced. Romagnani has suggested that the ratio of antigen-driven IFNγ:IL-4 synthesis is much more important than the absolute amount of either cytokine in determining hypersensitivity versus clinical unresponsiveness (Romagnani, 1993). In the present study, in addition to the increased IL-4 and decreased IFNγ production, we observed that, although the IFNγ response to grass pollen stimulation remains dominant even in most allergic individuals, the ratio of IFNγ:IL-4 synthesis was consistently lower in all allergic patients than that of normal donors, indicating a pronounced shift towards increased Th2-like activity in the circulating immune repertoire of grass pollen sensitive individuals. Thus, our data further confirmed that the imbalanced production of antagonistic

IL-4 and IFNγ, rather than the absolute intensity of either response, is decisive in developing of hypersensitivity.

One point in particular needs to be emphasised. Although the cytokine synthesis pattern differed quantitatively between normal and allergic individuals, the vast majority of subjects studied demonstrated significant IFNγ and/or IL-4 production following allergen in vitro restimulation. This suggests that the determining factor in the decision between allergy and tolerance to environmental allergens lies hinges on which form of responsiveness is induced, specifically whether a Th1- or Th2-like pattern of cytokine response is triggered, rather then whether a immune response is mounted or not. Most individuals, both atopic and non-atopic, are all in fact responsive to allergen challenge, with the responses of non-allergic individuals acting to prevent hypersensitivity.

2.3 Differential IL-2 requirements in induction of IL-4 and IFNγ synthesis

Identification of factors which are able to influence the commitment of Th1 or Th2 cells is of central importance for understanding the mechanism(s) underlying the development of allergy and further immunological therapy of such allergic diseases. Up to date, still little is known concerning the T cell differentiation pathways, however, cytokines, most notably IL-4 and

IL-12, are suggested to play the most important role in this process. IL-2, which has pleiotropic functions on many cell types, is also one of the several candidates.

Although IL-4 mediated T cell proliferation was reported as IL-2-independent by several groups (Lichtman, 1987; Hergen, 1987; Lorre, 1990), the weight of evidence clearly supports the requirement of IL-2 in the early stages of developing IL-4 responses by small, resting, naive T cells (Powers, 1988; Weinberg, 1990; Seder, 1991; Ben-Sasson, 1990; Paul, 1990). However, since long-term in vitro T cell stimulation was used by these previous studies to analyze IL-4 gene expression, it was unable to separate the role played by IL-2 in the early clonal expansion of IL-4 producing cells from that in IL-4 gene expression itself.

By analyzing murine IL-4 gene expression following short term in vitro polyclonal- or antigen-specific-stimulation, Yang and HayGlass recently proposed that IL-4 response may be divided into two stages in terms of IL-2 requirements: IL-2 dependent and IL-2 independent stages (Yang & HayGlass, 1993). They observed that IL-2 was necessary for IL-4 synthesis induced by anti-CD3, while allergen-specific IL-4 production by cells from immunized mice was IL-2 independent. Based on the consideration that the nature of T cell populations targeted by different stimulus are different, ie. anti-CD3 preferentially activates resting, naive cells while the cells targeted by specific antigen are most likely those previously

activated and memory CD4 T cells, they suggested that IL-2 is necessary for the initial activation and clonal expansion of IL-4-producing cells, but the stage of production and release of biologically active IL-4 is independent of IL-2. Aiming at further exploring this concept in our human allergy study, we examined the IL-4 gene expression by fresh human PBMC following short term in vitro allergen-specific stimulation. We found that the frequency of allergen-induced IL-4 producing cells remains unaffected by the addition of rIL-2 to the culture. Thus our results further confirm the IL-2 independency in terms of IL-4 gene expression.

In contrast to IL-4, IFNγ synthesis has been demonstrated by several investigators to be strongly IL-2 dependent (Kelly, 1987; Pestka, 1987; Wilson, 1988; Yang and HayGlass, 1993). In agreement with these reports, we observed that the frequency of IFNγ producing cells was increased by at least 2-fold upon addition of exogenous IL-2 to the culture (n=17). This observation may reflect subjects in which endogenous IL-2 production or consumption is a factor influencing IFNγ production.

This difference in IL-2 dependence in IL-4 and IFNγ synthesis suggests a differential requirement for cytokine gene expression by distinct T cell subsets. Although it is important for the presence of IL-2 in the growth and differentiation of precursor T cells destined to produce IL-4, IL-2 does not appear to affect the effector phase of IL-4 gene expression in antigen-specific

responses. The most biologically relevant situation that comes to mind is the ongoing IgE response observed in allergic individuals. Further work needs to be done in this area.

This finding may be significant in understanding the persistent IL-4 dominant responses in allergic patients. Under such circumstance, a high level of IL-4 is consistently produced by Th2 cells and possibly other local cells (such as mast cells) independent of IL-2. This may further result in (1) recruitment and differentiation of more Th2 cells which are capable of synthesizing IL-4, (2) inhibition of IFNγ synthesis as a consequence of crossregulation by IL-4 directly and indirectly, possibly through suppression of IL-2 synthesis. Supporting evidence was provided by quite a number of reports which indicate that IL-4 has the capacity to: inhibit expression of IL-2R (Martinez, 1990), suppress IL-2-dependent T cell proliferation (Martinez, 1990; Karry, 1988), suppress IL-2 and/or IFNγ induced cytokine gene expression (Gautam, 1992), and inhibit IL-2, IFNγ synthesis directly (Peleman, 1989; B-Fernandez, 1991). Consequently, the ongoing IL-4 responses may serve to maintain the commitment of Th2-dominant response.

III. Differential response to IL-12 observed in normal and allergic individuals

3.1 IL-12 induces strong IFNγ production by both unstimulated and allergen-stimulated PBMC and synergizes with IL-2 in this effect

IL-12 is a recently described cytokine that has a unique heterodimeric structure. It was initially identified and purified from the conditioned medium of EBV-transformed human B lymphoblastoid cell lines (Kobayashi, 1989), but the majority of IL-12 is produced by macrophage/monocyte following appropriate stimulation. In the past several years, extensive studies have been focused on investigating the biological effects of IL-12. In particular, it is suggested that IL-12 can (1) enhance the cytolytic activity of a number of effector cells including T cells (Gately, 1992), NK cells and LAK cells (Kobayashi, 1989; Wolf, 1991; Naume, 1993; Robertson, 1992; Chehimi, 1992 and 1993; Gately, 1992; Zeh, 1993) and macrophages (Michael, 1994); (2) increase proliferation of activated NK and T cells (Zeh, 1993; Gately, 1991; Perussia, 1992; Bertagnolli, 1992; Andrews, 1993), (3) induce production of cytokines, most notably IFNγ (Gubler, 1991; Wolf, 1991; Chan, 1991 and 1992; Wu, 1993; Naume, 1993); and (4) inhibit IL-4 induced IgE production (Kiniwa, 1992). Based on these potent immunomodulatory activities, IL-12 has been proposed to play an important role in promoting the generation of Th1 responses in vivo (Trinchieri, 1993; Hsieh, 1993; Manetti, 1993, Germann, 1993).

Since the objective of the present study is to investigate the cytokine synthesis pattern which is characteristic of atopic patients, factors capable of influencing cytokine gene expression are also invaluable parameters to be evaluated. In this concern, we primarily considered to examine (i) IFNγ-inducing activity of IL-12

not only to confirm the results obtained by other studies, but also to extend the observation to our antigen-specific system, (ii) IL-12 production by human PBMC of allergic and normal individuals, and (iii) the responsiveness of allergic and nonallergic individuals to IL-12. Because of some unsolved technical problems concerning the detection of IL-12 production (which is still under active investigation), we initially examined the capacity of IL-12 in inducing IFNγ synthesis and compared that between grass pollen allergic and non-allergic individuals.

Our results demonstrated that IL-12 induces IFNγ production by unstimulated PBMC (without the presence of grass pollen) in a dose-dependent fashion. Also in keeping with the results of previous studies, IL-2 is shown clearly synergizing with IL-12 in triggering IFNγ production. Most importantly, in addition to other IL-12 synergizers studied, most of which are polyclonal activators such as PHA, anti-CD3 and phorbol diesters which have been previously well documented (Chan, 1991; Wu, 1993), we observed that IL-12 significantly amplified allergen-stimulated IFNγ synthesis.

It is well recognized that regulation of IFNγ production during inflammation or an immune response is of central importance to mechanisms of both adaptive and nonadaptive resistance (Trinchieri, 1985; Perussia, 1987; Cassatella, 1985; Nathan, 1983). Thus, the ability of IL-12 to induce IFNγ production from PBMC may represent one of the most biologically significant function of this cytokine,

and might be particularly important in determining the nature of response induced in vivo. Since IL-12 may be produced by monocytes/macrophages and possibly B cells at biologically active concentration (D'Andrea, 1992) during an ongoing immune response, it is reasonable to speculate that locally produced IL-12 may both induce IFNγ synthesis by naive T and NK cells and further augment allergen-stimulated IFNγ production which may then significantly affect the response to environmental allergen by inducing prevalent differentiation of Th1 cells. Further evidence also suggested that IL-12 play a major role in regulating the induction of effector response either by directly facilitating the development of Th1 cells (Hsieh, 1993; Trinchieri, 1993; Manetti, 1993; Germann, 1993) or by inhibiting the production of IL-4 (Kiniwa, 1992).

3.2. Impaired responsiveness to IL-12 observed in allergic individuals

Inspired by our observation that rIL-12 significantly amplified allergen-stimulated IFNγ synthesis, we next examined the responsiveness to IL-12 in terms of IFNγ production in grass pollen allergic and normal subjects. We believed this may underline, at least in part, the mechanism(s) controlling the differential effector responses induced. In line with our hypothesis, we observed a significantly different response to rIL-12 stimulation between allergic and normal individuals. Specifically, the majority of allergic patients (5 out of 7) produced little if any IFNγ in

response to IL-12 stimulation even at very high IL-12 concentration used (100 pg/ml). However, under the same experimental conditions, PBMC from normal donors synthesized significantly higher level of IFNγ (5-9 folds higher) (Table 10). This result was further confirmed by similar, independent experiments (Table 11). Thus, our initial data indicates an impaired responsiveness to IL-12 in allergic individuals.

We speculate that this finding might be of great significance for understanding the differential effector response, ie. hypersensitivity vs. clinical unresponsiveness, observed in allergic and normal individuals following exposure to environmental allergens. IL-12 and IFNγ have been well documented to be the most important cytokines in determining the differentiation of CD4 T cells to Th1 subsets. It was reported that IFNγ inhibits the proliferation of Th2 clones and this effect is sufficient to limit the clonal expansion of Th2 clones (Fernandez-B, 1988; Gajewski, 1988; Fitch, 1993). In addition, IFNγ is able to inhibit most of the effects of IL-4 on B cells, i.e., antibody class switch to IgE, activation for B cell growth and differentiation, and the expression of some cell surface molecules such as MHC class II and the Fc receptor for IgE (CD23) (Street and Mosmann, 1991). Furthermore, IFNγ promotes differentiation of Th-precursor to Th1 cells, both in vitro (Gajewski, 1989) and in the Leishmania major infection model in vivo (Coffman RL, 1991; Locksley RM, 1991; Scott P, 1991). There is also growing evidence that IL-12 plays a major

role in induction of Th1 response in both murine and human system (Schmitt, 1994; McKnight, 1994; Trinchieri, 1993; Hiseh, 1993; Manetti, 1993, Germann, 1993). In an in vitro model utilizing ovalbumin-specific CD4 T cells derived from TCR-transgenic mice, Hiseh et al have shown that antigen combined with murine rIL-12, but not a number of other cytokines, preferentially induced the development of Th1 cells from naive T cells and addition of neutralizing anti-IL-12 antibody to the cultures abolished this Th1-inducing activity (Hiseh, 1993). In a human study, CD4 T cell lines, produced from atopic individuals specific to Dermatophagoides pteronysinus, generally exhibited a Th2-like phenotype. However, cell lines generated in the presence of human rIL-12 exhibit a Th0 or Th1-like cytokine profile (Manetti, 1993). In contrast, in the presence of anti-IL-12 antibody, PPD-specific T cell lines which usually exhibit a Th1-like phenotype, produce both IL-4 and IFNγ. Furthermore, in one report presented by Romani et al, IL-12 was suggested to be more important than IFNγ in inducing Th1 differentiation (Romani, 1994). Thus, it seems that IFNγ and IL-12 play a critical role in generating Th1, and inhibiting Th2, responses. As discussed earlier, IL-12 was also demonstrated to be a very strong inducer for IFNγ synthesis. Our observation that allergic patients exhibited much poorer response to IL-12 in IFNγ production suggests that deficient responsiveness to IL-12 may account for the pathogenesis of allergic diseases. It is reasonable to speculate that normal

responses to allergen are characterized by dominant generation of Th1 cells which are promoted by certain cytokines, of which IFNγ and IL-12 play the most important role. IL-12 further facilitates this process indirectly by enhancing allergen induced IFNγ production (our data) and also possibly by inhibiting IL-4 synthesis (Kiniwa, 1992; Morris, 1994). However, in allergic patients, the capacity of IFNγ production and the frequency of allergen-specific IFNγ producing PBMC (this thesis) are impaired, at least partly due to the decreased response to IL-12. Possibly together with some other currently unknown reasons, a dominant Th2 response is thus generated which lead to overproduction of IL-4 and hypersensitivity occurs as the consequence of the IL-4 induced IgE production. It should be noted that a comparison of the ability of IL-12 production between allergic and normal individuals would be necessary at this stage.

Collectively, our study suggests that an imbalanced Th1/Th2-like cytokine synthesis, ie. decreased IFNγ and increased IL-4 production, by circulating pollen-reactive cells is associated with the occurrence of immediate hypersensitivity observed in allergic patients following exposure to grass pollen. IFNγ, but not IL-4, synthesis by antigen-stimulated PBMC is IL-2 dependent. Moreover, IL-12 is demonstrated not only to be able to induce IFNγ synthesis by unstimulated PBMC, but also significantly enhances the allergen-induced IFNγ production, and IL-2 is synergistic with IL-12 in its IFNγ-inducing activity. Furthermore, an impaired response to IL-12

was observed in grass pollen allergic individuals, which may represent at least one reason for the decreased ratio of IFNγ: IL-4 production exhibited in allergic patient in response to environmental allergens.

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