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MODULATION OF CYTOKINE SYNTHESIS PATTERNS AND IgE RESPONSES IN VIVO WITH CHEMICALLY MODIFIED ALLERGEN

by

XI YANG

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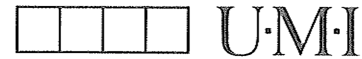

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XI YANG

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To My Motherland,
China

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ABBREVIATIONS

$Al(OH)_3$	:	Aluminum hydroxide, alum
anti-μ	:	Rabbit anti-mouse IgM antibody
APC	:	Antigen presenting cells
BNHS	:	D-Biotin-e-aminocaproic acid N-hydroxysuccinimide ester
BSA	:	Bovine serum albumin
CD40L	:	CD40 ligand
CFA	:	Complete Freund's adjuvant
Con A	:	Concanavalin A
CO_2	:	Carbon dioxide
CTL	:	Cytotoxic T cell
d	:	Day
dpm	:	disintegrations per minute
DNA	:	Deoxyribonucleic acid
DTAF	:	dichlorotriazinyl amino fluorescein
DTH	:	Delayed type hypersensitivity
ELISA	:	Enzyme linked immunosorbent assay
FACS	:	Fluorescence activated cell sorter
Fc	:	Constant fragment of immunoglobulin
FCS	:	Fetal calf serum
Fig	:	Figure
FITC	:	Fluorescein isothiocyanate
g	:	Gram

GA	:	Glutaraldehyde
GM-CSF	:	Granulocyte-macrophage colony-stimulating factor
h	:	Hour
^{3}H	:	Tritiated
Hb	:	Hemoglobin
HBSS	:	Hank's balanced salt solution
IFN	:	Interferon
Ig	:	Immunoglobulin
IL	:	Interleukin
IL-2R	:	Interleukin-2 receptor
i.p.	:	Intraperitoneal
i.v.	:	Intravenous
L.	:	Leishmania
LFA	:	Lymphocyte function-associated factor
log	:	Logarithm
LPS	:	Lipopolysaccharide
LT	:	Lymphotoxin
M	:	Molar
mAb	:	monoclonal antibody
mg	:	Milligram
MHC	:	Major histocompatibility complex
min	:	Minute
ml	:	Milliliter
MLR	:	Mixed lymphocyte reaction
MNCs	:	Mononuclear cells

mM	:	Millimolar
mPEG	:	monothoxypolyethylene glycol
mRNA	:	Messenger RNA
M_r	:	Relative molecular mass
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NK	:	Natural killer
NRIg	:	Normal rat immunoglobulin
NRIgG	:	Normal rabbit immunoglobulin G
OA	:	Ovalbumin
OA-POL	:	Glutaraldehyde-polymerized ovalbumin of M_r 3.5×10^7
O.D.	:	Optical density
PAF	:	Platelet-activating factor
PBS	:	Phosphate buffer saline
PCA	:	Passive cutaneous anaphylaxis
PK	:	Prausnitz-Kuestner
PPD	:	Purified protein derivative
PSF	:	Penicillin, streptomycin and fungzone
r-IL	:	Recombinant interleukin
rpm	:	rotations per minute
S.D	:	Standard deviation
s.c.	:	Spleen cell
SEM	:	Standard error of the mean
sIg	:	Surface immunoglobulin
TCR	:	T cell receptor

TES	:	*Toxocara canis* excretory-secretory antigen
TGF-β	:	Transforming growth factor-β
Th	:	T helper
TNF	:	Tumor-necrosis factor
Ts	:	T Suppressor
U	:	Unit
μCi	:	Microcurie
μg	:	Microgram
v	:	Volume
vs	:	Versus

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SUMMARY

The pattern of cytokine production elicited after in vivo exposure to antigen largely determines the type of effector immune responses that develops. Adminstration of high M_r (3.5×10^7) chemically modified ovalbumin (OA-POL) inhibits murine IgE and increases IgG_{2a} responses. This activity was previously demonstrated to be CD4 T cell dependent and reversible upon concurrent anti-IFNγ mAb treatment in vivo, suggesting the critical role of CD4 T cells as well as the cytokines produced by these cells in the regulation of these immune responses.

The objective of the present study was to elucidate the mechanism by which a chemically modified allergen (OA-POL) induces antigen-specific, isotype-selective IgE suppression. Our hypothesis was that the balance between Th1 and Th2-like cells in vivo is changed as a consequence of administration of this chemically modified, but not native, allergen. Specifically, we examined the possibility that in vivo exposure to this modified protein directed cytokine production to a Th1-like dominated pattern, thereby inhibiting native protein primed IgE synthesis.

Using a system of short term bulk culture of murine spleen

cells immediately ex vivo, we demonstrated:

1) the rapid induction of allergen specific IL-4 producing CD4 cells following OA alum immunization, a finding which suggests that CD4 T cells capable of producing IL-4 constitute a normal component of the mature extrathymic murine T cell repertoire. The choice of adjuvants used for in vivo immunization was shown to be critical in determining the cytokine production profile induced. Moreover, antigen specific in vitro restimulation was more representative than polyclonal T cell stimulation for the measurement of in vivo cytokine synthesis patterns.

2) differential IL-2 requirements for the induction and maintenance of IL-4 vs IFNγ responses. Although IL-2 was required for induction of IL-4 responses, IL-4 gene expression in established responses was independent of IL-2. In contrast, the IFNγ response, both in the initial stages of development and later gene expression, was largely IL-2 dependent.

3) differential patterns of cytokine gene expression are induced by in vivo exposure to chemically modified and native allergen. Administration of chemically modified allergen (OA-POL) elicited predominantly cytokines characteristic of Th1 cells, whereas native allergen

induced cytokines not uniquely characteristic of Th1- or Th2-like cells. In both de novo induced and established hypersensitivity, this chemically modified allergen (OA-POL) treatment altered the ratio of antigen driven synthesis of IL-4 to IFNγ, consequently inhibited the IgE responses.

4) an apparent involvement of distinct antigen-presenting cell (APC) populations in processing and presentation of native and chemically modified allergen leading to consequent effects on cytokine and antibody production. B cells play an important role in presentation of unmodified OA as suggested by a failure to prime Th2-like cells in B cell deficient mice. In contrast, the administration of chemically modified OA (OA-POL) primed CD4 T cells capable of inhibiting induction of IgE responses (i.e. functionally Th1-like) in vivo despite the absence of B cells.

These observations are helpful for the understanding of the signals involved in initiating and sustaining of Th1-like and Th2-like cytokine responses. Moreover, these observations may ultimately be useful in clinical settings including allergy where the ability to select specific patterns of cytokine gene expression would be advantageous.

INTRODUCTION

I. LITERATURE REVIEW

1.1 Allergy and IgE

About 12-20% of the population suffers from various forms of allergy, a state of immediate hypersensitivity due to excessive IgE mediated responses to common environmental substances. The antigens which can induce allergic responses are called allergens (the term of antigen and allergen is used interchangeablely in this thesis). It is believed that allergic diseases tend to increase with advancing civilization and are commoner today than they used to be. Medical statistics reveal that they occur more often in industrialized countries than in agrarian countries, and more often in large towns than in the countryside. Whether this reflects increased incidence or the availability of more comprehensive medical care is a subject of ongoing debate.

Allergic diseases have been documented for centuries. John Bostock described the classical symptoms of seasonal allergic rhinitis (hay fever) in 1819. 40 years later, Salter observed that the etiologic agents for this condition were environmental and that sensitivity to these environmental agents could be tested by an erythematous

skin reaction. The more scientific description of allergic response was made by Prausnitz and Kustner in the early 1920s. They succeeded in provoking an allergic reaction by transferring the serum of Kustner, who was reacting to fish, to Prausnitz, who was allergic to pollen. Local reinjection of fish antigen to Prausnitz immediately triggered a wheal and a flare at the site of reinjection. However, the mediators (antibodies) responsible for the initiation of this reaction were not clear, leading to them being named reagins (Coca, 1943).

A major breakthrough in allergy happened 40 years later. In 1966, the Ishizakas and colleagues demonstrated that the so-called "reaginic" antibodies in hay fever patients belonged to a unique, previously unrecognized immunoglobulin class, which they referred as γE, being renamed as IgE in 1969 (Ishizaka, 1966, Bennich, 1969). Subsequently, an atypical myeloma protein was proven to share the same antigenic structure with the polyclonal reaginic antibodies. Abnormal IgE responses, have since been documented in numerous independent studies and are accepted as the key for immediate hypersensitivity.

IgE antibodies share with the other immunoglobulins (Ig) two light and heavy chains but, like IgM, display five instead of four domains in the heavy chains. They are

extremely heat labile in that they lose their ability to sensitize human skin for the Prausnitz/Kustner (PK) reaction after heating at 56° C for 4 hours. Loss of activity in the PK reaction following mild treatment with 2-mercaptoethanol indicated the presence of labile S-S bonds within their Fc domains. IgE is unable to fix complement or to cross the placenta.

IgE plays its role in allergic responses by sensitizing mast cells and basophils via binding with a high affinity receptor (Fc_ϵ receptor) on these cells. The reaction of allergen to cell-bound IgE antibodies induces production and/or release of a variety of mediators which cause a cascade of allergic reactions. These mediators include histamine, serotonin, chemotactic factors, heparin, leukotrienes, prostaglandins, platelet-activating factor (PAF) and a variety of enzymes. Common potential allergens include grass pollens, animal dander, molds, insect venins, dust constituents, some food proteins and a wide variety of other substances. Some drugs, notably penicillin, can cause severe immediate hypersensitivity.

IgE antibodies have been found in the human, monkey, rabbit, rat , mouse, guinea pig, dog, sheep, pig, and cow, but the presence of their counterpart in avian species is of some doubt. It has been suggested that in humans, the

higher the blood levels of total IgE, the greater the predisposition to allergy (Tada, 1975).

1.2 Allergen immunotherapy

The current practice for treatment and prophylaxis of allergic disease consists of (i) environmental control, (ii) pharmacologic management and, in some cases, (iii) allergen immunotherapy (AI). Although avoidance of allergen is the most efficient means by which to prevent allergic reactions, it is frequently difficult to achieve. Even food allergens, which are easier to avoid than pollens, molds etc, are difficult to eliminate in that so many commercial food products are multi-component. For example, milk proteins are used in variety of foods that might not be suspected of containing milk, such as nondairy creamers and margarines, bread, canned tuna fish and hot dogs (Sampson, 1992).

Commonly used drugs for allergy therapy include topical corticosteroid, bronchodilators, antihistamines, and other antagonists of vasoactive mediators released from mast cell and basophils. Application of these drugs has proven to be highly effective in preventing and controlling the allergic symptoms. For example, topical corticosteroids largely diminish the effects of allergic events through inhibition

of several processes, including lysosomal enzyme release, phagocytosis, accumulation of inflammatory cells, and the synthesis or release of many chemical mediators of inflammation. The range of pharmacologic agents currently available is capable of providing excellent symptomatic control, even to highly allergic individuals.

Since allergy reflects an inappropriate immunological reaction, there has been a longstanding interest in therapeutic approaches related to immunology. The initiation and, largely empirical, development of immunotherapy was the result of these efforts. In 1909, Noon initially reported treatment of grass pollen induced rhinitis using serial injections of pollen extracts to allergic patients. Influenced by the immunological theories then current, he attributed the success of this "active immunization" to antitoxin immunity (Noon, 1911).

This immunotherapeutic approach, known at various times as desensitization or hyposensitization, is currently practised under the term "allergen immunotherapy". Acting by mechanisms which remain unresolved, it decreases clinical sensitivity. In general, immunotherapy consists of the administration of slowly increasing quantities of antigen over an extended period of time. Its aim is to induce a degree of clinical tolerance, or lowered

sensitivity to the antigen as evidenced by a decline in clinical symptoms and medication requirements. Immunotherapy is recommended clinically in some cases when it is not possible to avoid the allergen and poor results or significant side effects from pharmacologic treatment occur (Evans, 1992). Immunotherapy is effective for patients with anaphylactic reactions to insect stings. Its success in patients with allergic rhinitis or asthma is quite variable and it is not recommended for food allergy.

Despite its stated efficacy in properly selected cases, immunotherapy has from the beginning presented major problems. Controlled trials in 1960s demonstrated that the effects of immunotherapy on allergen induced symptom-medication score reduction are dose related. Larger doses were more likely to elicit clinical improvement but greatly increased the risk of anaphylactic reactions. The potential for local and systemic reactions, including anaphylactic shock, has limited its clinical application. To overcome these difficulties much effort has been taken to modify allergenic extracts with the aim of diminishing their allergenicity while retaining or improving their therapeutic effectiveness.

Allergens have been chemically modified by many different approaches including (1) alum precipitation; (2)

conjugation with monothoxypolyethylene glycol (mPEG); (3) polymerization by glutaraldehyde; (4) formaldehyde denaturation; (5) conjugation with dextran; and (6) photooxidation, among others (Norman, 1982). Clinical trials with these materials suggest that their effectiveness is equivalent to that of aqueous extracts. However, among these modified agents only alum adsorbed extracts and some glutaraldehyde polymerized allergens are commercially available for clinical application. In addition, various other alternatives have been in use, including skin titration testing and treatment (the Rinkel method), subcutaneous provocation and neutralization testing, and sublingual provocation testing. All of these techniques use very small doses of antigen, and therapeutic benefit has frequently not been shown when they are subjected to controlled trials (Ohman, 1992).

The basis of allergen immunotherapy is not clear. It has become apparent that no single mechanism is responsible for all immunotherapeutic effectiveness, but rather that many types of immune alterations may occur during immunotherapy. The first observation and a commonly measured clinical indication of immunological change is the striking rise in IgG or "blocking" antibody. This preferential IgG response is postulated by some to reduce allergic symptoms by preventing the antigen from combining with mast cell-bound

IgE. There is some evidence for this in insect sting allergy. Passive administration of hyperimmune IgG preparations to patients in whom systemic reactions to immunotherapy are common and patients intolerant to conventional immunotherapy regimens reduced the frequency of systemic reactions in well-controlled trials. Studies in inhalant allergy, however, show a weak relationship between the degree of rise in IgG and symptomatic benefit (Bousquet, 1990; Sadan, 1969).

Another alteration following prolonged immunotherapy is the frequently observed decrease of allergen specific IgE synthesis (Van Meter, 1979; Reid, 1986). This IgE reduction is thought likely to decrease allergen sensitivity. However, IgE antibody levels do not seem to change consistently in immunotherapy even when there is clinical benefit. In some cases, IgE antibody actually rises at a time when clinical benefit is observed (Bousquet, 1988; Cockroft, 1977).

More recently, the effect of immunotherapy on prevention of allergic responses has, in many cases, been suggested to be related to the activation of certain T cell subsets (Hseith, 1989, Takata, 1990, Wilkinson, 1987). In light of the importance of T lymphocytes in the regulation of immune responses, the role played by T cells in immunotherapy will

certainly receive more attention in the study of the mechanisms underlying immunotherapy.

The complexity of the mechanism of action of immunotherapy may be partially due to the heterogeneity of the allergen preparations used. For example, depending on the proteins used and reactions employed, glutaraldehyde treatment of allergic proteins forms molecules markedly different in size and extent of polymerization. Some polymers lose the immunogenicity of native proteins while other low molecular weight molecules become immunogenic upon polymerization. Obviously, consistent results and a clear understanding of the mechanism of immunotherapy require the availability of relatively "homogeneous" allergen preparations.

1.3. Egg allergy and chemically modified ovalbumin (OA-POL) induced IgE suppression

Hypersensitivity to different foods is a common cause of a variety of allergic symptoms. Egg white is one of the important food items that frequently induces allergy, particularly in children. Hen's eggs have been used as a nutrient throughout the evolution of man. It is the most "complete" animal food naturally processed for the growth of the embryo. Egg is today an inherent ingredient in human and animal food and it is most difficult to be totally

excluded from meals.

Allergens responsible for egg allergies have been well characterized. It has been shown that among 40 different egg proteins, only 3 are major allergens, namely ovalbumin (OA), ovomucoid and ovotransferrin. OA is the most abundant of the proteins, forming 58% (w/w) of the whole egg white (Langeland, 1982, 1983).

The complete amino acid sequence of hen ovalbumin comprising 385 residues, has been deduced from analysis of cyanogen bromide fragments and peptides derived by digestion with a number of proteolytic enzymes. The molecular weight of the polypeptide chain is 42,699 dalton. Ovalbumin mRNA has been isolated and the sequence has been determined, which is in complete agreement with the amino acid sequence previously observed (Nisbet, 1981). The crystal structure of OA has also been characterized (Stein, 1990).

Due to the clear understanding of the molecular structure of OA and its nature as a "real" allergen, the OA system is obviously a useful model for allergic disease investigations. Moreover, many animal species including mice and rats primed with low doses of the human allergen OA (using alum as adjuvant) consistently exhibit IgE

responses, thereby providing a valuable model system for the study of human immediate hypersensitivity.

In prior work, HayGlass and Strejan developed a murine model in which administration of glutaraldehyde polymerized OA could inhibit the induction of an IgE responses normally induced by native OA. By modification of the reaction conditions, they successfully obtained a glutaraldehyde polymer of 35 x 10^6 daltons of limited-heterogeneity as assessed by gel filtration. Although the variation in physicochemical spectrum (ways of inter- and intra molecular bonds formed by GA) were still present, the elution profile of OA-POL on gel filtration and, more importantly, the immunologic properties of these preparations were practically identical from batch to batch (HayGlass & Strejan, 1983, 1984).

The administration (i.p.) of this soluble, high M_r polymerized OA (termed OA-POL) to the 14 strains of mice tested, suppressed (90-99%) primary and secondary IgE responses elicited by immunization with native OA (alum). This inhibition was essentially isotype-specific for IgE, with 0-5 fold decreases in IgG1, minor changes in allergen-specific IgA, IgM and 100-fold increase in IgG_{2a} production. Moreover, OA-POL induced long-term IgE suppression by a single course of in vivo (three 80 μg i.p. injections)

treatment. The resulting isotype-specific inhibition of IgE responsiveness was maintained for at least 1 year in the absence of further treatment in C57BL/6 mice, despite multiple re-exposures to the sensitizing allergen. Most significantly, OA-POL treatment suppressed not only de novo but also well-established IgE responses in this mouse strain (HayGlass, 1990, 1991a, 1991b, 1991c).

Although the suppression effect on the IgE response had been clearly demonstrated, the mechanism(s) responsible for this were still obscure. The main focus of the present study was to elucidate the nature of these changes. Previous work showed that OA-POL induced IgE suppression and IgG_{2a} elevation is CD4 T cell dependent. Anti-IFNγ treatment at the time of immunization of mice with OA-POL prevented the suppression of the IgE response and elevation of the production of IgG_{2a} antibody (HayGlass, 1991d). Collectively, these data suggested the involvement of regulatory CD4 T cells and of the cytokines they produced in the antigen-specific isotype specific immunosuppression observed in vivo following administration of this class of chemically modified allergen.

1.4 Cytokine production and IgE regulation

1.4.1 CD4 T cell subsets

T lymphocytes are defined by the surface expression of T cell receptor (TCR) complexes. There are presently two defined types of TCR: one is a heterodimer of two disulphide-linked polypeptides (α and β), the other is structurally similar but consists of γ and δ polypeptides. Both receptors are associated with a group of polypeptides making up the CD3 complex (γ, δ, ϵ, ζ, ω). These T cells can be further subdivided into those expressing either CD4 or CD8 molecules.

1.4.2. Early studies of CD4 T cell functional variability

Many T cell functions were characterized before the definition of CD4 and CD8 T cell subsets. When anti-CD4 and anti-CD8 antibodies became available, these antibodies were used to determine the type of responses mediated by different T cell subsets. Although CD4 T cells are commonly referred to as helper T cells, numerous studies revealed that cells bearing CD4 may not only provide help for antigen-specific and polyclonal antibody responses and induce B cell proliferation by antigen or various mitogenic stimuli, but also mediate delayed-type hypersensitivity (DTH) responses, kill target cells and suppress antibody production.

More recent studies have demonstrated that expression of CD4 molecules correlates almost absolutely with the recognition by the T cell receptor of antigenic peptides bound to class II major histocompatibility complex (MHC) molecules. This class II MHC specificity is probably a consequence of the binding of class II/antigen complex by CD4 and the function of CD4 as an active signalling component of the T cell receptor for antigen-class II complex. Therefore, what such populations of cells have in common, in addition to the expression of CD4, is their MHC class II restriction rather than functional commitment.

Such studies at the population level demonstrated the functional complexity of CD4 T cell function. They, however, did not resolve a critical question: could all these functions be mediated by a single CD4 cell type, or did each function reflect the activity of individual T cells with a high degree of functional specialization? As well, what is the molecular nature by which CD4 cells regulate these responses?

1.4.3 Classification of Cloned CD4 T cells into distinct subsets on the basis of the spectrum of secreted cytokines and of their functional activities

The availability of cloned T cell lines made it possible to

compare the cytokine profile and functional attributes of different T cell clones. Studies in several laboratories classified cloned T cell lines into at least three major types. Two major distinct patterns of cytokine synthesis were initially recognized in a panel of long-term mouse T helper cell clones. These two patterns appeared to correlate with the induction of delayed-type hypersensitivity (DTH) or help for most antibody responses, thus providing a possible explanation for the often separate and reciprocal regulation of cellular and humoral responses (Parish, 1972; Brestcher 1981). Th1 but not Th2 cells secrete interleukin 2 (IL-2), interferon-γ (IFNγ) and lymphotoxin (LT). In contrast, Th2 but not Th1 clones secrete IL-4, IL-5, IL-6 and IL-10, and express mRNA for the p600 (IL-13) gene. Both Th types express IL-3, granulocyte-macrophage colony--stimulating factor (GM-CSF), tumor-necrosis factor (TNF) and three members of the macrophage inflammatory protein family. Most of the "common" cytokines are expressed in significantly greater amounts by Th1 clones, but IL-3 is expressed at similar levels, and prepromet enkephalin is frequently expressed more strongly by Th2 cells (Mosmann & Coffman, 1989).

Distinct Th1 and Th2 cytokine producing patterns have been documented in large numbers of independently derived murine T cell clones, which constitute the main portion of cell

clones derived in in vitro culture. However, a number of other cytokine secretion phenotypes are also displayed in tissue culture, and some of these phenotypes are also stable. Th0, another putative CD4 subset or stage of differentiation, expresses most or all of the cytokines made by Th1 and Th2 clones (Kelso, 1988; Firestein, 1989). Another relatively common pattern is the synthesis of IL-2, IL-4 and IL-5, but not IFNγ.

The development of the Th1/Th2 notion and its value in the interpretation of many immunological experiments carried out in mouse models, focused great interest for its possible relevance to understanding the human immune system. Initial reports indicated that human T cell subsets are more variable in cytokine profile than that of mouse. More T cell populations have the profile of Th0 subsets which produce IL-2, IL-4 and IFNγ. In 1991, Romagnani successfully derived Th1 and Th2 cell clones from different antigen primed human T helper cell cultures. They found that all purified protein derivative (PPD)-specific T cell clones established from normal individuals produce IL-2 and IFNγ but undetectable levels of IL-4 and IL-5, whereas TES (*Toxocara canis* excretory-secretory antigen)-specific T cell clones established from atopic donors produce IL-4 and IL-5 but no, or limited amounts of, IFNγ and IL-2 (Romagnani, 1991; del Prete, 1991). A number of groups

reported similar findings in independent model systems (Maggi, 1991; Parronchi, 1991; Kapsonbeg, 1991). The similarity in cytokine patterns observed in distinct mouse and human T cell subsets strengthens the notion that these subsets are a central feature of immune regulation.

Studies using long term T cell clones has greatly enriched our understanding of the regulatory processes that control T cell functions. The clonal T cell populations formerly used in immunological study, including T lymphoma and T cell hybridoma, are excellent for investigation of T cell signal transduction and TCR characterization, but their neoplastic nature restricted their application in the study of immunoregulation. Obviously, in terms of T cell physiology and regulation of cytokine gene expression, T cell clones are much more relevant to in vivo cells in nature than these tumor cells. Therefore, regulatory mechanisms can be studied rather readily using these clones that retain "normal" phenotypic characteristics.

Many observers have reported that in the process of T cell cloning, culture conditions significantly influence the nature of the clones obtained as assessed by cytokine production and function. Consequently, the data obtained from studies of long term cultured clones may not represent accurately in vivo responses. Therefore, studies using long

term cultured T cell clones alone are insufficient for clear elucidation of the mechanisms underlying immunoregulation. From this point of view, the properties of "fresh" cells as soon as they are obtained from an immunized animal (ex vivo cells), are considered to be more relevant to ascertaining the properties of normal cells in vivo because of the absence of long term artificial in vitro manipulation. Therefore, the utilization of these cells, in combination with brief (overnight, thus testing gene expression in the absence of clonal expansion) in vitro antigenic stimulation, would be more favourable for the study of cytokine production patterns following antigen exposure in vivo, at least at population level. It should be noted that "fresh" cells also have some disadvantages, including (1) heterogeneity, (2) low frequency of antigenic responders, (3) variations between animals and immunizations, and (4) variations between cell preparations.

1.4.4 Factors involved in cytokine spectrum determination

The nature of lymphokines produced in response to different infectious agents can often determine whether the response elicited will be protective or not. Thus, understanding the factors that determine which lymphokines will be produced is of considerable importance for immunological

manipulation whether for the purpose of induction (i.e. vaccination) or redirection (i.e. allergen immunotherapy) of immune responses.

Many possible mechanisms are thought to act independently or together to commit a naive T cell population to produce particular cytokines upon antigen stimulation. (a) Cytokines themselves may regulate the ability of naive T cells to become committed to the production of various cytokines. (b) Different types of APC may express distinct surface molecules or produce different costimulatory factors that may affect the priming for distinct cytokines. (c) Perhaps there is a direct link between the specificity of the TCR and the type of cytokines that the cell may be primed to produce.

The exclusive production of certain cytokines by Th1 and Th2 clones suggests their autocrine activity in cell activation and differentiation. It has been demonstrated that IFNγ promotes differentiation of Th precursors into Th1 cells both in vitro and in the *Leishmania* model in vivo (Scott, 1991), while IL-4 has the reciprocal activity, inducing differentiation into Th2 cells both in vitro and in vivo (Seder, 1992; Chatelain, 1992). Moreover, it is documented that cytokines produced by each subset can be capable of inhibiting the differentiation, growth,and/or

function of other subsets. IL-4, produced by Th2 cells, inhibits IL-2 receptor expression, IL-2 dependent proliferation, IFNγ induced cytokine gene expression and IL-2 as well as IFNγ synthesis. Therefore, IL-4 added to bulk lymphocyte cultures shifted the differentiation of CD4 T cells from Th1 to Th0 or even Th2 phenotype (Maffi, 1992). IL-10 inhibits antigen-presenting cell (APC)-induced lymphokine production by Th1 but not Th2 cells.

Mouse IL-10, produced by Th2 also, inhibits cytokine synthesis by Th1 cells indirectly via effects on macrophages. These inhibitory effects were predominantly observed on production of cytokines such as IFNγ and IL-3, which are produced relatively late after T cell activation, whereas the levels of cytokines like GM-CSF, lymphotoxin (LT) and TNF-α which are produced at an early stage, are unaffected or are only slightly affected (Fiorentino, 1991). Human IL-10 inhibits IFNγ, GM-CSF, TNFα, LT and IL-3 production by peripheral blood mononuclear cells activated by anti-CD3 mAbs or phytohemagglutin. IL-10 acts preferentially on macrophages/monocytes and does not appear to affect the ability of B cells to stimulate lymphokine production by Th1 clones. Human IL-10 also has direct effects on human T cells. The antigen-specific proliferative responses of human T-cell clones were inhibited by IL-10 when mouse L cells were transfected with

the relevant class II MHC molecules and employed as APCs.

IFNγ, produced by Th1 cells among others, inhibits the proliferation of Th2 cells, efficiently limiting the clonal expansion of such cells. Although IFNγ does not directly inhibit lymphokine production by stimulated Th2 cells, it does inhibit many of the agonist effects of those secreted lymphokine, such IL-4-dependent B cell differentiation (Fitch, 1993).

IL-12, previously known as natural killer cell stimulating factor, has been demonstrated to increase IFNγ synthesis by Th1 and NK cells. IL-12 is produced neither by Th1 nor Th2 cells, but its Th1 enhancing and Th2 inhibitory activity suggests it may play an important regulatory role in determining the balance of Th1 and Th2 activity in vivo (Manetti, 1993).

Although characterization of the role played by many of these cytokines in antigen-stimulated in vivo responses is still to be determined, it is reasonable to hypothesize that these interactions may tend to stabilize CD4 responses once they have been established as predominantly Th1 or Th2, making therapeutic redirection of cytokine gene expression patterns a major difficulty. The concept of "cross-regulation" provides a basis for understanding the

well-documented reciprocal relationship between cellular and humoral immune responses (Parish, 1972).

Antigen-induced T cell activation is initiated by the recognition of class II MHC-associated peptides derived upon processing of the antigen. Various types of antigen-presenting cells may be involved in the processing and presentation of these antigens, including macrophages, B-lymphocytes, dendritic cells, Langerhans cells, eosinophils and human T cells. The type of APC which process the antigen, or the costimulatory factors produced or surface molecules expressed by the APC cells themselves, may determine the nature of T cell subset activation. Indeed, some Th2 clones are stimulated more efficiently for cytokine production by purified B cells in in vitro culture while some Th1 cells are more easily activated by macrophages (Gajewski, 1991). The mechanism for this selective effect of different APC populations is not clear at present and is being actively investigated. It is possible that surface molecules important for cellular interactions or cell signalling either in Th1 or Th2 clones are differentially expressed on distinct APC populations. Alternatively, B cells may be lysed by some Th1 cells, therefore losing the ability to present antigenic peptides to this population. Certainly, this distinction is not clear cut and there are many exceptions in this rapidly

progressing area. In addition, costimulatory requirements are observed in some proliferative responses such as the necessity of the production of IL-1 for the proliferation of some cells belonging to Th2 clones. These observations demonstrate that multiple factors influence the responses of different Th cell subsets to different APC populations. Importantly, the accessory cell requirements for established clones may change with culture conditions, and may differ from the requirements for stimulating unselected CD4 T cells. In fact, studies of the effect of distinct APC populations on the initial in vivo priming of different T cell subsets are quite limited.

The developmental relationship between various CD4 T cell subsets is currently not clear. Though the concept was initially appealing, no solid evidence was found to support a linkage between the cytokine pattern and antigen specificity i.e. differential TCR V region expression. Rather, it seems there is now strong evidence that these different subsets come from a common cell lineage. For example, CD4 cells from mice expressing transgenes encoding a T cell receptor specific for pigeon cytochrome C peptide (88-104) differentiate to Th1 or Th2 subsets under different in vitro culture conditions (Robert, 1992). More direct analysis of the T cell receptor repertoire used by mice infected with *Leishmania major* revealed predominant

expansion of Va8-JaTA72, Vb4 expressing cells in both progressive and healing mice, producing IL-4 and IFNγ dominated responses respectively (Steven, 1993). These results demonstrate that a T cell subset with a relatively limited receptor repertoire can manifest distinct cytokine gene expression patterns leading to opposite functional phenotypes in vivo. Since strongly Th1- and Th2-like phenotypes are found most easily after vigorous immunization or chronic infection, it seems likely that the Th1 and Th2 cells represent phenotypes that have differentiated in reaction to strong and repeated stimulation, whereas the Th0 or the IL-2,IL-4,IL-5 patterns may represent cells at an intermediate stage of differentiation or, perhaps, may reflect typical gene expression patterns induced in in vivo responses to most conventional antigens. It should be noted that these studies of Th1-like or Th2-like responses in vivo evaluate cytokine gene expression patterns at the CD4 T cell population level. It is as yet unresolved whether normal CD4 T cells express Th1 or Th2 phenotypes at the single cell level in vivo.

Many other factors may be involved in selective activation of specific CD4 T cell subsets. The degree of stimulation through the TCR may cause the cell to differentiate down one or another pathway. Factors contributed by the antigen

(or by the "agent" bearing the antigen) may be deterministic, by means of selectively acting on one or another of the cells that participate in the interaction. Obviously, such an action may proceed via indirect pathways in that it recruits one of the mechanisms discussed above.

1.4.5 Cytokine profile of CD8 clones

At the establishment of Th1/Th2 concept, Mosmann and Coffman examined the cytokine producing profile of mouse CD8 cytotoxic T cells (CTL). It was observed that CTL clones had cytokine producing profiles similar to those of Th1 cells, predominantly IL-2 and IFNγ synthesis but minimal or undetectable IL-4, IL-5, and IL-6 production (Mosmann & Coffman, 1989). Recently, some investigators reported that cytokines produced by CD8 T cells play a role in the regulation of immune responses.

Salgame and his colleagues reported that human CD8 T cell clones obtained from individuals with lepromatous leprosy infections were strong IL-4 producers. As well, they showed that IL-4 produced by CD8 T cell clones following receptor stimulation is a necessary condition, in vitro, for suppression of proliferation of Th1 T cell clones. In contrast, they found all the CD4 T cell clones obtained from tuberculoid lesions responded to *M. leprae* antigen by

producing IL-2 and IFNγ, but low amounts of IL-4, whereas the CD8 T cell clones from lepromatous lesions predominantly produced IL-4 but not IL-2 or IFNγ. Interestingly, a single antigen-responsive CD4 clone from a lepromatous lesion had the same profile of cytokine and suppressive activity as the CD8 T cell clones (Salgame, 1991; Bloom, 1992a, 1992b).

These data have led to a consensus that the pattern of cytokines produced by T cells in response to antigen stimulation is more important than the surface phenotype in defining regulatory T cells. Therefore, expanding the paradigm initiated by Mosmann and Coffman, Bloom et al suggested that T cells should be classified as type 1 and type 2 T cells based on their cytokine profile rather than Th1, Th2 or Tctl , or Ts. Regardless of the terminology used, these studies make it clear that definition of the regulatory role played by activated T cells will require careful examination of the relationship between cytokine gene expression and effector function.

1.4.6. Parasitic models of cytokine-mediated immunoregulation

Although the distinction of cytokine synthesis between Th1 and Th2 cells is based on the profile of long-term clones,

a lot of evidence has shown the importance of T cell subsets and cytokine producing patterns in the regulation of immune responses at the population level. Most clear cut are responses to parasitic stimulation. This is partially because the study of parasitic infections has the advantage of: 1) persistent antigenic stimulation resulting from chronic infection, often leading to polarization of T cell-subset populations and extreme (and therefore prominent) immunoregulatory states; 2) the biological diversity of the different parasites and their in vivo habits which, in turn, leads to distinct forms of antigen presentation and T-cell activation; and 3) the genetic control of many parasitic infections, which appears to reflect polymorphism that controls host processing of the organisms and their antigens.

The regulatory and effector roles of CD4 T cells are particularly well understood in murine *L. major* infections. Cutaneous infection of most inbred murine strains with *L. major* leads to localized lesions that heal spontaneously and confer resistance to reinfection. However, in a few strains, including Balb/c, infection progresses to disseminated visceral disease that is usually fatal. Resistance and susceptibility are strongly associated with the development of strong Th1 and Th2 responses respectively (Liew, 1989). Fresh CD4 cells and CD4 T cell

clones from nonhealing Balb/c mice produce high levels of IL-4 but little IFNγ, whereas cells from healing C57BL/6 and anti-CD4 treated Balb/c mice produce more IFNγ but not IL-4 (Heinzel, 1989, 1991). The types of effector responses (cell-mediated vs humoral) are also different in healing and nonhealing animals. Healing disease is accompanied by strong DTH reactivity, but poor antibody responses, whereas nonhealing mice have strong antibody (including IgE) responses, but no DTH reactivity. Moreover, *Leishmania*-specific Th1 lines can transfer protection to Balb/c mice, whereas the transfer of Th2 lines exacerbates the nonhealing process (Scott, 1989).

The requirement for IFNγ in the development of healing, Th1 response to *L. major* was confirmed by in vivo blockade of its activity by mAbs. A single injection of anti-IFNγ mAb converted a resistant into a susceptible mouse, provided the antibody treatment was carried out during the first one or two weeks after parasitic infection. It was speculated that IFNγ acts indirectly for preferential stimulation of Th1 development, perhaps on antigen-presenting cell function or homing behaviour (Belosevic, 1989).

Similar experiments revealed that IL-4 was required for the development of *L. major* susceptible Th2 responses. Treatment of Balb/c mice with anti-IL-4 led to development

of a Th1 response and healing whereas injection of IL-4 into C3H mice induced IL-4 production and inhibited IFNγ production in this early response. Again, the alteration in cytokine expression induced by anti-IL-4 was apparent as early as 4 days after infection (Sadick, 1990).

In contrast to the permanent changes in Th subset response caused by injection of neutralizing antibodies to IL-4 or IFNγ, administration of either IL-4 or IFNγ failed to cause reversal of the long-term response or the disease outcome. This may either reflect the fact that cytokines have very short half-life in vivo or suggest that IFNγ and IL-4 are necessary but not sufficient for the development of Th1 and Th2 responses (Sadick, 1991, 1990).

Correlation of Th1 responses with healing and Th2 response with nonhealing disease are interpretable in terms of the regulation of specific effector functions by the cytokines characteristic of each subset. Macrophage killing of *Leishmania* is most effectively stimulated by IFNγ, and can be further enhanced by TNF. In contrast, IL-3 and IL-4 can inhibit this activation, and IL-10 can be an inhibitor of IFNγ production by Th1 cells. Thus, the cytokines produced by the antileishmanial Th1 or Th2 response, in addition to cross-regulating the development of the opposite CD4 subset, may determine the fate of the parasite inside the

macrophage that *Leishmania* obligately infects (Liew, 1989; Fiorentino, 1989).

Although similar protective Th1 activity has been shown in some other parasitic models such as *Trypanosoma cruzi* and *Toxoplasma gondii*, protective responses are characterized by dominant Th2 stimulation in rodents infected with *Heligmosomoides polygyrus*. IL-4 production in this host is a protective factor which favors resolution of disease (Urban, 1991). Therefore, what these parasitic models demonstrate, is not the particular role of Th1 or Th2-like patterns (inherently beneficial or harmful), but rather the importance of specific cytokine patterns in the regulation of particular immune responses and determining clinical outcome.

1.4.7. Regulation of IgE responses by cytokines

Since Isakson and Vitetta (1982) discovered that a T cell derived substance favored induction of LPS-stimuated in vitro IgG1 synthesis, much attention has been paid to examining the effects of cytokines on isotype selection. IL-4 is well characterized as the most important cytokine in IgE regulation. Murine IgE synthesis by LPS stimulated B cells in vitro was demonstrated to be IL-4 dependent. Similar results were obtained in T cell dependent systems

in which B lymphocytes are cultured with antigen specific T cells in the presence of appropriate antigens (Coffman, 1986a, 1986b). Anti-IL4 mAb injection revealed that endogenous IL-4 was required for the primary, and most of the secondary, murine IgE responses in vivo (Finkelman, 1988). Similarly, human IL-4 added to cultures of mononuclear cells (MNCs) derived from peripheral blood, tonsils or spleen induced IgE synthesis in the absence of polyclonal activators. Moreover, maintenance of human IgE responses in vitro is totally dependent on IL-4 (Vercelli, 1989).

IL-4 promotes IgE synthesis by isotype switching (reviewed by Li, 1991). Cloned surface IgM+ B cells may be induced to proliferate and to switch to IgE with high frequency in the presence of activated CD4 T cells and IL-4. Besides those that produce a single isotype, some murine B cell clones produce multiple isotypes including IgE, suggesting that during clonal expansion, switching might proceed from IgM to IgG1 and from IgG1 to IgE in the presence of activated T cells and IL-4 (Matsuoka, 1990; Yoshida, 1990). Recently, Mandler at 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. Cloned surface IgM+, surface IgE-, EBV-transformed B-cell lines and BL cells can frequently be induced to switch to IgE producing cells when cocultured with activated allogenic CD4 T cell clones and IL-4. This induction of ϵ-chain switching was shown to occur through a recombination deletion event and not through differential RNA processing (Jabara, 1990). These observations corroborate the report that ϵ-chain switching induced in normal B cells by infection with EBV reflects gene rearrangement through recombination deletion (Thyphronitis, 1991).

It should be noted that induction of IgE synthesis requires two signals. IL-4, as one signal, can not induce IgE synthesis by purified B cells, as cognate interaction of B cells with activated autologous T cells is also necessary (Blackwell, 1988). The costimulatory signals provided by activated CD4 T cells critical for B cell isotype switch remain to be clearly defined. IgE responses are blocked by mAbs against CD2, CD4 and lymphocyte function-associated antigen (LFA)-1, indicating that these adhesion molecules are associated with the cellular interactions required for productive IgE synthesis. Further investigation of the T

cell signal revealed that the CD4 clones must be activated and physical T-B cell interactions are required, because resting T cells and T cell clones separated from the B cells by semiporous membranes failed to induce IgE synthesis in the presence of IL-4. Membranes of activated CD4 T cells induce IgE synthesis by B cells in the presence of IL-4, the same as intact CD4 T cells. Thus, the costimulatory signal provided by CD4 T cell clones is mediated through a membrane-associated component that is induced after activation of T cell clones.

The costimulatory signal provided by CD4 T-cell clones can be replaced by anti-CD40 mAbs (Jabara, 1990; Zhang, 1991; Gascan, 1991, Splawski, 1993). Stimulation of purified B cells through CD40 resulted in IgE synthesis specifically in the presence of IL-4 (Shapirask, 1992). The successful cloning of the gene encoding CD40 ligand (CD40L) in activated CD4 T cells and the replacement of anti-CD40 mAb activity by CD40-ligand transformed cells suggests the potential importance of the CD40-CD40L interaction in IgE switching. Another molecule, TNF-α, the membrane form of which is expressed on activated CD4 T cells, was also revealed to have costimulatory activity. Neutralization of TNF-α by anti-TNF-α mAbs completely blocked IL-4 dependent IgE synthesis in cocultures of purified B cells and CD4 T cell clones or T-cell membranes (de Vries, 1991). These

data suggest the involvement of multiple membrane molecules in the induction of IgE responses.

IL-4 has been thought to be the only cytokine capable of inducing IgE class switching. However, Punnonen and his collegues recently reported that IL-13 induced IL-4 independent IgE synthesis, indicating an alternative pathway for the induction of IgE switching (Punnonen, 1993). As well, many other cytokines are involved in the regulation of IgE responses. IL-5, IL-6 and TNF-α are enhancing factors for IL-4 induced IgE synthesis. IL-5 enhances IgE synthesis by MNCs but only when IL-4 is present at suboptimal concentration. The mechanism by which IL-5 enhances IgE synthesis in vitro is unclear. It should be noted that IL-5 is not effective in modulating in vivo IgE responses as demonstrated by the finding that administration of neutralizing anti-IL-5 mAbs to parasite infected mice did not affect IgE levels in these mice, while anti-IL-4 mAbs blocked IgE production (Pene, 1988).

IL-6 strongly enhanced IL-4-induced IgE synthesis in vitro, while neutralizing antibodies against IL-6 completely blocked IL-4-induced IgE synthesis in culture of MNCs or highly purified B cells stimulated via anti-CD40 mAb and IL-4, indicating that IL-6 has an important amplifying function on IL-4-induced IgE synthesis. IL-6 acts at

relatively late stages of B-cell differentiation without isotype preference, promoting the growth of plasma cells (Vercelli, 1989b).

TNF-α probably acts directly on B cells because it has been shown to enhance IL-4 induced germline ϵ-chain transcription in purified B cells, suggesting that increased germline ϵ-chain transcription results in increased IgE synthesis and confirming the notion that there is a quantitative correlation between the levels of germline ϵ-chain mRNA synthesis and IgE production (de Vries, 1991).

Both IFN-γ and IFN-α are capable of blocking IL-4 induced IgE synthesis in murine and human in vitro systems (Pene, 1988, Del Prete, 1988). In vivo administration of recombinant IFNγ has been shown to enhance IgG_{2a} synthesis and, at very high doses, decreases IgE and IgG1 synthesis. In contrast, in vivo injection of anti-IFNγ antibody enhanced polyclonally stimulated IgG1 and IgE responses and blocked IgG_{2a} responses. It should be noted that although IFNγ can enhance IgG_{2a} secretion in the presence of an additional stimulus, it is not absolutely required for the induction of IgG2a responses. It is still unclear whether IFNγ plays its role by promoting isotype switch to IgG_{2a} at the DNA level or by selectively promoting the growth or

differentiation to Ig secretion of B cells that are already committed to this isotype. The interspecies (mouse and human) similarity in regulation of IgE synthesis by IL-4 and IFNγ indicates that their relative production could play a major role in determining the extent of an immune responses including IgE production (Finkelman, 1990).

More recently, it was demonstrated that induction of IgE synthesis can be blocked by transforming growth factor-β (TGF-β). This inhibitory activity works in early stages of cell differentiation. TGF-β blocks the induction of transcription of germline DNA encoding the ϵ-chain , which precedes IgE switching. Because germline ϵ-chain transcription is required for IgE synthesis, it is probable that TGF-β inhibits IL-4 induced IgE synthesis by inhibition of this transcription. Interestingly, IFNγ and IFNα, which block IgE synthesis in cultures of MNCs, were ineffective in blocking IgE production induced by anti-CD40 mAbs and IL-4, i.e. in the absence of T cells. Furthermore, IFNγ and IFNα failed to inhibit germline ϵ-chain transcription induced by IL-4 in purified B cells. Thus, IFNγ and IFNα appear likely to mediate their inhibitory effect through CD4 T cells.

II. SCOPE OF THE PRESENT STUDY

The primary goal of this study was to elucidate the mechanism by which a chemically modified allergen (OA-POL) induces antigen specific, isotype selective IgE suppression following allergen (OA-alum) immunization. Our hypothesis was that the balance between Th1 and Th2-like cells in vivo is changed as a consequence of administration of this chemically modified allergen while administration of native allergen, in a model of conventional allergen immunotherapy, was hypothesized to be of minimal impact on OA stimulated cytokine production. Specifically, we examined the possibility that in vivo exposure to this modified protein steered the cytokine production to a Th1-like dominated pattern, consequently leading to inhibition of native protein primed IgE production. This hypothesis was developed based on the following evidence: (1) OA-POL treatment inhibited the development of IgE while concomitantly increasing IgG_{2a} synthesis, (2) OA-POL induced immunoregulatory activity is CD4 T cell dependent, and (3) in vivo administration of anti-IFNγ antibody treatment reversed the isotype regulatory activity of OA-POL.

The reason for this Th1-like cytokine pattern bias is speculated to be related to selective processing and

presentation of modified molecules by the antigen-presenting cell (APC) populations other than the one presenting native OA molecules. Specifically, native OA preferentially activate Th2-like CD4 T cell subset gene expression in vivo may be preferentially presented by B cells while OA-POL may be optimally presented by cells other than B lymphocytes. This hypothesis is based on the facts that (1) the greatly decreased antigenicity of OA-POL compared to native OA may decrease its opportunity to be captured and presented by OA-specific B cells; (2) the greatly increased molecular size of OA-POL may make it more easily picked up in vivo by phagocytic cells, including macrophages.

The approach developed for analysis of cytokine gene expression patterns in the present study is short term bulk culture of spleen cells immediately ex vivo. This technical choice was based on the consideration of previous studies (1) using long term T cell clones which may differ in cytokine patterns and function from T cells in vivo, and (2) use of polyclonal rather than antigenic stimulation in in vitro cultures may reflect the potential of cytokine synthesis by cells in the culture instead of the pattern induced by antigenic exposure in vivo.

The present study demonstrates (1) rapid induction of

allergen specific IL-4 producing CD4 cells in vivo; (2) differential IL-2 requirements in the initial activation and continued maintenance of Th1-like and Th2-like cytokine secretion patterns; (3) differential patterns of cytokine gene expression are induced by in vivo exposure to chemically modified and native allergen; (4) a determinant effect of chemically modified allergen treatment on de novo and established cytokine and antibody responses; (5) preliminary evidence suggesting an apparent involvement of distinct APC populations in processing and presentation of native and chemically modified allergen, leading to consequent effects on cytokine and antibody production.

In light of the numerous reports current at the initiation of this project that IL-4 producing cells in vivo exist solely as precursor cells requiring extended cycles of in vitro stimulation and clonal expansion for their development, and our observation that most of these experiments were performed using complete Freund's adjuvant (which actively inhibits IgE production) (Kishimoto, 1982), we initially compared the effects of different adjuvant usage on the induction Th1-like and Th2-like cytokine synthesis following allergen immunization. Consistent with previous reports, we found that the use of CFA as adjuvant induced antigen-specific cells capable of IFNγ and IL-2 synthesis with minimal IL-4 production. In contrast, OA-

alum immunization, which elicited strong in vivo IgE responses, consistently induced antigen-specific cells capable of producing IL-4. Importantly, this difference in cytokine patterns was only observed upon antigen driven restimulation whereas polyclonal stimulation (immobilized anti-CD3 or Con A) failed to distinguish between cytokine patterns among OA-alum immunized, OA-CFA immunized and naive mice.

Characterization of the factors involved in development and maintenance of antigen driven IgE responses is an important prerequisite for therapeutic modulation of allergic reactions. To elucidate the role played by IL-2 in the development and maintenance of IL-4 and IFNγ synthesis and its consequent effect on maintenance of hypersensitivity, we studied the effect of IL-2 on developing and established IL-4 and IFNγ responses. It was demonstrated that although IL-2 was required for the initial development of IL-4 responses, IL-4 gene expression in established responses was independent of IL-2. In contrast, the IFNγ response, both at the stage of developing and gene expression, was strongly IL-2 dependent.

Then we examined the cytokine patterns induced by in vivo exposure to native and chemically modified allergens. Administration of chemically modified allergen (OA-POL)

elicited Th1 dominated cytokine synthesis (characterized by preferential IFNγ secretion with minimal amounts of IL-4 and IL-10) whereas the pattern of cytokine synthesis induced by native allergen reflected a balance in Th1-like and Th2-like cytokine secretion (similar levels of IFNγ, IL-4 and IL-10). These results demonstrate that it is possible to intentionally select a particular cytokine pattern in antigen driven responses for experimental or clinical purposes.

In order to investigate the effects of treatment with chemically modified protein (OA-POL) on subsequent antigen exposure, we then examined the cytokine patterns and antibody responses elicited by allergen (OA) immunization in the presence of alum adjuvant following modified protein (OA-POL) administration. It was shown that although the IFNγ increase induced by OA-POL was largely masked by the strong stimulation with alum adjuvant, the IL-4 secretion in OA-POL pretreated group was constantly low compared to control mice. More significantly, the ratio of IFNγ/IL-4 definitely increased upon OA-POL pretreatment, which correlate well with OA-POL induced IgE suppression. However, native OA treatment under similar conditions, although sometimes leading to a slight increase in IFNγ production, did not change the ratio of IFNγ/IL-4 due to a comparable increase in IL-4 production, which is consistent

with the failure of native OA pretreatment to suppress OA-alum induced IgE responses. These results further demonstrate the importance of the balance of Th1 and Th2 cytokine synthesis rather than a particular cytokine level in the determination of the class\subclass of antibody produced.

The effects of OA-POL treatment on pre-established IgE responses and its relationship with cytokine pattern alteration were then investigated in this study. As demonstrated previously (HayGlass, 1991c), three courses of OA-POL treatment inhibit ongoing IgE responses induced by OA-alum immunization. Similar to its effects on de novo IgE responses, OA-POL treatment given post-allergen immunization shifted the antigen-driven cytokine production to a Th1 dominated pattern (increased IFNγ/IL-4). This seems encouraging in that it implies it is not only possible to pre-select a cytokine pattern in naive individuals but also possible to reeducate established cytokine patterns, at least, at the population level.

To elucidate the mechanism by which modified protein preferentially elicits dominant cytokine patterns distinct from those induced by native antigen, we studied the role played by distinct APC populations in the in vivo presentation of native and modified allergen. By use of a

B cell deficient mouse model (chronic anti-μ antibody treatment), we demonstrated that antigen presentation of native OA, but not chemically modified OA (OA-POL), requires the presence of functional B cells or their products.

These observations are helpful for the understanding of the signals involved in initiating and sustaining of Th1-like and Th2-like cytokine responses. Moreover, these observations may ultimately be useful in clinical settings, including allergy, where the ability to select specific patterns of cytokine gene expression would be advantageous.

MATERIALS AND METHODS

I. MATERIALS

1.1. Animals

C57BL/6 mice, 6-12 weeks old, were bred at University of Manitoba breeding facility or were purchased from Charles River Canada, St Constant, PQ, Canada.

Female S-D rats were purchased from Charles River, Canada.

All animals used in this study were maintained and used in strict accordance with the guidelines issued by the Canadian Council on Animal Care. Mice were routinely monitored for antibodies to mycoplasma, Sendai virus and rodent coronaviruses, including MHV, by ELISA (Murine ImmunoComb, Charles River).

1.2. Chemicals and reagents

Ovalbumin (5x recrystallized) was purchased from ICN Biochemicals (Montreal, Canada); Hydrochloric acid, ethyl alcohol, sodium azide, sodium phosphate monobasic, sodium carbonate, sodium bicarbonate and paraformaldehyde from CanLab (Winnipeg, MB); bovine serum albumin, p-nitrophenyl phosphate, 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, ON); glutaraldehyde and 2-mercaptoethanol from Kodak (Rochester, N.Y.); fetal calf serum from Intergen company (N.Y. USA); 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); lipopolysaccharide (LPS) from Difco Laboratories (Detroit, MI); 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). Complete Fluend's Adjuvant (CFA) and 0.05% Trypsin-EDTA from Gibco-BRL (Burlinto, ON); DMSO (Dimethyl sulfoxide), diethanolamine, acetict acid and 2-propanol from Fisher Scientific (Ottawa, ON); streptavidin-alkaline phosphatase, purified rat IgG, affinity-purified goat anti-rat IgG (Fc), and alkaline phosphatase conjugated goat anti-rat IgG (H+L) from Jackson Immuno Research Laboratories (West Grove, PA); normal rat Ig (NRIg) and FITC-conjugated goat anti-rat Ig from Biocan Scientific (Toronto, ON); readysafe scintillation fluid from Beckman (Mississauga, ON); rabbit anti-murine μ chain antibody (anti-μ) and normal rabbit IgG (NRIgG) were kindly provided

by Mr. R. Gieni (University of Manitoba, Winnipeg, MB). Hybridoma 145-2C11 (anti-CD3) was provided by Dr. J. Bluestone (University of Chicago, Chicago,IL); hybridomas GK 1.5 (anti-CD4), HO2.2 (anti-CD8), R4.6A2 (anti-IFNγ) and 7D4 (anti-IL-2R) were obtained from ATCC (American Type Culture Collection, Rockville, MD). Hybridomas YTS 191.1 (anti-CD4) and YTS 169.4.2.1 (anti-CD8) were kindly provide by Dr. H. Waldmann (University of Cambridge, Cambridge, UK). Hybridomas XMG 1.2 (anti-IFNγ), SXC1 (anti-IL-10), SXC2 (anti-IL-10), S4B6 (anti-IL-2) and recombinant IL-10 standard were kindly provided by Dr. T. Mosmann (University of Alberta, Edmonton, Alberta). Fibroblast LB and encephalomyocarditis virus were provided by Dr. F. Jay (University of Manitoba, Winnipeg, MB). Independent Lymphoma WEHI-279 was provided by Dr. M. Hagen (University of Iowa, Iowa City, IA) and Dr. A. Kelso (Walter and Eliza Hall Inst, Melbourne, Australia). International murine IFNγ reference reagent Gg02-901-533 was provided by Dr. C. Laughlin (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). Recombinant interleukin-2 (rIL-2) from Genzyme (Boston, USA). Recombinant interleukin-4 (rIL-4) was provided by Dr. R. Tepper (Dana-Farber Cancer Institute, Boston, MA) and Dr. W. Paul (National Institute of Health, Bethesda, MD). CT.4S cell line was provided by Dr. W. Paul (NIH, Bethesda, MD). HT-2 cells were provided by Dr. K. Rock (Harvard

Medical School, Boston, USA).

1.3. Equipment

Laminar flow biological safety cabinet (model Nu-408EM-400) was purchased from Nuair Inc. (Plymouth, MN). Water - jacketed incubator (model 3158) was purchased from Forma Scientific, Division of Mallinckrodt Inc. (Marietta, OH). Sorvall RT 6000 B refrigerated centrifuge was a product of Dupont Canada Inc. (Burnaby , B.C.). Refrigerated model PR. J centrifuge was a product of International Equipment Company (Needham, MA). 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 purchased from Cambridge Technology Inc. (Cambridge, MA). Liquid scintillation analyzer (Packard Tri-Carb 2200CA) was obtained from Packard Instrument Company (Downer Grove, IL). Thermix Stirring Hot Plate (model 301T) and Vortex-Genie were produced by Fisher Scientific (Ottawa, ON). A microscope was purchased from Carsen Medical Scientific Co. (Winnipeg, MB). Biogel A-50m gel filtration column was a product of Bio-Rad Laboratories (Mississauga, ON). Pharmacia Biopilot System was purchased from Pharmacia Fine Chemicals (Piscataway, NJ). CD4 enrichment polypropylene immunocolumns (Collect.plus) were

purchased from Biotex Laboratories Inc.(Edmonton, AB). Centriprep-30 concentrators were purchased from Amicon (Oakville, ON). 96 well ELISA plates, polystyrene flat-bottom tissue culture plates, u-bottom plates and 24 well tissue culture plates were obtained from Corning Science products (Missisauga, ON). EPICS V Fluorescence Activated Cell Sorter was a product of Coulter Electronics, Inc. (Hialeah, FL). Cell irradiation was done at Manitoba Cancer Foundation using a Cobalt 60 source.

II METHODS

2.1. Preparation of chemically modified OA (OA-POL)

OA was dissolved at 25 mg/ml in sodium acetate/acetic acid buffer (0.1 M, pH 5.3), 0.5 pH unit above its isoelectric point. Glutaraldehyde (6% in 0.15 M NaCl, Eastman Kodak Co., Rochester, NY) was added dropwise with stirring over a period of several minutes to obtain a final molar ratio of 200:1 GA/OA. The reaction was allowed to proceed for 5 h, in order to yield high molecular weight OA polymers. After three dialysis changes against borate buffered saline (0.1 M, pH 8.3), the solution was applied to a Biogel A-50m gel filtration column (2.5 x 90 cm) for characterization and purification. The polymerized protein was recovered as a single sharp symmetric peak (Ve/Vo of 1.4-1.55) eluting

at an average M_r of 3.5×10^7 (Vo of Biogel A-50m is 5.0×10^7).

2.2. Immunization and treatment of Mice.

2.2.1. Adjuvant and antigen

$Al(OH)_3$ adjuvant (alum) was prepared as follows. 20 ml 10% $AlK(SO_4)_2$ was added with 2 drops of 0.2% phenol red during stirring followed by dropwise addition of around 20 ml 0.5 N NaOH until the solution just got slightly pink. The solution was left to sit at room temperature for 10 min with subsequent spinning down at 1000 rpm for 3 min. The supernatant was decanted and the precipitate was resuspended with 40 ml of 0.15 N NaCl followed by centrifugation for 3 min. This washing process was repeated once more and the final precipitate was resuspended with 40 ml 0.15 N NaCl. This final solution containing $Al(OH)_3$ at 10-12 mg/ml was stored at room temperature. For use, protein antigen was absorbed onto alum by sitting at room temperature for 10 min. Before injection this preparation was neutralized by adding same volume of Hank's balanced salt solution (HBSS).

Complete Freund's adjuvant (CFA). CFA was gently shaken to resuspend mycobacteria before being used. Protein antigen

was dissolved in 0.85% NaCl and mixed with same volume of CFA in a small beaker. The mixture was emulsified by repeatedly drawing the contents of the beaker into the syringe and expelling them until the emulsion was well formed by the criterion that a drop of the emulsion placed on water remains intact and does not spread.

2.2.2. Immunization and treatment.

Immunization consisted of administering 2 or 0.2 μg OA absorbed onto 2 mg alum adjuvant or 100 μg OA emulsified with 100 ul CFA intraperitoneally (i.p). A course of chemically modified OA (OA-POL) or native OA treatment consisted of three 80-μg i.p injections in saline (0.5 ml, no adjuvant) every other day. To inhibit de novo IgE responses, OA-POL was administered 10, 12, and 14 days prior to immunization with 2 μg OA (alum). To abrogate established IgE responses three courses of OA-POL treatment were given following 0.2 μg OA-alum immunization.

2.3. Staining of cells with fluorescent-conjugated antibodies

To assess the purity of enriched cell populations following in vivo antibody treatment, cell preparations were stained with antibodies to specific cell markers and/or fluorescent

antibody conjugates and analyzed by FACS. Briefly, 4 x 10^5 cells were added to each well of 96 well U-bottom plates on ice. Plates were centrifuged 40 seconds at 1200 rpm in a refrigerated IEC centrifuge. Supernatants were removed with Pasteur pipet and appropriate volume (50-100 ul) of first reagent (antibody to specific cell marker) was added to each well. After 40 min incubation on ice, wells were filled with cold wash medium (5% FCS/RPMI 1640) and spun down for 40 seconds as above. Supernatants were decanted and cells were washed another two times. An appropriate volume (25-30 ul) of diluted second reagent (fluorescent conjugated antibody against first reagent) was added and plates were kept on ice in dark for 40 min. After two washes, cells were either analyzed immediately or fixed with 100 ul of 2% paraformaldehyde followed by supplement of 100 ul washing medium 5 min later. The plates were kept at 4° C in the dark until analyzed by FACS, usually within a week. Approximately 5,000 - 10,000 cells were analyzed per sample. For sIg+ cell testing, cells were stained by fluorescent conjugated rat anti-mouse Ig only and no second reagent was applied. In most experiments, duplicate preparations of each sample were independently stained and analyzed.

2.4. CD4 T cell depletion in vivo.

Mice were CD4 T cell depleted via three i.p. injections of 200-300 μg purified anti-CD4 mAb YTS 191.1. These anti-CD4 mAb injections were carried out two days before and one day after antigen immunization. Mice given normal rat Ig (NRIg) treatment under the same conditions were used as control. CD4 T cell depletion was assessed by spleen cell flow cytometry. The percentage of CD4 and CD8 cells was determined by treatment with anti-Ig panned splenic T cells (see below) with anti-CD4 (GK 1.5) or anti-CD8 (HO2.2) tissue culture supernatant followed by staining with affinity purified FITC-conjugated goat anti-rat Ig. The percentage of positive cells and their relative fluorescence intensity was determined using FACS.

2.5. Preparation of B cell deficient mice

B-cell deficient mice were prepared by chronic injection of rabbit anti-mouse μ chain antibody (anti-μ) as described (Taghi-Kilani, 1990). Briefly, newborn C57BL/6 mice were injected intraperitoneally, starting within 24 h of birth, with 150 ul polyclonal antibody containing 700 - 1000 μg of mouse red blood cell - absorbed, $(NH_4)_2SO_4$ precipitated, rabbit anti-mouse IgM antibodies per injection. The injection was given three times a week throughout the life of the mice. Control mice were injected with $(NH_4)_2SO_4$ precipitated normal rabbit IgG (NRIgG) in the same amount

as the anti-μ antibody. The percentage of spleen cells expressing cell surface Ig (sIg+) was determined by flow cytometry. Cells from anti-μ treated, normal rabbit IgM treated and age-matched normal mice were stained with affinity purified FITC-conjugated goat anti-mouse Ig. After extensive washing, these cells were analyzed using FACS.

2.6. Adoptive transfer of CD4 enriched T cells.

Antigen immunized or treated donor mice were killed by cervical dislocation and single spleen cell suspensions were normally prepared (see below). Cells were first treated with 0.85% NH_4Cl for 1 min at room temperature to remove red blood cells. Then, the cells were panned with immobilized sheep anti-mouse Ig to deplete B cells (see below). The recovered cell populations were then stained with anti-CD8 mAb and goat-anti-mouse Ig polyclonal antibody and run through the murine CD4 T cell enrichment immunocolumn (see below). The CD4 T cell enriched spleen cells ($\geq$ 85% CD4 by flow cytometry) were injected i.v. in a volume of 0.5 ml to normal syngeneic recipients. All recipients were boosted with 2 μg OA-alum 4 - 6 h after cell transfer.

2.7. Determination of antigen-specific antibody production.

IgE: levels of anti-OA IgE were determined by 48-h passive cutaneous anaphylaxis (PCA). Briefly, Female S-D rats were injected intradermally with 0.15 ml of serial dilutions of murine sera. 48 h later, the rats were challenged by the i.v. injection of 200 μg ovalbumin in 1.0 ml physiological saline and 2% Evan's blue. The highest dilution giving a significant blueing reaction was taken as the end point. Means of duplicate or triplicate analysis, rarely differing by more than one two-fold dilution in repeat assays, are presented.

IgG_{2a}: anti-OA IgG_{2a} was determined by ELISA. Briefly, 96 well plates were coated with 100 ul/well of OA at 10 μg/ml in coating buffer (0.05 M $NaHCO_3$ buffer, pH 9.6) overnight at 4° C. Excess protein binding sites were then blocked (0.2 ml/well of 1% BSA in PBS, pH 7.4) for 2 h at room temperature. After extensive washing (0.05% Tween 20 in PBS), serum samples and a polyclonal lab standard IgG_{2a} were prepared in dilution buffer (0.5% Tween 20, 0.5% BSA in PBS) and added at 0.1 ml/well. Following 3 h incubation at 37° C and subsequent washing, biotinylated goat anti-mouse IgG_{2a} mAb was added at 1:2000 dilution for overnight incubation at 4° C. The next day, the plates were washed, streptavidin-alkaline phosphatase was added (0.1 ml/well, 1 h, 37° C) at the recommended dilution, the plates were washed again and 100 ul/well of p-nitrophenyl phosphate in

0.5 mM $MgCl_2$, 10% diethanolamine, pH 9.8) was added. 100 min later the plates were read by automatic ELISA reader. The highest dilution giving an optical density ($O.D_{410}$) of 0.5 was defined as the end point.

2.8. Determination of protein concentrations

2.8.1. Lowry Protein assay

Serial dilutions of protein standard and samples were supplemented with 0.15 N NaCl to 1 ml. Lowry solutions A (1% $CuSO_4.5H_2O$, 0.5 ml) and B (2% NaTartrate 0.5 ml) were mixed first, followed by addition of solution C (2% Na_2CO_3) to a final volume of 50 ml. The final solution was mixed well and used immediately after being prepared. 5 ml of this solution was add to each tube and vortexed well. After sitting for 10 min at room temperature, 0.5 ml 1 N phenol reagent was added to each tube. Absorbanes were read at 720 nm 30 min later using a spectrophotometer. Protein concentrations in samples were calculated with reference to the standard curve.

2.8.2. BCA protein assay

Bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL) was used for protein quantitation of purified mAbs. The modified microtiter plate protocol is as follows. 20 ul of serially diluted protein standard (BSA),

identically diluted samples and blank solutions were applied into 96 well ELISA plates followed by the addition of 200 ul of working reagent (mixture of reagent A and reagent B , provided by manufacturer). After 30 seconds shaking on a microtiter plate shaker, the plate was covered and incubated at 37° C for 30 min. Absorption at 570 nm was read with a UV max microtiter plate reader and protein concentration in each sample was determined using the standard curve plotted according to the protein concentration and optical absorption.

2.9. Preparation and purification of mAbs

2.9.1. Hybridoma growth.

Hybridomas XMG 1.2 (Rat IgG1), R4.6A2 (Rat IgG1) and 11B11 (Rat IgG1) were set up in T75 tissue culture flasks at 2 x 10^5 cells/ml in complete culture medium (RPMI 1640 medium supplemented with 2 mM L-glutamine, 50 uM 2-mercaptoethanol, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 units/ml fungizone, and 10% heat-inactivated fetal calf serum). Cells were grown in a 37° C incubator (5% CO_2) until exhaustion (< 40% viability) and the supernatants were harvested by centrifugation for mAb purification.

2.9.2. Determination of rat IgG concentrations.

Determination of rat IgG concentrations, necessary in the purification and quantitation of the rat IgG1 mAbs, was carried out as follows. ELISA plates were coated overnight with affinity-purified goat anti-rat IgG Fc (10 μg/ml) as a capture reagent. After blocking and washing, dilutions of tissue culture supernatant (or fractions eluted from the ion exchange purification process) were incubated for 3 h at 37° C. After washing, alkaline phosphatase conjugated goat anti-rat IgG was incubated for overnight at 4° C. Purified rat IgG was used at 2.5×10^{-2} to 9.8×10^{-5} μg/ml as standard. The ELISA was completed by washing and addition of substrate as described above.

2.9.3. Purification procedures

Supernatants were purified by ion exchange chromatography and gel filtration using the Pharmacia Biopilot system. Briefly, culture supernatants (0.5 to 3.5 L) were extensively dialysed against low ionic strength citrate buffer (0.01 M, pH 5.3), then concentrated via ion exchange chromatography using S-Sepharose 35/100. The mAb containing fraction was eluted using a gradient of increasing ionic strength (NaCl 0-0.8 M, Na citrate 0.01 M, pH 5.3), 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. The concetration of these mAbs were determined by ELISA. The final purity of the rat mAbs

obtained by this approach was typically >85% as determined by comparison of total protein present in the purified material (determined by BCA protein assay) compared with the concentration of total rat IgG (determined by ELISA).

2.10. Spleen cell culture

2.10.1. Preparation of spleen cell suspensions

Mouse spleens were removed aseptically, and single cell suspensions were prepared using a glass tissue homogenizer. Debris was removed by passing the cell suspensions through nytex filters into centrifuge tubes. Cells were spun down at 400 g for 5 min and washed once with serum free RPMI 1640 medium (no supplements). Red blood cells were lysed by adding 0.85% NH_4Cl for 1 min at room temperature with subsequent centrifugation. After another wash, cell pellets were resuspended in complete culture medium. The number and percentage of viable cells was determined by staining cell preparations with trypan blue and counted using a hemocytometer.

2.10.2. Preparation of T lymphocyte subpopulations

Spleen cells from immunized or normal mice were enriched for T cells by panning with affinity purified sheep anti-mouse Ig coated plates to remove B lymphocytes. Briefly,

100 x 15 mm sterile tissue culture petri dish was coated with 5 ml sheep anti-mouse Ig (50 μg/ml) at 4° C overnight. After 1 h blocking with 10 ml 5% FCS-PBS at room temperature and subsequent washing, 1 x 10^8 spleen cells in a volume of 5 ml were applied to the dish. The dish was incubated for 60 min at room temperature. The nonadherent population, harvested with a Pasteur pipette after gently rocking the dish, was predominantly T cell (75 - 95% Thy 1+ by flow cytometry). These cells were further selected for CD4 or CD8 positive cells by flow cytometry or CD4 or CD8 enrichment immunocolumns:

Flow cytometry. In some experiments, CD4 cells were positively selected by staining of T-cell enriched spleen cells with fluorescein conjugated anti-CD4 mAb (DTAF-YTS 191.1.2), or negatively selected by staining with anti-CD8 mAb (YTS 169.4.2.1) and DTAF goat anti-mouse IgG (heavy and light chain) polyclonal antibody. Cells were sorted by an EPICS V FACS at 3000 cells/second. Aliquots of sorted populations were reanalysed and typically exhibited $\geq$97% purity of the desired cell population.

CD4 or CD8 enrichment immunocolumn. T-cell enriched spleen cells were stained with anti-CD4 mAb (YTS 191) (for CD8 enrichment) or anti-CD8 mAb (YTS 169) (for CD4 enrichment). 1-1.25 x 10^8 stained cells were loaded to a

polypropylene immunocolumn which contains prepared glass beads, coupled with goat anti-mouse IgG (H+L) and sheep anti-mouse IgG (H+L) polyclonal antibody. Stained T cells and sIg+ cells (B cell) were removed by the column and eluted cells were enriched T cell subsets. This technique removes more than 95% B and CD8 (for CD4 enrichment) or CD4 (for CD8 enrichment) cells from lymphocyte preparations under optimal conditions. The purity of the enriched cells was typically $\geq$ 85%.

2.10.3. Irradiation of spleen cells

Spleen cells to be used as APC in vitro were suspended in complete culture medium, irradiated with 2500 rads in a ^{60}Co irradiator, washed, and resuspended in complete medium prior to culture.

2.10.4. Culture protocols

Spleen cell culture. Mice were killed at various times following in vivo treatment or/and immunization. Except where specified, spleen cell suspensions were cultured at 7.5 x 10^6/ml (2 ml/well) alone or with different concentrations of OA, concanavalin A (Con A) or plate-bound anti-CD3 mAb (145-2C11) in 24 well tissue culture plates at 37° C in complete medium. Duplicate cultures were established from the spleen cells of individual mice in each group. Culture supernatants were harvested at

different time intervals and stored at -20° C until cytokine production was analyzed.

Sorted cell culture. Positively or negatively selected CD4 or CD8 spleen T cell populations were cultured at 3-6 x 10^6/ml (0.2 ml/well) with 5 x 10^6/ml irradiated naive spleen cells in the presence or absence of OA using 96 well, flat-bottom plates at 37° C in complete medium. Triplicate cultures were set up and supernatants were harvested at different times.

2.11. Spleen cell proliferation

2.11.1. T cell proliferation

Unfractionated spleen cells were cultured in triplicate at 5 x 10^5 cells/0.1 ml/well using 96 well flat-bottom plates in the presence or absence of specific antigen or Con A. Plates were incubated in 5% CO_2 at 37° C for 24 h followed by pulsing with 1 μCi/well tritiated thymidine for 18 h. ^{3}H-thymidine uptake was determined by liquid scintillation counting.

2.11.2. B cell proliferation

Spleen cells were cultured in triplicate at 5 x 10^5

cells/well in 96 well flat-bottom plate in the presence of LPS at a final concentration of 5 μg/ml in complete medium. Cultures were incubated at 37° C for 48 h, pulsed with 1 μCi/well ^{3}H-thymidine, and harvested 18 h later. ^{3}H-thymidine uptake was determined by liquid scintillation counting.

2.12. Cytokine determinations

2.12.1. IL-2.

HT-2 cells are highly responsive to IL-2. 5 x 10^{3} cells in 50 ul fresh complete medium were added to each well which containing 100 ul of two fold dilutions of tissue culture supernatants in 96 well, flat-bottom plates. Cultures were incubated in 37° C for 24 h and pulsed with 1 μCi tritiated thymidine/well for 6 to 8 h. ^{3}H-thymidine uptake was determined by liquid scintillation counting. The specificity of this bioassay for IL-2 was assured by analysis of replicate samples in the presence of a cocktail of tissue culture supernatants containing anti-IL-2 mAb S4B6 and anti-IL-2R mAb 7D4. An internal standard of murine rIL-2 $\pm$ anti-IL-2/IL-2R mAb was run in each assay. This HT-2 subline does not proliferate in response to IL-4 concentrations below 800 U rIL-4/ml.

2.12.2 IL-4.

CT.4S, a CTLL derived cell line highly responsive to IL-4 was used (Hu-li, 1989). CT.4S bioassay was carried out by ^{3}H-thymidine uptake or the MTT method. Briefly, for ^{3}H-thymidine uptake, 5 x 10^3 cells/0.2 ml/well were cultured in 96 well, flat-bottom plates with serially diluted IL-4 standard and tissue culture supernatants for 48 h prior to an 18 h pulse with 1 μCi ^{3}H-thymidine/well. Cells were harvested with a PHD cell harvester and ^{3}H-thymidine uptake was determined by liquid scintillation counting; 2) MTT assay: 5 x 10^3 CT.4S cells in 50 ul of complete medium were added to triplicates of two-fold dilutions of 50-ul culture supernatant aliquot and cultured for 24-36 h at 37° C. A total of 50 μg MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) in 10 ul RPMI 1640 were then added to each well. After a further 4-h incubation, 190 ul of stop solution (2:1 2-propanol:H_2O made 0.04 N in HCl) was added to each well. Each well was vigorously pipetted, the plate was left at 37° C for 10 min, and A_{570} was determined. Each assay was calibrated against murine rIL-4 standards with IL-4-deficient medium used as negative control. The specificity of this bioassay for IL-4 was determined by parallel analysis of replicate supernatants in the presence of purified anti-IL-4 mAb (11B11) at concentrations sufficient to neutralize the activity of

>200 U/ml of rIL-4.

Using the ^{3}H-thymidine uptake method, the CT.4S assay detects IL-4 at 0.5-1.0 U/ml and quantifies amounts $\geq$ 2 U/ml. MTT assay improved sensitivity to detect IL-4 at levels of 0.15 to 0.30 U/ml and readily detected amounts $\geq$ 0.6 U/ml, with standard error less than 5%.

2.12.3. IL-10

A dual mAb based ELISA was used for IL-10 determination as described. Briefly, 50 ul purified anti-IL-10 mAb SXC1 was used to coat 96 well ELISA plates overnight at 1 μg/ml. After blocking, dilutions of culture supernatants and rIL-10 standard were incubated for 3 h at 37° C followed by incubation with biotinylated anti-IL-10 mAb SXC2. Streptavidin-alkaline phosphatase and p-nitrophenyl phosphate were used for development. The lower limit of detection is 0.2 U/ml, with quantitation of cytokine $\geq$ 0.5 U/ml. Standard error was <10% in most experiments.

2.12.4. IFNγ

2.12.4.1. Inhibition of viral cytopathic effect bioassay

A mycoplasma free subline of mouse L-929 fibroblasts,

designated LB, was used for the assay. Actively proliferating cultures were trypsinized (0.05% Trypsin-EDTA), resuspended as a single cell suspension in complete medium at 4 x 10^6/ml and plated in 96 well flat bottom plates at 100 ul/ml then cultured for 3 h at 37° C to re-establish an adherent monolayer. At this time, recombinant and natural IFNγ standards were added as 100 ul samples of two-fold dilutions in complete medium. Following 18 h additional incubation at 37° C, the capacity of IFNγ to protect LB cells from viral cytopathic effect was determined by addition of 100 ul of diluted encephalomyocarditis virus (a gift of Dr. F. Jay, University of Manitoba), in complete medium, to all except unchallenged control wells. Virus infected fibroblasts cultured in IFNγ deficient, complete medium were used as negative controls. To maximize sensitivity, virus was used at the predetermined minimum concentration necessary to elicit a 100% cytopathic effect in unprotected, actively proliferating fibroblast cultures. The plates were cultured for a final 24-30 h at 37° C, after which multiwell plates were emptied by rapidly flicking out the supernatant and blotting them firmly on paper toweling. Approximately 100 ul crystal violet solution (0.25% crystal violet, 0.9% NaCl, 20 mM Tris HCl, 20% methanol v/v, pH 7.5) was added to each well and, after 10-30 minutes at room temperature, the plates were gently rinsed with running tap water.

Specificity in this bioassay was determined by concurrent analysis of replicates in the presence of purified anti-IFNγ mAb XMG 1.2 at concentrations sufficient to block the activity of >200 U/ml IFNγ. Supernatant activity was attributed to IFNγ if $\geq$ 90% of protection from viral cytopathic effect was abolished by the inclusion of this anti-IFNγ mAb. The concentration of biologically active IFNγ was expressed as a reciprocal titre based on the sample dilution at which 50% inhibition of viral cytopathic effect was observed. This assay detects IFNγ at 2 - 4 U/ml.

2.12.4.2. WEHI-279 bioassay

IFNγ concentrations were determined on the basis of its ability to inhibit WEHI-279 proliferation essentially as described (Reynolds, 1987), but using an abbreviated assay. In brief, WEHI-279 cells in log phase growth (viability >99%) were seeded at 1 x 10^4 cells/0.1 ml culture medium in each well of 96 well flat-bottom plates. 0.1 ml of two fold dilutions of culture supernatants were added and the plate was incubated at 5% CO_2, 37° C for 48 h. Internal standards of IFNγ containing, con A-stimulated mouse spleen cell supernatants, calibrated against World Health Organization-National Institute of Allergy and Infectious Diseases international murine IFNγ reference reagent Gg02-901-533 were included in each assay. Cells cultured in IFNγ

deficient medium were used as a negative control. ^{3}H-thymidine (1 μCi/well) was added for an additional 6-8 hours of culture and ^{3}H-thymidine incorporation was determined by concurrent analysis of replicates in the presence of purified anti-IFNγ mAb XMG 1.2 at concentrations sufficient to block the activity of >200 U/ml IFNγ. Inhibition of WEHI-279 growth was attributed to IFNγ if $\geq$ 90% of growth inhibition was abolished by the inclusion of anti-IFNγ mAb. Data are presented as mean dpm $\pm$ SEM.

2.12.4.3 IFNγ ELISA

Although the WEHI-279 bioassay is sensitive for IFNγ analysis, it has the disadvantage that non-specific inhibitory effects from metabolites present in exhausted tissue culture supernatants can be inhibitory. To overcome the disadvantages of complexity of the inclusion of replicate cultured in the presence and absence of neutralizing antibodies for each sample as well as the restriction of cell viability for optimal assay quality and time-consuming procedures, we developed a simple and highly specific as well as sensitive ELISA for murine IFNγ using two mAb that are readily available. This ELISA system was used for most of the experiments presented with some results confirmed for biological activity by the WEHI-279

bioassay.

2.12.4.3.1. Biotinylation of R4.6A2

Purified R4.6A2 was dialysed against $NaHCO_3$ (0.1 M, pH 8.15) and concentrated to 1-2 mg/ml using a Centriprep-30 concentrator . A biotin-spacer conjugate (BNHS) was dissolved in anhydrous DMSO at 2 mg/ml and immediately added to the antibody at the ratio of 1:4 (antibody:BNHS, 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. This reagent was stable for at least 8 months at 4° C in NaN_3 (0.01%).

2.12.4.3.2. IFNγ ELISA procedure

In a manner similar to the antibody ELISAs, 96 well ELISA plates were coated with XMG 1.2 at 5 μg/ml in coating buffer. After overnight incubation at 4° C, plates were blocked for 2 hours at room temperature and washed extensively. Culture supernatants and an internal IFNγ standard were serially diluted and added to the plate. Plates were incubated at 37° C for 3 hours, then washed three times. Biotinylated R4.6A2 was added for an overnight incubation at 4° C. The plates were then washed next day

and streptavidin-alkaline phosphatase was added for 1 h at 37° C, the plates were washed again and p-nitrophenyl phosphate in $MgCl_2$, 10% diethanolamine was added. The plates were read by automatic reader at 30, 60, 90 and 120 min. This assay commonly detects IFNγ activity $\geq$ 0.1 U/ml and quantitates it $\geq$ 0.5 U/ml, with SEM less than 5%.

2.13. Statistical analysis

PCA and ELISA titres were log transformed, following which geometric means were compared using unpaired two-tail Student's t tests. Cytokine production was also analyzed by two-tail Student's t test.

RESULTS

I. DEVELOPMENT OF SENSITIVE CYTOKINE MEASUREMENT ASSAYS

The intention of the present study was to examine cytokine production patterns in an animal model of human immediate hypersensitivity. Considering the limitations in interpreting the data of studies using long term cultured CD4 T cell clones and polyclonal T cell activators, we decided to use an alternative approach in this study. Specifically, we chose to characterize antigen-driven cytokine synthesis by spleen cells from protein antigen primed animals directly ex vivo in 14-36 h primary cultures. Because antigen-driven cytokine synthesis in short term cultures is typically two or more orders of magnitude below that observed following activation of long term T cell clones or hybridomas, a prerequisite for successful detection of cytokine synthesis in this investigation was the availability of highly sensitive and specific cytokine measurement techniques. Indeed, some groups have reported a failure to detect IL-4 synthesis by freshly isolated T lymphocytes from polyclonal or antigen primed animals unless such cells were carried in culture for up to 12 days with multiple cycles of antigenic stimulation. This may partially have been due to the low sensitivity of IL-4 determination techniques then available. Since IFNγ and IL-4 are critical in IgE

regulation and the methods available were not ideal for determination, we initially made efforts to optimize techniques for quantitative analysis of small amounts of antigen driven cytokine protein production.

1.1. Development of a dual mAb based murine IFNγ ELISA and its comparison with bioassays

A variety of bioassays have been used to quantitate IFNγ production in tissue culture supernatants derived from murine primary cultures. These include viral inhibition assays and inhibition of WEHI-279 cell proliferation, MHC class II induction, and others (Havell, 1992; Reynolds, 1987). Most of these assays offer a high degree of sensitivity. However, they share the disadvantage of all bioassays that other cytokines present in the sample may synergize with or antagonize the effects of the analyte on the bioassay's target. Moreover, particularly in the case of inhibition assays, non-specific inhibitory effects from metabolites presenting in exhausted tissue culture supernatants can be problematic. Although the inclusion of neutralizing antibodies to the cytokine can largely discriminate this non-specific inhibition, the higher variation in the detection of low amounts of cytokine limits their utility for antigen-drive cytokine

quantitation. Therefore, we developed a simple ELISA for murine IFNγ determinations using two mAbs that are readily available. It markedly improves murine IFNγ detection in terms of sensitivity, specificity and reproducibility. Here, IFNγ ELISA is compared with two of the most widely used bioassays.

1.1.1. Inhibition of viral cytopathic effect assay

The capacity of IFNγ containing tissue culture supernatants to protect mycoplasma free L-929 (LB) cells from lysis by virus was evaluated. As shown in Fig. 1, this widely used assay offers reasonable sensitivity with a detection limit of 2-4 U/ml IFNγ
under optimal conditions. However, its main problem in practical use is a lack of specificity. IFN$\alpha\beta$, which often exists concurrently in tissue culture supernatants with IFNγ, can also protect L-929 cells from lysis by virus (Fig. 2). The discrimination of the activity of different isotypes of interferon requires that all samples be tested in the presence and absence of neutralizing anti-IFNγ antibodies. The utility of this assay is also restricted by other considerations. The method is somewhat cumbersome in that there is a need to optimize the concentrations of fibroblasts used and virus added for maximal sensitivity. Moreover, fibroblasts need to be continuously maintained in

culture, with the sensitivity of the assay highly dependent on the fibroblasts being in log phase growth.

This assay offers the least precise approach of the three examined, yielding a titre based on 50% inhibition of viral cytopathic effect. As such, it provides a rather subjective analysis of IFNγ production.

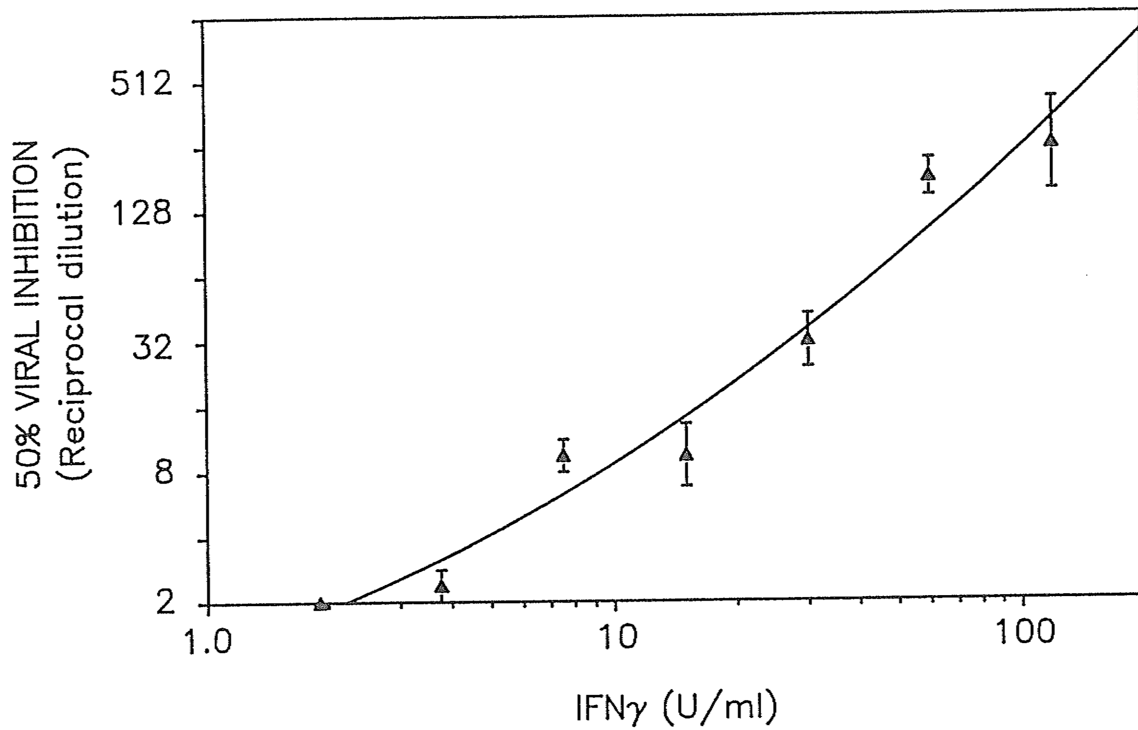

Fig 1: Sensitivity of IFNγ quantitation by inhibition of viral cytopathic effect. A rIFNγ standard was used and the end point (50% inhibition) for protecting L-929 cells from viral cytopathic effect was tested. Data are presented as reciprocal titres ± SEM derived from one of the five independent assays.

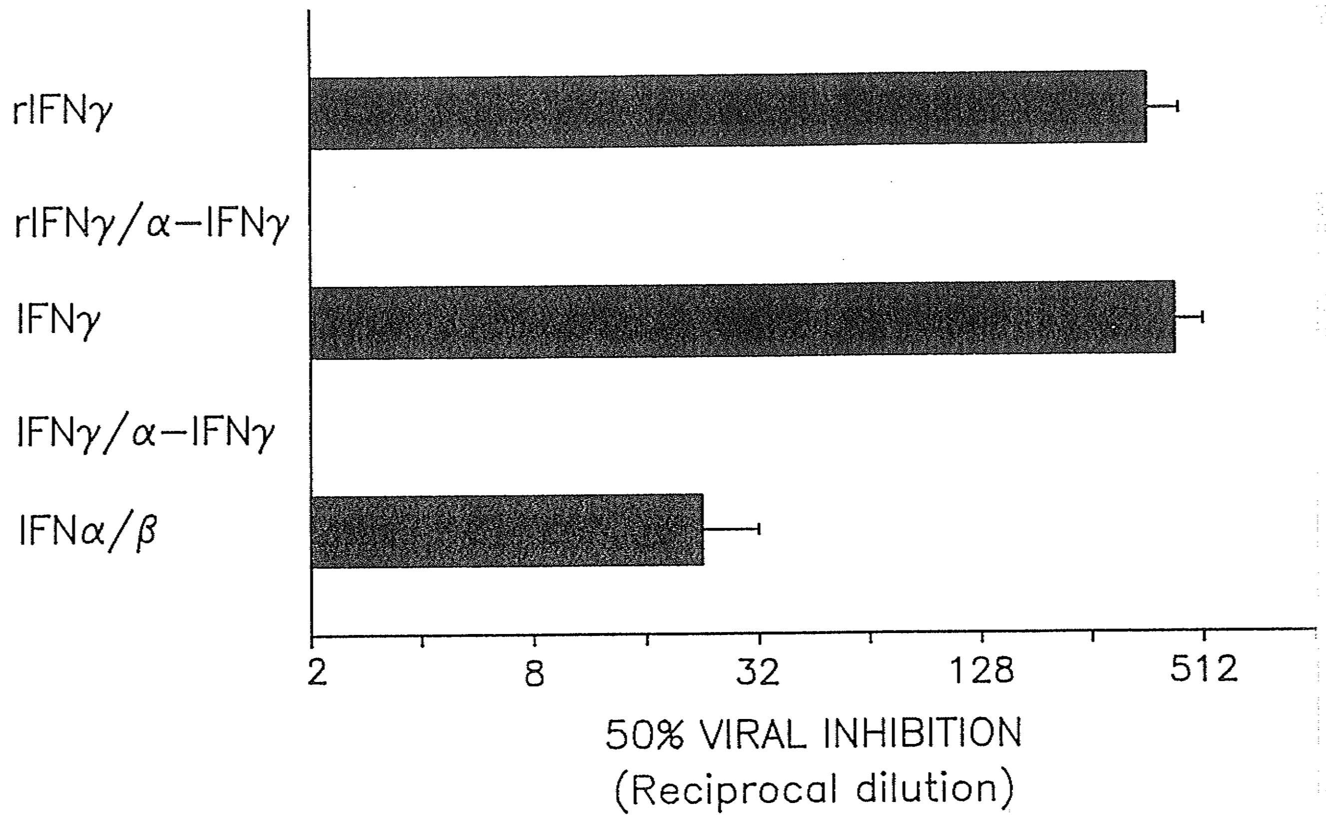

Fig 2: Specificity of IFNγ quantitation by inhibition of viral cytopathic effect. Anti-IFNγ mAb (XMG 1.2) were added to cultures concurrently with 100 U/ml rIFNγ, natural IFNγ, or IFNαβ. The protection conferred by the addition of IFNαβ was not affected by the addition of anti-IFNγ mAb (not shown).

1.1.2. IFNγ dependent inhibition of WEHI-279 cell growth

B cell proliferation is markedly sensitive to IFNγ activity. This sensitivity has been exploited in assays based on the dose dependent inhibition of B hybridoma WEHI-279 proliferation. Using ^{3}H-thymidine uptake, this approach reproducibly quantifies IFNγ concentrations at and above 2 U/ml (Fig. 3). Using the method described here, we found this assay to exhibit excellent sensitivity and assay to assay reproducibility. The measurement of natural or rIFNγ was not interfered with by addition of the functionally related (IFN$\alpha\beta$) or unrelated cytokines tested (Fig. 4). However, addition of acidic, exhausted tissue culture supernatants elicited variable inhibition of WEHI-279 B cell growth which was not neutralizable by anti-IFNγ mAb (data not shown). This non-IFNγ dependent inhibition limits the utility of this assay. A further disadvantage is that the sensitivity of the assay is exquisitely dependent on the WEHI-279 cells being in log phase growth, with 2-5 fold decreases in sensitivity resulting from use of cultures containing 3-5% non-viable cells. Finally, although this assay is not sensitive to the presence of T cell polyclonal activators such as anti-CD3 or Con A, the presence of mitogens such as LPS or PHA in the samples renders the assay useless.

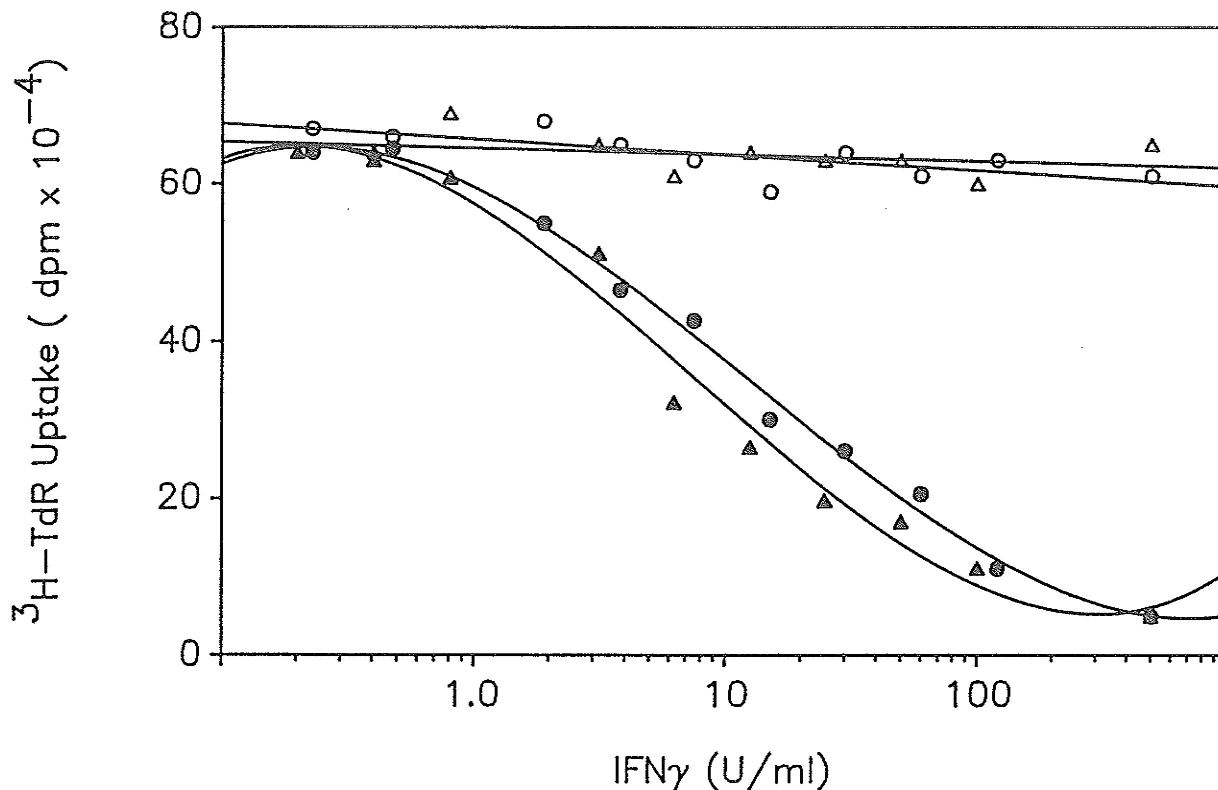

Fig 3: Sensitivity of WEHI-279 growth inhibition for IFNγ quantitation. Natural IFNγ (● ○) or rIFNγ (▲ △) were serially diluted in the absence (closed symbols) or presence (open symbols) of purified anti-IFNγ mAb. The effect of IFNγ on WEHI-279 proliferation was detected by measuring ^{3}H-thymidine uptake. In this assay, SEM ranged from 6-13% and are omitted for clarity.

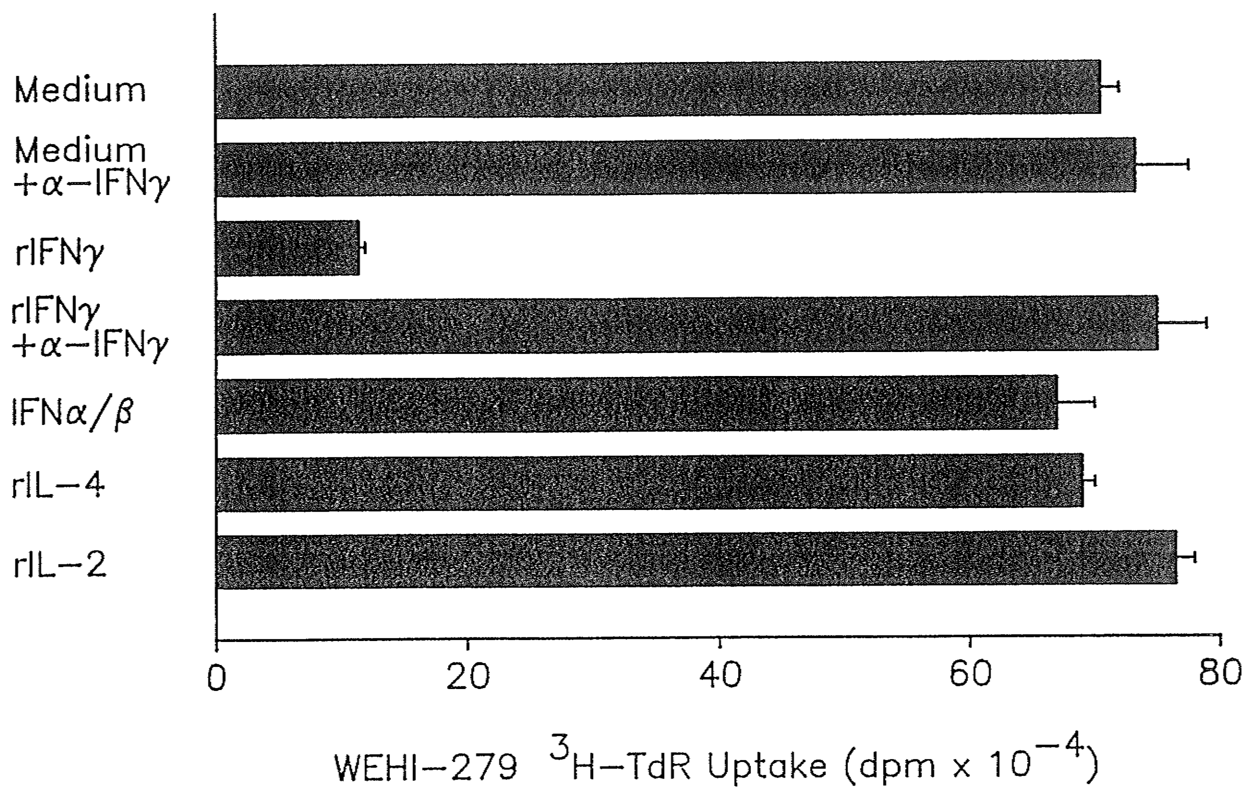

Fig 4: Specificity of WEHI-279 growth inhibition for IFNγ quantitation. Addition of rIFNγ (100 U/ml) but not IFNαβ (100 U/ml), rIL-2 (500 U/ml) or rIL-4 (500 U/ml), leads to inhibition of WEHI-279 proliferation which is reversible by the addition of anti-IFNγ mAb.

1.1.3. IFNγ ELISA development

The inherent advantages of ELISAs prompted us to establish an IFNγ specific, dual mAb based assay using two mAbs which recognize spatially distinct epitopes on natural IFNγ. The assay can be performed in a single microtitre plate in 8-10 h, although for convenience it is typically carried out over 24 h. Unlike the bioassays, it does not require continuous culture of IFNγ responsive cells nor optimization of their growth prior to bioassay. It yields the most consistent results of the three assays examined.

Using purified anti-IFNγ mAb XMG 1.2 and biotinylated R4.6A2 as capture and developing mAbs respectively, this assay quantitates from 0.5 U/ml to 100 U/ml. The specific upper and lower limits of the linear portion of the standard curve, hence the useful portion of the assay, are determined by the length of time the alkaline phosphatase/substrate step is allowed to proceed. The slopes of the linear portion of the curves generated with natural IFNγ (Fig. 5A) and a large number of primary tissue culture supernatant samples were almost invariably parallel, an essential requirement for analyte quantitation.

This assay yields small standard errors, frequently <5%. Thus the detection limit of the assay, defined as three standard deviations above the A410 of control wells lacking only IFNγ, is commonly 0.05 to 0.1 U/ml (Fig. 5B). This highlights the advantages of this assay in applications requiring highly sensitive IFNγ detection such as limiting dilution analysis.

Most importantly, this assay is highly specific for IFNγ, independent of inference from other cytokines, including IFN$\alpha\beta$, or non-specific variables (i.e., waste metabolites in culture supernatants) (Fig. 6). Moreover, it has the advantage of being much more rapid and simple in operation than the two bioassays tested. The sole limitation of this assay, in common with all ELISAs, is that it does not directly assess biological activity.

1.1.4. Variability of IFNγ assays

Intra-assay variation was determined in each of the three assays via following quintuplicate analysis of rIFNγ and natural IFNγ standards. As seen in Table 1, a major advantage with the ELISA was that it exhibited the lowest variability of the three assays examined. The mean intra-assay coefficient of variation for the ELISA was 0.077, approximately one fifth of those obtained in the two

bioassays. Similarly, comparison of the interassay coefficient of variation for the three assays tested identifies the ELISA as the assay of choice (Table 2).

An alternative ELISA for murine IFNγ, based on capture with anti-IFNγ mAb R4-6A2 and use of biotinylated polyclonal rabbit antiserum, streptavidin-enzyme conjugates as developing reagents, has been reported (Curry, 1987). The limitation of this approach lies in the requirement for a source of polyclonal antibody highly specific for IFNγ or micro- to milligram quantities of purified IFNγ for immunization. These considerations have acted to limit its use. Therefore, the development of readily established sandwich immunoassay provides an optimal tool for determining murine IFNγ responses at physiological levels.

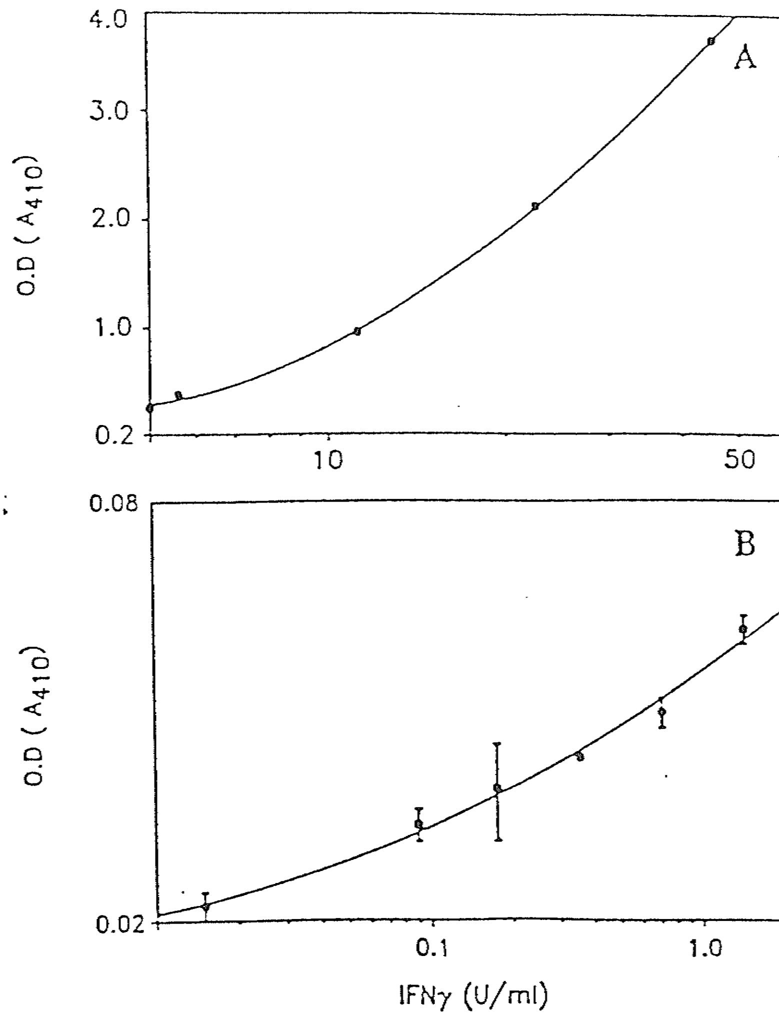

Fig 5: Sensitivity of dual mAb IFNγ ELISA. Panel A, optical absorbence at IFNγ concentrations 2 - 50 U/ml; panel B, sensitivity at low concentrations (0.01-2 U/ml). Data shown are ± SEM. Note expanded Y axis in panel B.

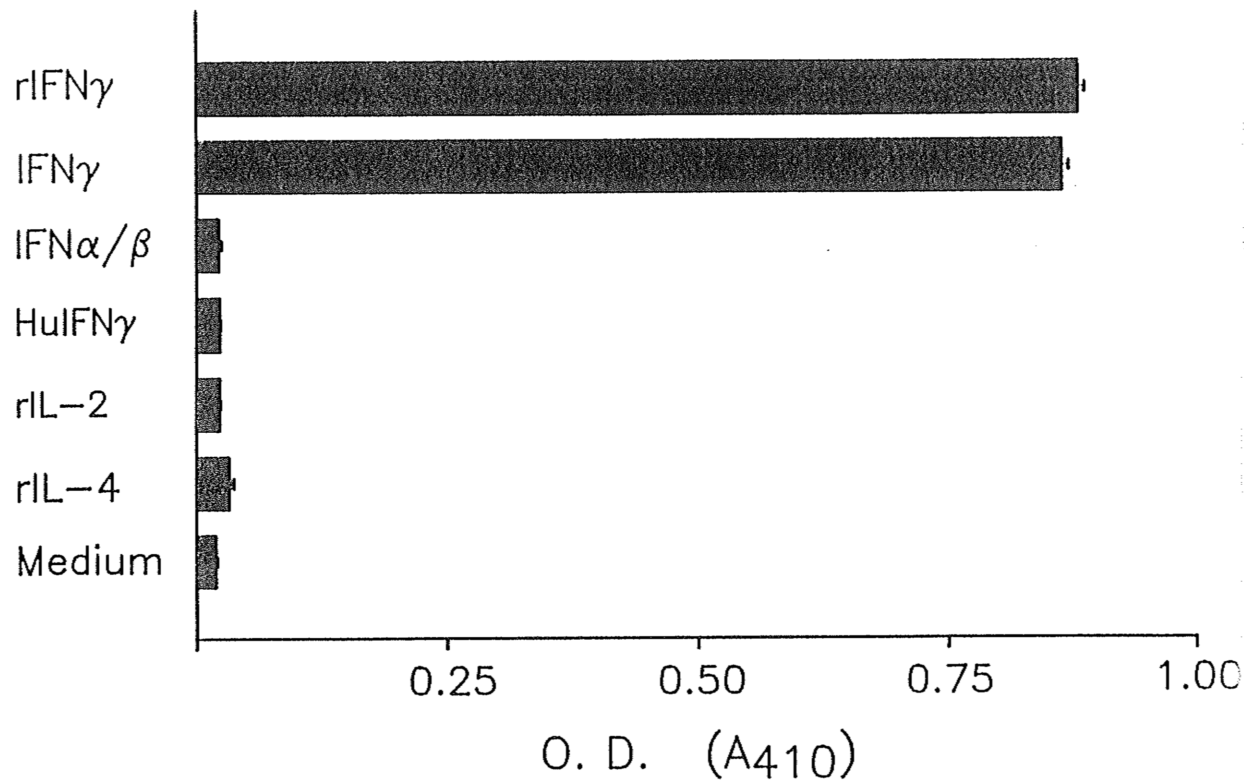

Fig 6: Specificity of IFNγ ELISA. Addition of IFNαβ (100 U/ml), Human IFNγ (500 U/ml), murine rIL-2 (500 U/ml) or rIL-4 (500 U/ml) failed to result in a signal above background. Natural and rIFNγ were used at 10 U/ml in this assay. Data shown are ± SEM.

Table 1

Intra-assay Variation in IFNγ Determinations

IFNγ added (U/ml)	ELISA Mean (C.V.)	WEHI assay Mean (C.V.)	Viral inhibition Mean (C.V.)
60	59.8 (.031)	77.7 (.299)	48 (.33)
30	32.0 (.073)	33.9 (.352)	21 (.390)
15	13.6 (.125)	13.3 (.462)	18 (.370)
7.5	6.8 (.090)	8.5 (.462)	6 (.340)
3.75	3.1 (.068)	2.2 (.358)	4.5 (.660)
1.88	1.88 (.069)	2.7 (.825)	N.D.

Legend: Cytokine concentrations were calculated from data obtained in the ELISA, WEHI-279 growth inhibition and inhibition of viral cytopathic effect assays carried out as described at Materials and Methods. For each IFNγ concentration, the mean of five replicates (and the associated coefficient of variation) is provided. N.D., Not done as this concentration was below the level of detection in this assay.

Table 2

Inter-assay Variation in IFNγ Determinations

IFNγ added	ELISA	WEHI assay	Viral inhibition
(U/ml)	Mean (C.V.)	Mean (C.V.)	Mean (C.V.)
60	57.5 (.121)	51.8 (.430)	45.8 (.260)
30	33.5 (.076)	24.4 (.364)	23.3 (.194)
15	12.1 (.145)	13.1 (.217)	18.4 (.168)
7.5	5.4 (.124)	6.1 (.331)	7.3 (.265)
3.75	3.0 (.119)	3.1 (.369)	1.4 (.289)
1.88	1.7 (.678)	1.8 (.851)	N.D.

Legend: The mean cytokine concentration and coefficient of variation obtained from four replicates of each assay at each IFNγ concentration is shown. N.D., not done.

1.2. Analysis of antigen-driven IL-4 responses using MTT versus ^{3}H-thymidine uptake CT.4S bioassay

CT.4S, a subline of CTLL, is highly responsive to IL-4 but poorly responsive to IL-2 at or below 100 units (Hu-LI, 1989). Other cytokines tested, including IL-1, IL-3, IL-6, CSF-1 G-CSF, and GM-CSF, do not significantly interfere with CT.4S cell proliferation. Since IL-2 levels in most in vitro culture systems are much lower than 100 U/ml, CT.4S bioassay in combination with replicate samples containing anti-IL-4 mAb, provides a valuable approach for IL-4 quantitation. The published method widely used for CT.4S assays was measurement of ^{3}H-thymidine uptake. This assay, as reported,
detects IL-4 at concentrations above 3-10 U/ml. Since antigen driven-IL-4 response in short term culture, especially cells from mice primed with allergen in the absence of adjuvant, were expected
to be weak, a more sensitive assay was required.

The MTT tetrazolium colorimetric assay introduced by Mosmann to measure cytotoxicity and cell proliferation was developed as a more sensitive, rapid and economical alternative to radioactive assays. The primary advantage of this approach is that instead of measuring cellular DNA

synthesis, the MTT assay measures the energy metabolism of living cells, which is expected to be more sensitive in the sense that it reflects cell viability rather than proliferation. In collaboration with Mr. R. Gieni in our laboratory, the CT.4S radioactive bioassay (^{3}H-thymidine uptake) was converted to an MTT assay which significantly (approximately ten fold) increased the assay sensitivity.

As shown in Fig. 7, both radioactive and MTT assays yield standard curves with wide linear portion for IL-4 quantitation. Although the upper limit of the linear portion of the radioactive assay is slightly higher than that of the MTT assay (100 vs 50 U/ml), the MTT assay offers a much more sensitive lower limit than the radioactive assay (0.2 vs 2 U/ml). The standard error of the MTT assay, even at very low IL-4 concentrations, is less than 5% , which is also a big improvement over the conventional CT.4S assay (radioassay has a standard error of 10-15%).

In addition, this MTT assay has several practical advantages. It is simpler and more rapidly performed. Since the substrate does not interfere with the measurement of the product there are no removal or washing steps, and this helps to increase the speed of the assay and minimize variability between samples. Moreover, it avoids the use of

hazardous radioisotope and decreases material and labour costs.

II. ALLERGEN-DEPENDENT INDUCTION OF CYTOKINE SYNTHESIS IN VIVO

A primary objective of this study was to characterize the patterns of cytokine synthesis induced by administration of chemically modified and native allergens. Although IL-2 and IFNγ synthesis by normal T cells were well characterized at the initiation of this study (1989), the requirements for induction of IL-4 synthesis in vivo were very controversial. At that time, most groups reported that "fresh" CD4 T cells, examined directly ex vivo, fail to produce IL-4 upon short-term culture. Induction of IL-4 mRNA and secretion of detectable quantities of IL-4 was focused in vitro only after cells are primed and restimulated with Con A or anti-CD3 antibody. The consensus from several groups was that CD4 T cells with the potential to become IL-4 secretors exist in vivo in the form of precursors which require 4-12 days of culture and one or more cycles of rest and restimulation with mitogen or antigen before they become detectable as lymphokine-secreting cells (Swain, 1988; Powers, 1988; Hayakawa, 1989; Fox, 1989; Powell, 1990; Weinberg, 1990, Rocka, 1991). On the other hand, publication of experiments from this (Wang,

1991) and other (Scott, 1990) laboratories utilizing in vivo administration of polyclonal T cell activators demonstrated rapid , sequential IL-2 *and IL-4 gene expression* in naive mice.

Although IL-4 mRNA synthesis following primary in vivo stimulation with low-dose anti-CD3 mAb was interpreted as consistent with the presence of T cells able to synthesize IL-4 in the preimmune lymphocyte pool, there were three important reservations to this approach: (1) the impact of environmental antigens in prepriming of IL-4 synthesizing precursors, even in nominally germ-free mice, is difficult to assess following polyclonal T-cell activation; (2) the possibility of translational regulation of cytokine gene expression was not addressed in those studies which solely examined IL-4 mRNA synthesis, and, most importantly, (3) polyclonal stimulation (e.g. with anti-CD3 mAb) may not be representative of T-cell responses elicited by antigenic stimulation.

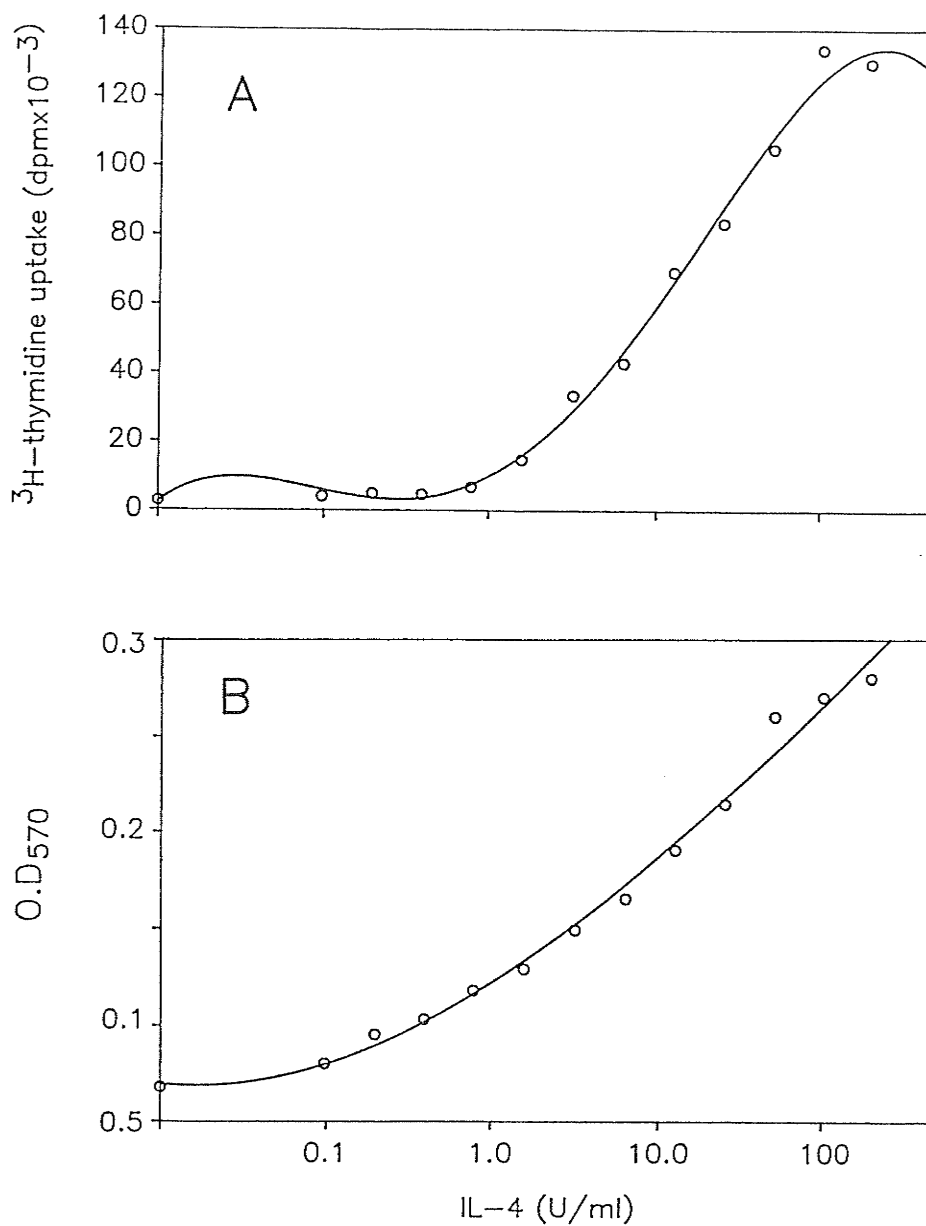

Fig 7: Sensitivity of CT.4S radio- and MTT bioassay for IL-4 detection. Panel A, CT.4S proliferation measured by ^{3}H-thymidine uptake. Panel B, CT.4S viability measured by MTT assay.

We hypothesized that previous failures to detect IL-4 synthesis directly ex vivo following in vivo immunization may have reflected the experimental conditions selected rather than an inherent absence of IL-4 secreting cells in the extrathymic T-cell repertoire. We therefore compared allergen specific antibody production with cytokine secretion in naive, OA-alum and OA-CFA immunized mice using overnight cultures derived from mice immunized 1-21 days prior to sacrifice. This comparison of different adjuvants was initiated based on the facts that CFA, used in several of the studies cited above, is an excellent stimulator of humoral and cell-mediated response but very poor or inhibitory for IgE production (Kishimoto, 1982).

2.1 OA-alum and OA-CFA immunization elicit reciprocal antibody responses

Mice were immunized with OA in alum or CFA and bled 4, 7, 10, and 14 days later. As shown in Fig. 8, strong OA-specific IgE responses, peaking 10 days after immunization, were elicited by OA-alum immunization. The IgG_{2a} responses, however, were poor following this mode of immunization. In contrast, OA-CFA immunized mice generated weak IgE responses and strong IgG_{2a} responses ranging from 16 to 30-fold higher than those seen in OA-alum immunized groups.

These results confirm the effects of different adjuvant in the selection of antibody isotypes.

2.2 Kinetics of cytokine synthesis and optimal stimulation conditions

To compare cytokine producing patterns of different groups, a characterization of the kinetics of cytokine gene expression was necessary. Mice were killed at 1, 2, 3, 4, 5, 6, 8, 12, and 21 days following OA-alum immunization. Spleen cells were cultured in the presence of polyclonal (anti-CD3 or Con A) or antigen-specific (OA) stimulation. Fig. 9 demonstrates that IL-2, IFNγ and IL-4 gene expression occurred at roughly the same time, i.e. 4-5 days after immunization, with peak cytokine synthesis observed at d.5-6. The cytokine synthesis subsequently decreased with minimal levels detectable at d.12.

Study of the kinetics of cytokine secretion in in vitro cultures demonstrated that IL-2, IL-4 and IFNγ proteins were readily detectable in culture supernatants 14 - 16 h following antigen specific stimulation and are maximal or near-maximal at 24 h. IL-2 and IL-4 levels were dramatically decreased after 24 h culture whereas IFNγ levels in supernatants were steady or slightly increased until 72 h culture (Fig. 10).

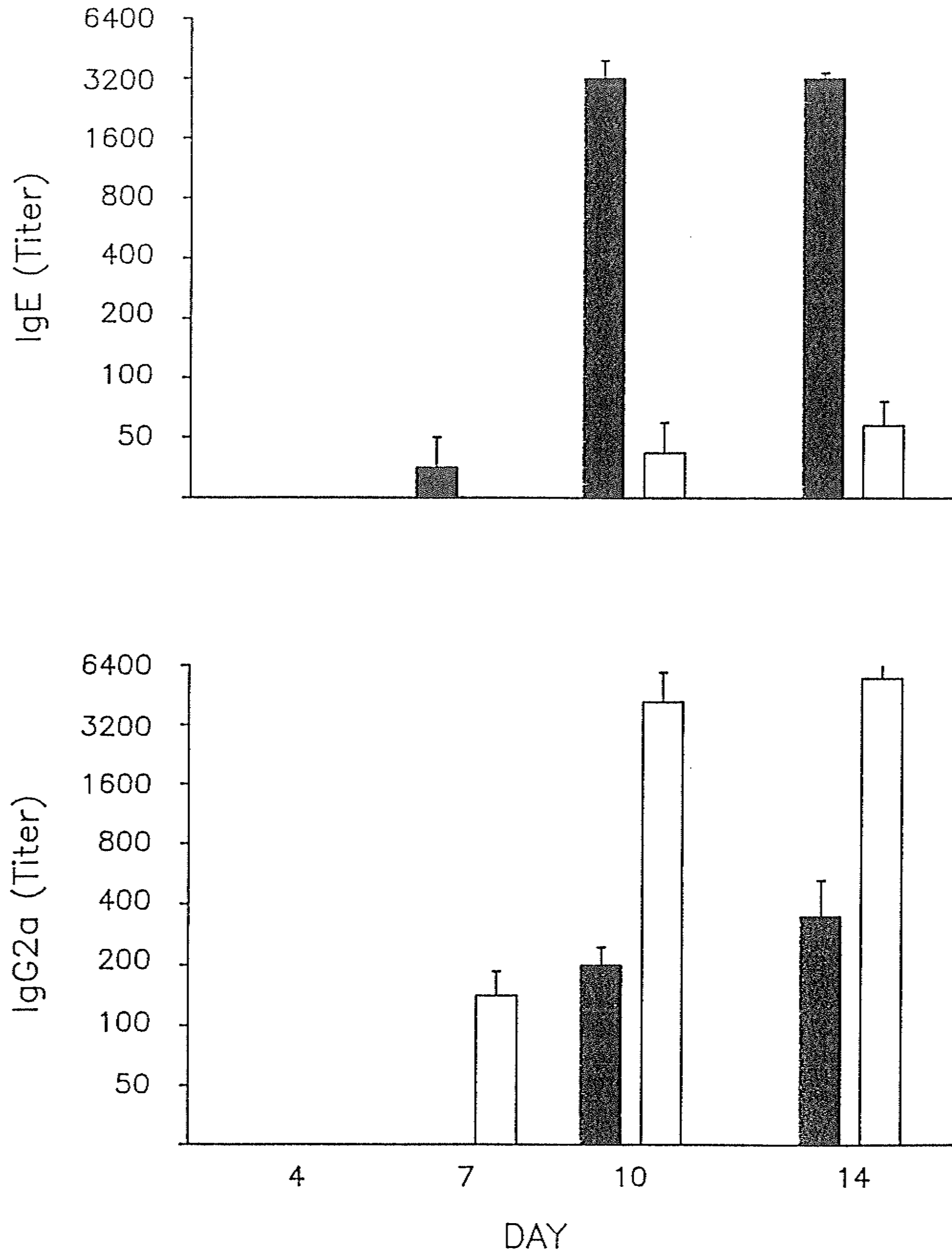

Fig 8: OA-alum and OA-CFA immunization elicit reciprocal antibody responses. Mice were immunized with OA-alum (■) or OA-CFA (□). OA-alum induced strong antigen-specific IgE, and weak IgG_{2a} responses while OA-CFA immunization led to strong IgG_{2a} but poor IgE response (IgE, $p<0.001$; IgG2a, $p<0.005$). One typical experiment is shown.

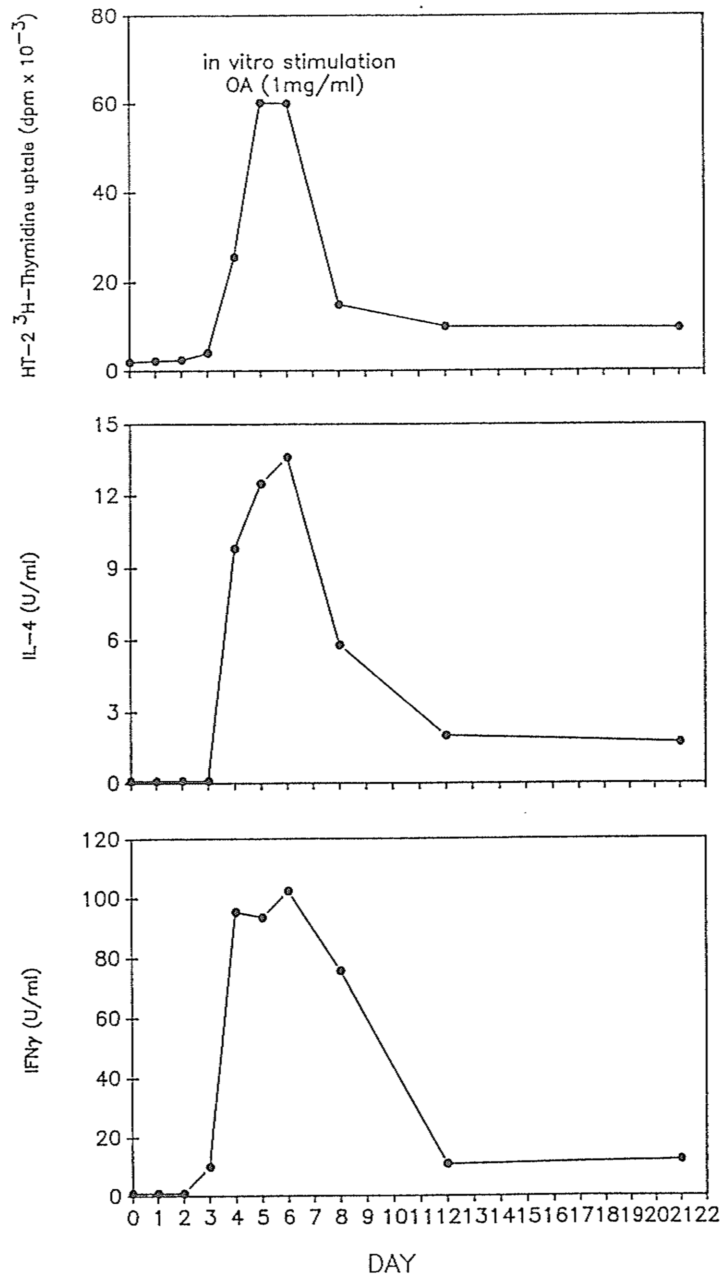

Fig 9: Kinetics of antigen-driven cytokine synthesis following in vivo immunization. Mice were immunized with OA-alum and killed at different times. Spleen cells were stimulated with OA (1 mg/ml) and cytokine production in 24 h culture supernatants was determined. IL-2 was tested by HT-2 proliferation assay, IL-4 by MTT CT.4S assay and IFNγ by ELISA.

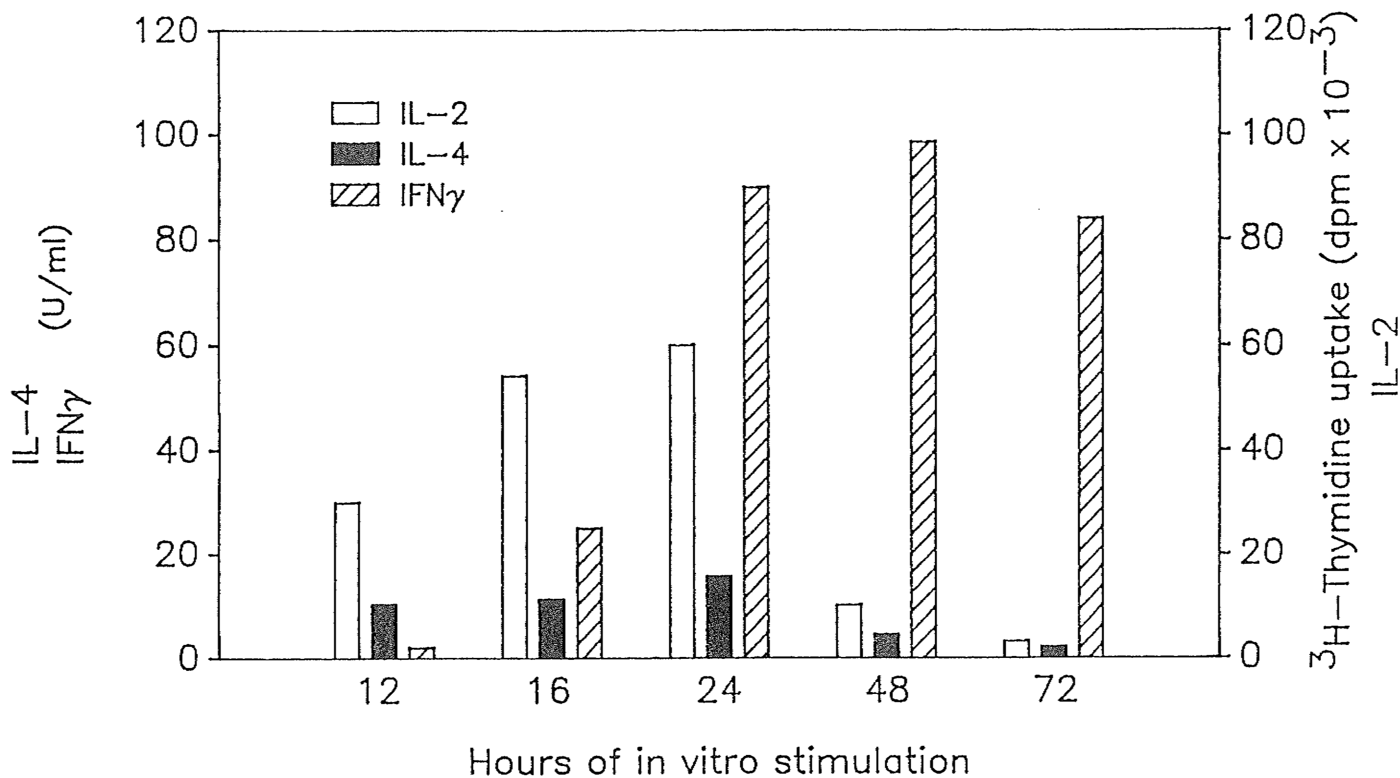

Fig 10: Kinetics of antigen-mediated cytokine synthesis in vitro. Spleen cells from mice immunized with OA-alum were stimulated in vitro with OA (1 mg/ml). Tissue culture supernatants were harvested at different times and cytokine levels were determined as described for Fig 9.

For in vitro restimulation, OA at 300 $\mu g/ml$ consistently elicited detectable cytokine responses and 1 mg/ml is the optimal concentration for most experiments (Fig. 11). Spleen cells in 24 well plates were cultured at 0.3, 3.0 and 15 million cells per well (2 ml). The results of three identical experiments are summarized in Table 3 which shows that 7.5 x 10^6 cells/ml gave maximal in vitro cytokine synthesis. Therefore, except where specified, all the experiments involving cytokine gene expression in this study examined the cytokine profile of spleen cells from mice that were immunized in vivo 5 days before being killed following 24 h in vitro restimulation at 7.5 x 10^6 cells/ml.

2.3 Comparison of cytokine patterns induced by OA-alum and OA-CFA immunization

The relationship between specific antibody responses and cytokine production in response to antigen-specific stimulation was examined by comparison of cytokine patterns induced following OA-alum and OA-CFA immunization. Spleen cells from OA-alum-immunized, but never from OA-CFA-immunized or naive, mice generated substantial IL-4 responses (Fig. 12). Injection of alum or CFA alone failed to elicit detectable IL-4 synthesis in vitro under any of

the conditions tested (Fig. 12). In about 60% of the experiments performed, substantial IL-4 production occurred following culture of cells from OA-alum immunized groups, but not naive groups or those immunized with antigen in CFA, without the addition of soluble antigen in vitro. The preferential induction of IL-4 synthesis by OA-alum immunization is consistent with the potent ability of OA-alum to induce IgE secretion.

In most experiments, IFNγ synthesis in OA-CFA-immunized groups was markedly higher than that of naive or OA-alum immunized groups cultured in the absence of in vitro antigen (Fig. 13). However, IFNγ levels were essentially identical in OA-CFA and OA-alum-immunized groups following antigen-specific restimulation in vitro (Fig. 13). IL-2 production was similar in OA-alum and OA-CFA groups (Fig. 14).

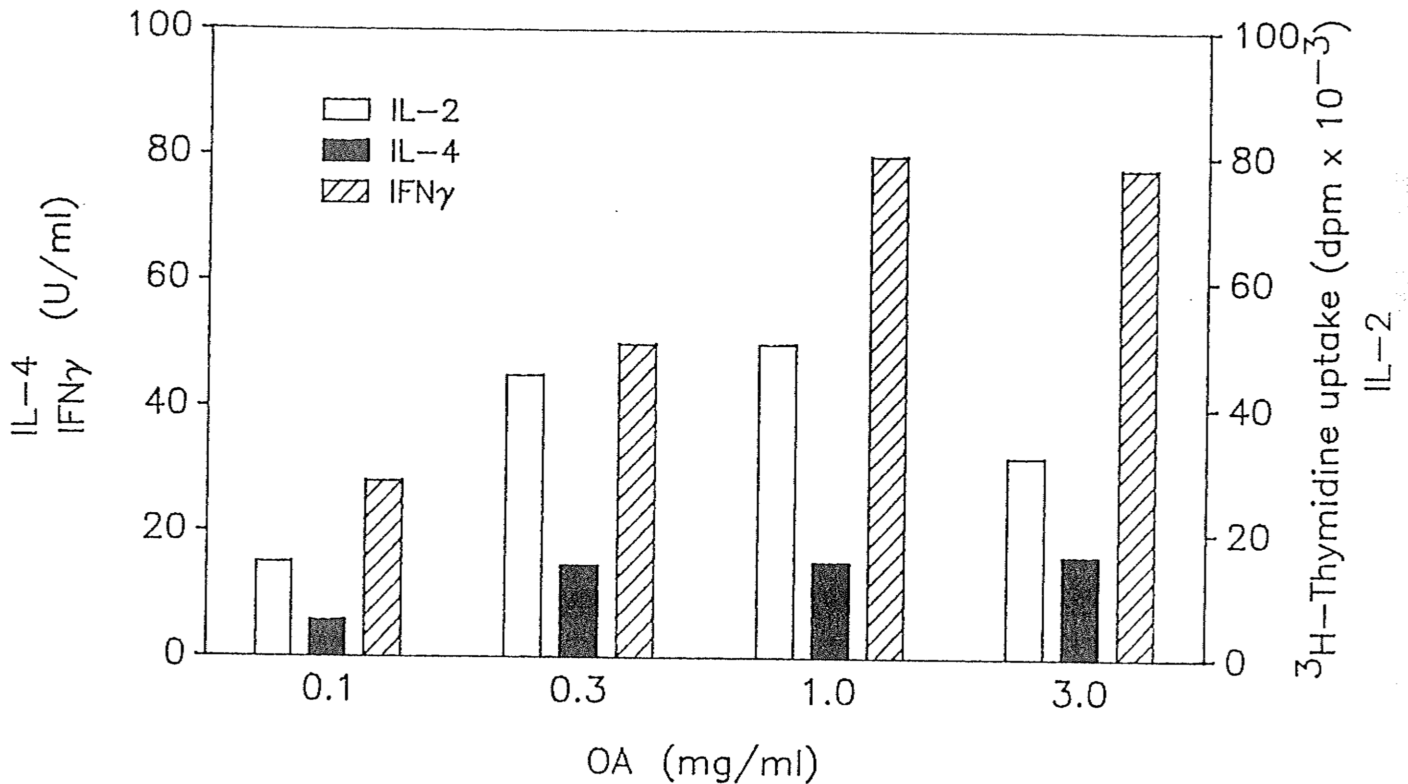

Fig 11: Optimal antigen concentration for in vitro stimulation. Spleen cells at 7.5 x 10^6 cells/ml were cultured in vitro in the presence of different concentrations of OA. Antigen-driven cytokine synthesis in culture supernatants were measured at 24 h using methods as in Fig 9.

Table 3

Optimal cell concentration for in vitro cytokine production

Cell Concentration (10^6/ml)	IL-4 (U/ml)		IFNγ (U/ml)	
	Expt.1	Expt.2	Expt.1	Expt.2
7.5	12.30(0.20)	12.25(0.95)	59.55(1.45)	113.20(1.20)
1.5	9.05(0.25)	3.10(0.14)	5.0 (0.50)	40.15(2.15)
0.15	<1	<1	<1	<1

Legend: C57Bl/6 mice were immunized with OA-alum (2 μg i.p.) 5 days before being killed. Spleen cells were cultured in different cell concentrations in the presence of optimal concentration of OA (1 mg/ml). 24 h culture supernatants were tested for IL-4 and IFNγ production and the results shown as mean ± SEM.

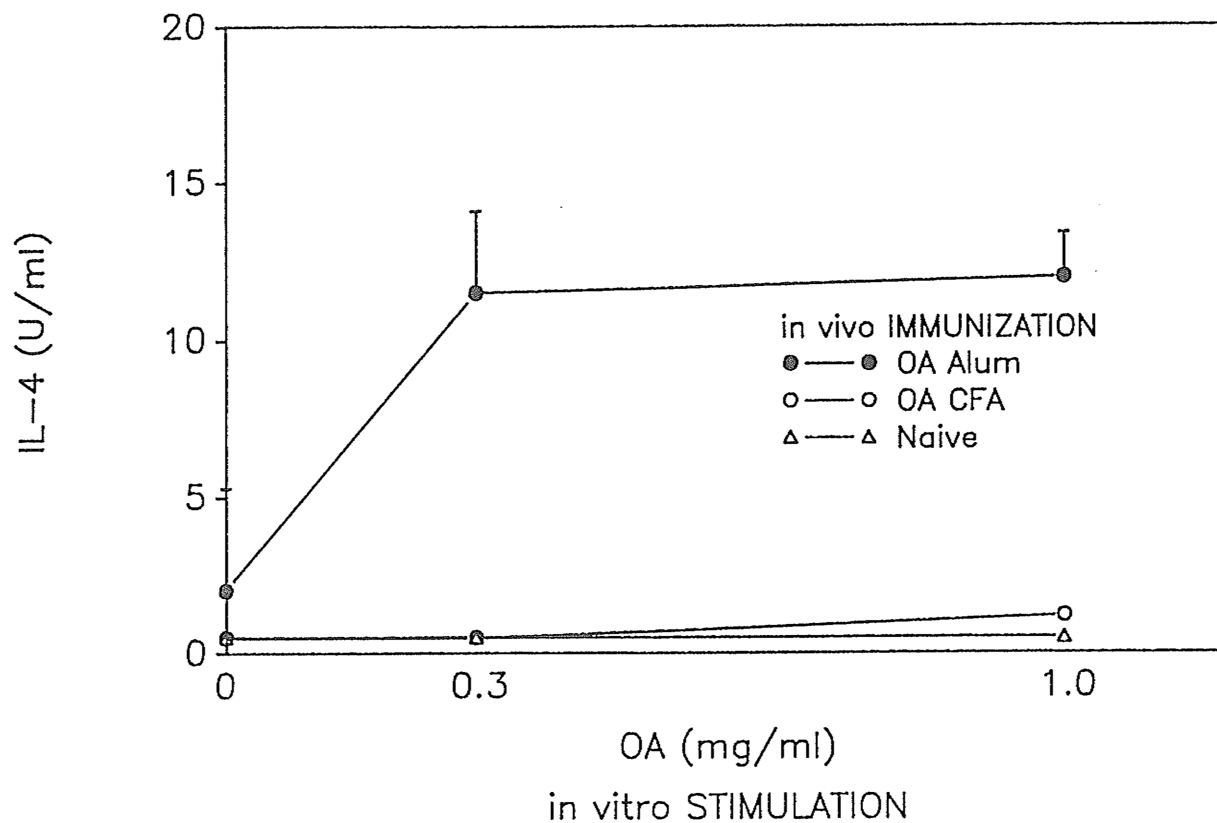

Fig 12: IL-4 production by spleen cell cultures freshly derived from OA immunized mice. Spleen cells from naive, OA-CFA and OA-alum immunized mice killed 5 days post-immunization were cultured in the presence or absence of OA for 24 hours. IL-4 content in the tissue culture supernatants was determined by CT.4S bioassay. Inclusion of purified anti-IL-4 mAb 11B11 reduced CT.4S proliferation to background in all groups.

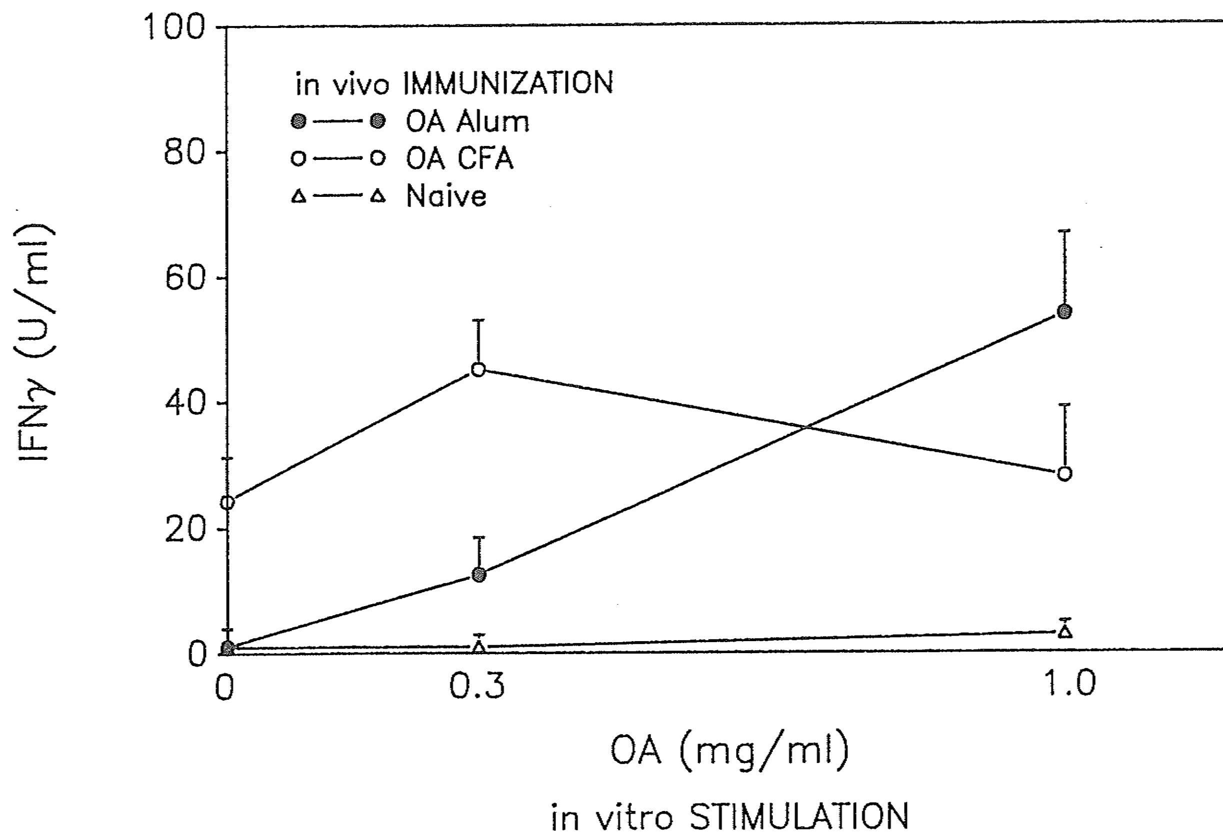

Fig 13: IFNγ production by spleen cell cultures established as described for Fig 12. IFNγ was quantitated with ELISA.

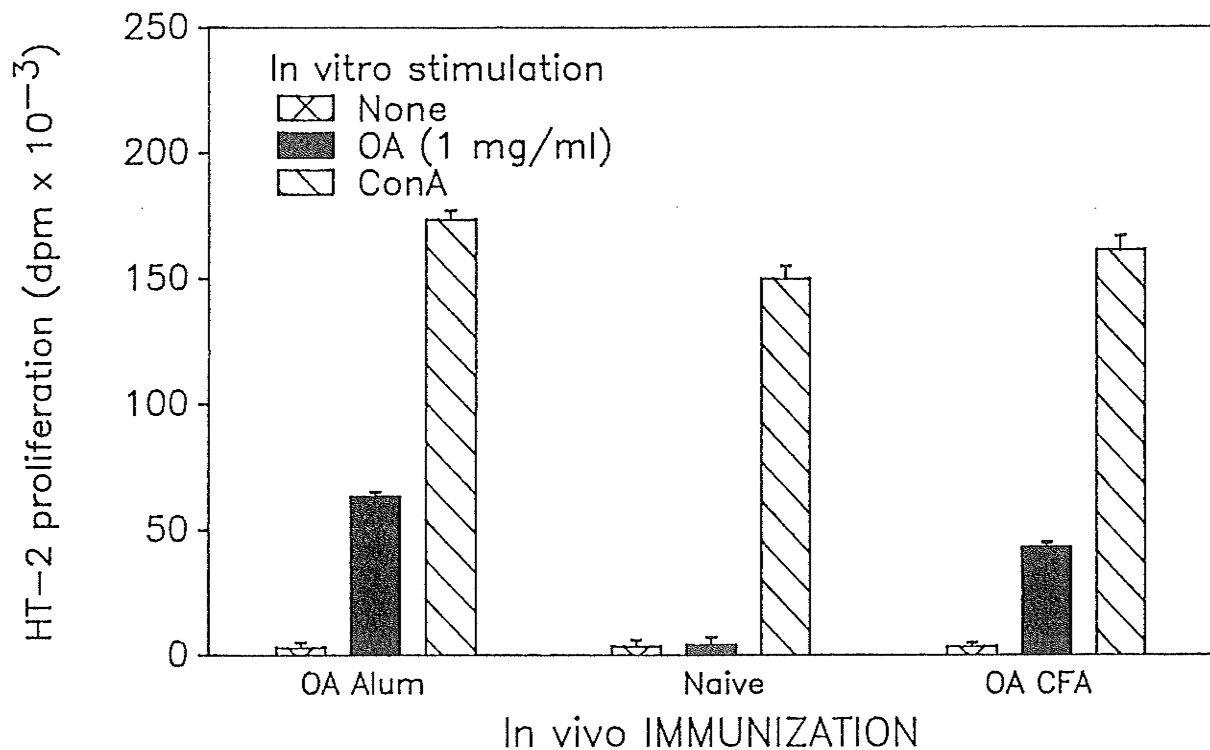

Fig 14: IL-2 production following antigen-specific (OA 1 mg/ml), polyclonal (Con A), or no in vitro restimulation of spleen cells from naive, OA-alum and OA-CFA immunized mice killed 5 days post-immunization, is expressed as mean anti-IL-2/IL-2 receptor mAb neutralizable proliferation of HT-2 cells ± SEM.

2.4 Antigen-specific and polyclonal in vitro activation yield different patterns of cytokine gene expression

Polyclonal in vitro activation represents a widely used strategy for characterization of CD4 T cell cytokine production in vivo. The implicit assumption is that reactivation of antigen primed lymphocytes by antigen or polyclonal activator will elicit no qualitative difference in the results obtained. Consequently, given the simplicity and more intense (i.e. more readily quantifiable) results obtained with this approach, it is widely used. Given our concern that the results obtained with these two strategies may not in fact be equivalent, we directly compared cytokine production following in vitro reactivation using antigen vs polyclonal activation.

In clear contrast to the results obtained following antigen-specific activation (Fig 12 and 13), there are no significant difference in IL-4 responses among naive, OA-alum and OA-CFA-immunized groups following immobilized anti-CD3 and Con A stimulation (Fig 15). Interestingly, although both anti-CD3 and Con A are polyclonal T cell activators, immobilized anti-CD3 seems more efficient in inducing IL-4 gene expression as reflected in higher IL-4 production (Fig 15). This implies that differences in activation pathways may influence cytokine gene expression.

Similar to IL-4, IFNγ responses following polyclonal in vitro activation failed to reflect the differences in cytokine synthesis which were obvious following antigen-specific stimulation (Fig. 13 versus Fig. 15).

Thus, the conclusions which may be drawn about the commitment towards a particular pattern of in vivo cytokine synthesis, based on the data obtained in vitro with "fresh" T cells, may be markedly different when evaluated with polyclonal versus allergen-specific stimulation, a finding which underlines the necessity of using antigen specific signals.

2.5 Allergen-specific IL-4 production in vitro is predominantly CD4 mediated

The results above, obtained with unseparated spleen cell populations, demonstrate that allergen stimulation elicits rapid induction of IL-4 synthesis. Allergen-induced IL-4 versus IFNγ production is quantitatively affected by the adjuvant selected, suggesting that previous failures to observe IL-4 production with "fresh" cells was attributable in large part to the adjuvant (CFA) selected, and that antigen-driven short term culture can be used to characterize cytokine production directly ex vivo.

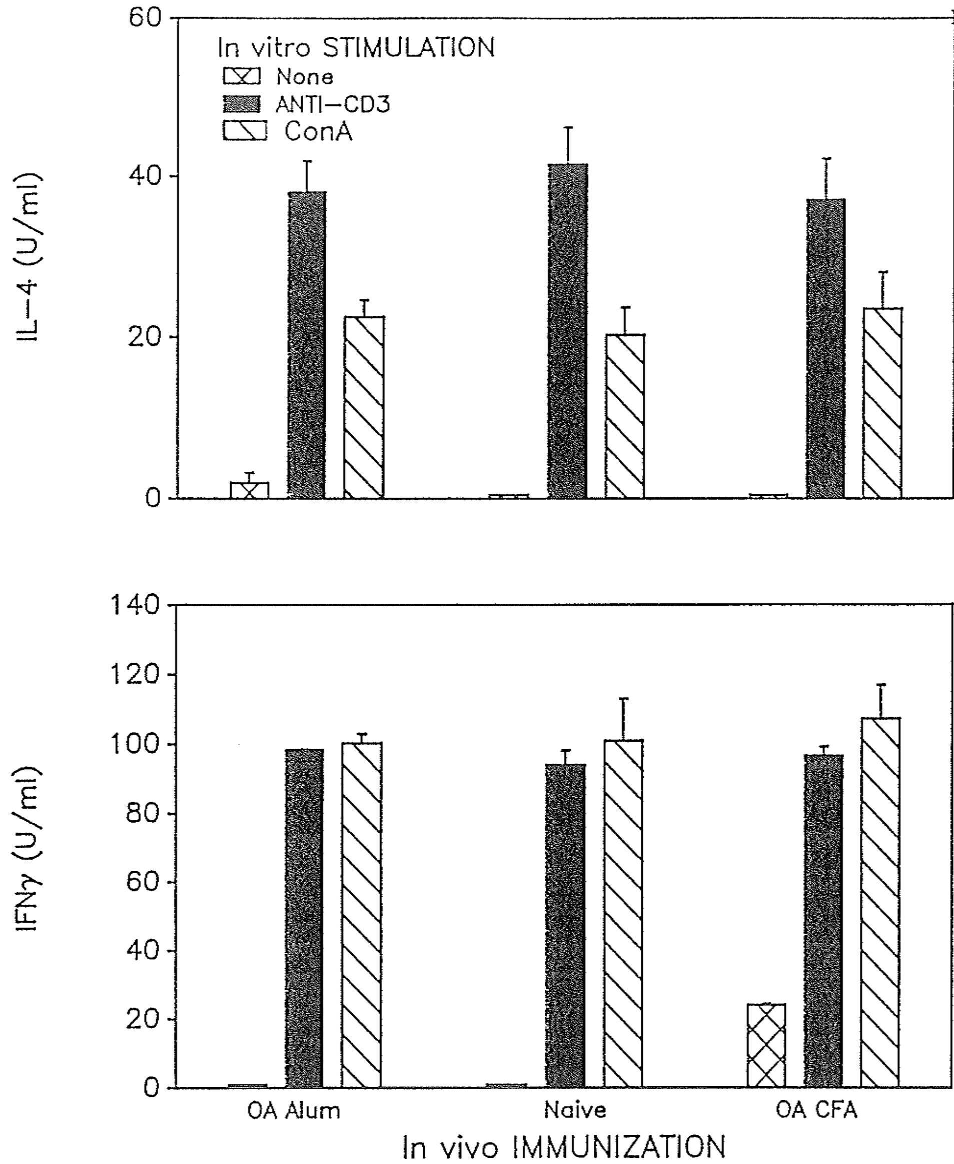

Fig 15: Polyclonal T-cell activation yields equivalent IL-4 and IFNγ responses in spleen cell cultures derived from naive, OA-CFA-and OA-alum immunized mice. Cultures established from the same individual mice as described for Fig 12 were cultured alone, with immobilized anti-CD3 mAb or with Con A (4 μg/ml) as indicated. Tissue culture supernatants obtained 24 h later were evaluated for IL-4 and IFNγ synthesis.

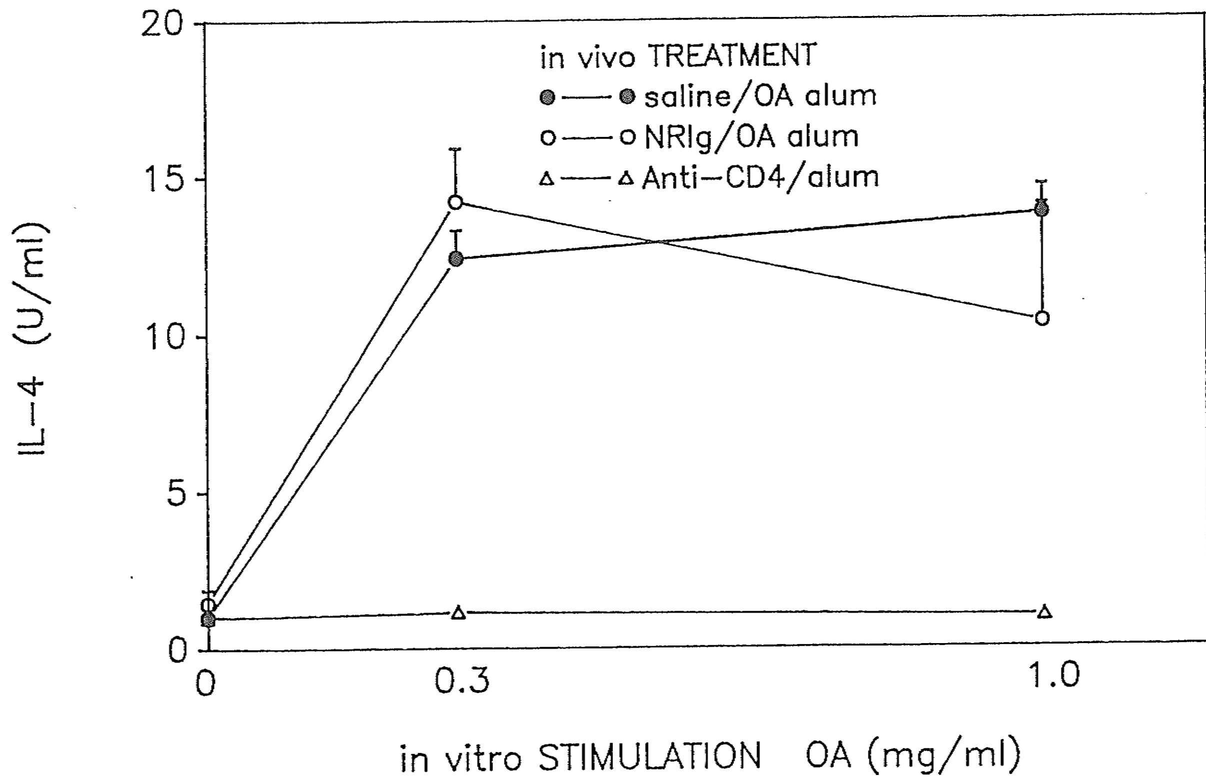

Fig 16: CD4 T cell dependence of IL-4 production by fresh spleen cells from OA-alum-immunized mice. Mice were treated with purified anti-CD4 mAb, normal rat Ig, or saline at d-1 and d1, OA-alum-immunized (d0) and killed (d5). IL-4 production in 24 h culture supernatents were determined by MTT CT.4S assay.

Table 4

Effect of anti-CD4 mAb treatment on T cell population in vivo

In vivo treatment	T cell subset (%)	
	CD4	CD8
Saline	25.96	35.24
N.RIg	24.86	18.46
anti-CD4	0.22	23.66

Legend: Mice were treated with anti-CD4 mAb, normal rat Ig or saline 6 and 4 days before being killed and tested for T cell subsets. Spleen cells were panned with coated sheep anti-mouse IgG to deplete B cells and then stained with FITC conjugated YTS 191 (anti-CD4) or YTS 169 (anti-CD8) mAbs. Percentage of T cell subsets were measured by flow cytometry.

However, several cell types in murine spleen are capable of producing IL-4 including CD4 T cells, CD8 T cells (Kesol, 1988; Seder, 1992), double negative $\alpha\beta$ T cells (Zlotnik, 1992), basophils, and non-B, non-T cell population (Ben-Sasson, 1990; LeGros, 1990; Kuehn, 1991). To exclude the possibility that other cells were responsible for the allergen-induced IL-4 synthesis observed above, we used three approaches to demonstrate that the IL-4 production in this system is CD4 T cell derived.

First, mice were treated with anti-CD4 or normal rat Ig in vivo immediately prior to OA-alum immunization. At death (day 5), CD4 cells constituted <2% of the spleen population in anti-CD4 mAb treated groups while CD4 cells in normal rat Ig treated group were at a level similar to normal control mice as measured by flow cytometry (Table 4). The data in Fig. 16 indicate that the majority of IL-4 production observed following OA-alum immunization was CD4 T cell dependent.

As an alternative approach, anti-CD4, anti-CD8 mAb or normal rat Ig were added to in vitro cultures to block the activity of the corresponding cell subset. As shown in Fig. 17, IL-4 synthesis was blocked in vitro after addition of anti-CD4 mAb, but not anti-CD8 or normal rat IgG, to

allergen-restimulated cultures derived from OA-alum immunized mice.

The dependence of IL-4 synthesis on CD4 T cells does not necessarily mean IL-4 is produced by these cells. Although the likelihood of CD4 cells cooperating in inducing IL-4 production by other cell types over the course of 24-h cultures is likely very limited, we addressed this possibility directly, using flow cytometry to negatively select CD4 cells (CD8-, sIg- but containing double negative T cells and non-B, non-T populations) (Fig. 18A), or to positively select CD4+ populations (Fig. 18B) from spleens of OA-alum-immunized mice 5 days after immunization. The resulting CD4-enriched and CD4-depleted cells were cultured 36 h in the presence of 1 mg/ml OA and irradiated spleen cells from naive mice. The results obtained in each of these approaches demonstrate that after short term, Ag-mediated restimulation of spleen cells obtained from mice shortly after Ag-specific immunization, all detectable IL-4 production was CD4 T cell derived. Using the same approach, Ag-driven IFNγ production was also shown to be CD4 derived (Table 5).

The results in this section clearly demonstrate the capability of CD4 T cells to produce IL-4 directly ex vivo. The reciprocal patterns of antibody and cytokine synthesis

elicited following OA-alum and OA-CFA immunization suggest that the nature of the antigenic stimuli quantitatively determines IL-4 induction in vivo. The difference of cytokine secretion patterns observed following antigen-specific and polyclonal T-cell activation argues that use of polyclonal T-cell activators, which stimulate both naive and primed cells, is likely to obscure changes induced in any given component of the T cell repertoire. Thus, these data strongly suggest that examination of cytokine responses via antigen-specific restimulation elicits weaker but more representative responses than those obtained following polyclonal activation.

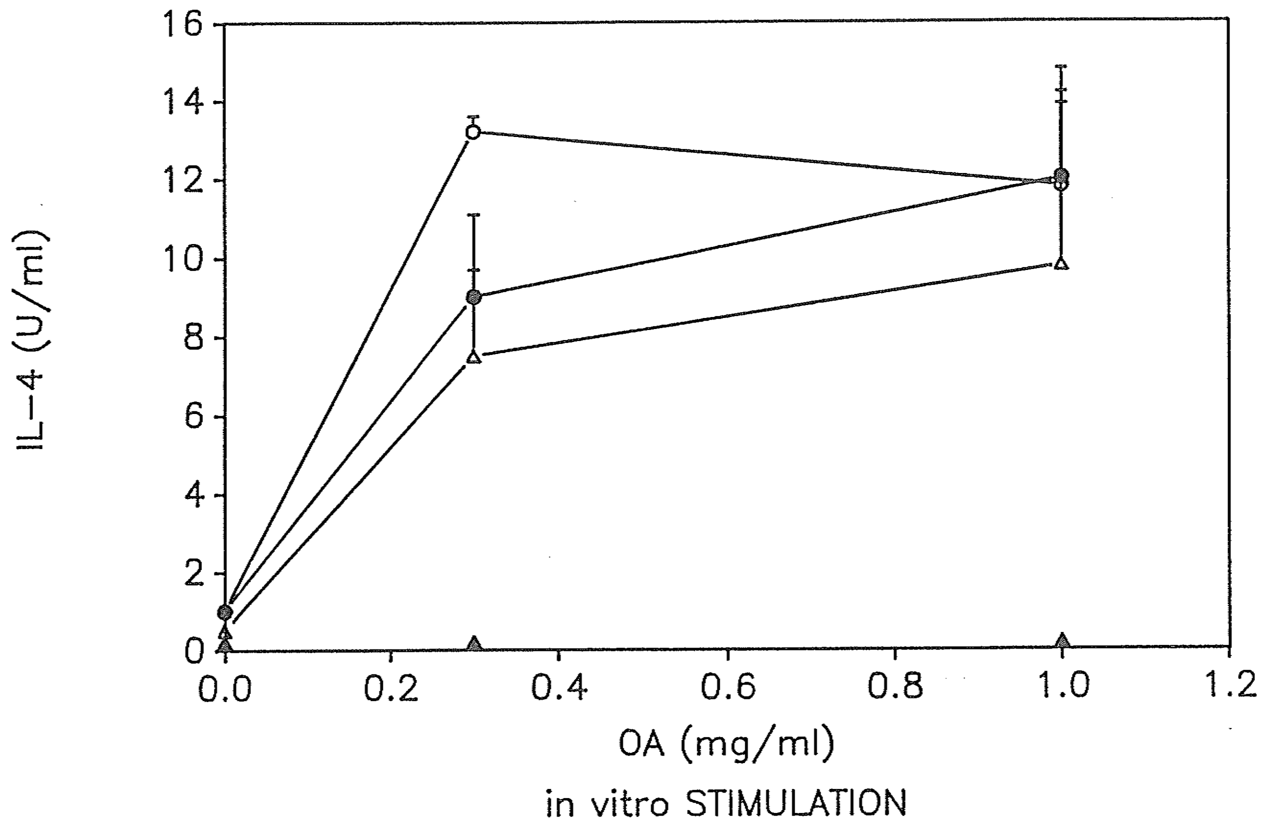

Fig 17: IL-4 synthesis is blocked by anti-CD4 mAb in vitro. Spleen cells from C57Bl/6 mice, OA-alum immunized 5 days prior to sacrifice, were stimulated with OA in the absence (●) or presence of anti-CD4 (▲), anti-CD8 mAb (○) or normal rat Ig (△). Culture supernatants were analyzed for IL-4 production by CT.4S bioassay.

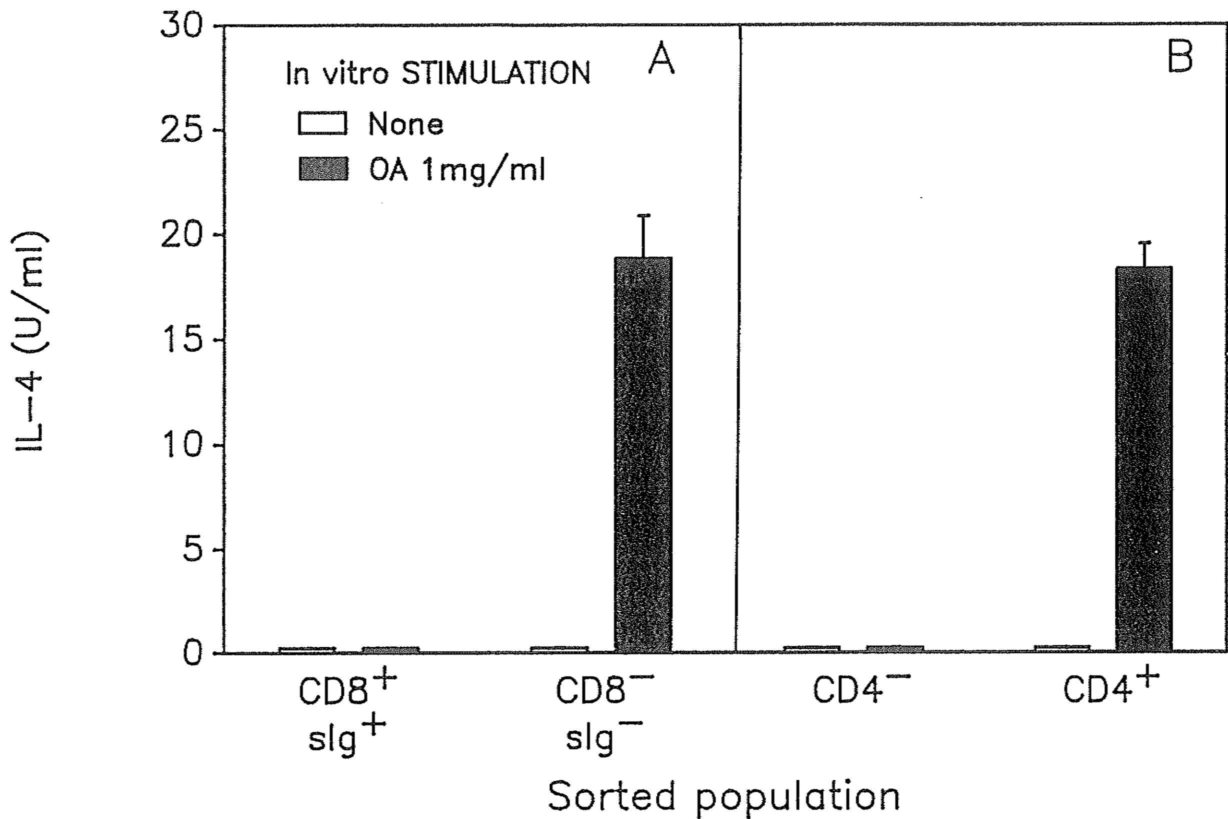

Fig. 18: IL-4 production is CD4 T cell derived in antigen driven responses. Panel A, CD4 enriched populations were obtained from mice identically immunized as Fig. 17, using negative selection by flow cytometry to obtained CD4 enriched and CD8, sIg-enriched populations. Sorted cells were cultured at 3 x 10^6/ml in the presence of 5 x 10^6/ml irradiated spleen cells with or without an optimal concentration of OA. Panel B, CD4 enriched cells obtained by positive selection using fluorescein conjugated anti-CD4 mAb were cultured as described for Panel A. Reanalysis of all sorted populations by flow cytometry demonstrated >97% purity of the selected population. Culture supernatants were analyzed for IL-4 production by CT.4S bioassay ± anti-IL-4 mAb.

Table 5

OA-stimulated IFNγ production is CD4 T cell derived

Cell Cultured	IFNγ (U/ml)
Spleen cells	22.3 ± 2.9
CD4 enriched s.c.	16.0 ± 0.6
CD8 enriched s.c.	<0.5
APC alone	<0.5

Legend: Mice were immunized with 2 μg OA (alum) and killed 5 days later. CD4-enriched or CD8-enriched spleen cell populations were cultured at 6 x 10^6/ml (200 μl/well) in the presence of 1 mg/ml OA and 5 x 10^6/ml irradiated spleen cells as a source of APC. Unseparated spleen cells were cultured at 10^7/ml with OA (1 mg/ml) in the absence of irradiated spleen cells. IFNγ production (± SEM) in culture supernatants was determined using ELISA.

III. DIFFERENTIAL REQUIREMENTS FOR IL-2 IN IL-4 AND IFNγ GENE EXPRESSION IN DEVELOPING AND ESTABLISHED RESPONSES

The demonstration of substantial in vivo IL-4 synthesis by CD4 T cells and antigen-specific IgE responses in OA-alum immunized mice suggests a pivotal role for IL-4 in induction of immediate hypersensitivity. To therapeutically modulate the patterns of cytokine production, and consequently block IgE secretion which is elicited in allergic individuals after exposure to allergens, it is necessary to characterize signals involved in initiating and sustaining of Th1 or Th2-like responses.

IL-4 is secreted by activated T cells, but weakly or not at all by resting T cells. The initial development of IL-4-secreting CD4 T cells from naive murine precursors requires both IL-2 and IL-4 and has been extensively studied using polyclonal T cell activators and 5 to 14-day cultures (Swain 1988; Rocken 1991; Le Gros, 1990; Ben-Sasson, 1990). Most studies carried out after Ag-specific or polyclonal stimulation in vivo indicate that IL-2 is required for induction of IL-4 responses (Powers, 1988; Weinberg, 1990; Seder, 1991). However, it remains unclear whether IL-2 is required for priming of IL-4 responses, expression of IL-4

or both.

Differences between use of polyclonal and antigen-driven activation to stimulate naive versus primed cell populations made it possible for us to dissociate cytokine priming and expression. Therefore, we examined the IL-2 requirement in polyclonal (predominantly de novo activation) and antigen-driven (predominantly recall of established responses in previously immunized mice) IL-4 and IFNγ synthesis.

3.1. Effects of endogenous IL-2 on polyclonally stimulated IL-4 and IFNγ production

To examine the role played by IL-2 in the synthesis of IL-4 by T cells in de novo and established responses, spleen cells obtained from naive mice and those immunized with OA-alum 5 days prior to sacrifice were compared in their capacity to generate IL-4 and IFNγ responses following in vitro restimulation with immobilized anti-CD3 mAb in the presence or absence of anti-IL-2/IL-2R mAb.

In the absence of in vitro stimulation, IL-4 and IFNγ responses by naive cells was undetectable, whereas <0.5 to 4.5 U/ml cytokine production was seen in unstimulated cultures derived from mice previously immunized with OA

(alum) in vivo (Fig. 19). Polyclonal in vitro stimulation resulted in intense IL-4 and IFNγ responses in all groups. It should be noted that prior in vivo immunization did not affect the intensity of polyclonally elicited IL-4 or IFNγ responses.

Consistent with the results of polyclonal activation in previous reports (Swain, 1988; Powers, 1988; Hayakawa, 1989), the bulk of this IL-4 production was IL-2 dependent (Fig. 19A). IFNγ production by spleen cells of naive or immunized mice was also found to be highly susceptible to the presence of anti-IL-2/IL-2R mAb over the course of polyclonal in vitro stimulation. (Fig. 19B).

3.2 Effects of endogenous IL-2 on Ag-stimulated IL-4 and IFNγ synthesis

To dissociate requirements for IL-2 in the initial activation of T cells from a putative requirement for IL-2 in antigen-driven IL-4 or IFNγ synthesis by primed cells, cultures were established using spleen cells obtained from mice immunized with OA-alum 5 days before being killed. Cytokine production was evaluated in culture supernatants obtained following antigen-specific stimulation in the presence or absence of IL-2/IL-2R mAb.

Antigen-mediated in vitro restimulation elicited pronounced IL-4 (Fig. 20) and IFNγ (Fig. 21) responses, although weaker than those after polyclonal stimulation (Fig. 19). Significantly, coculture with Ag and IL-2/IL-2R mAb to block endogenous IL-2 production was without effect on IL-4 synthesis but virtually abrogated IFNγ synthesis. These results demonstrate the independence of established IL-4 responses from IL-2 and identify differential requirements for IL-4 and IFNγ gene expression in established responses.

Because the demonstration that endogenous IL-2 activity in these cultures was effectively blocked by coculture in the presence of the anti-IL-2/IL-2R mAb cocktail used is critical to the interpretation of these data, IL-2 levels were directly determined (Table 6). As can be seen, the presence of this mAb cocktail over the course of in vitro antigen-mediated or polyclonal restimulation effectively neutralized IL-2, whereas culture in the presence of irrelevant antibodies was without effect.

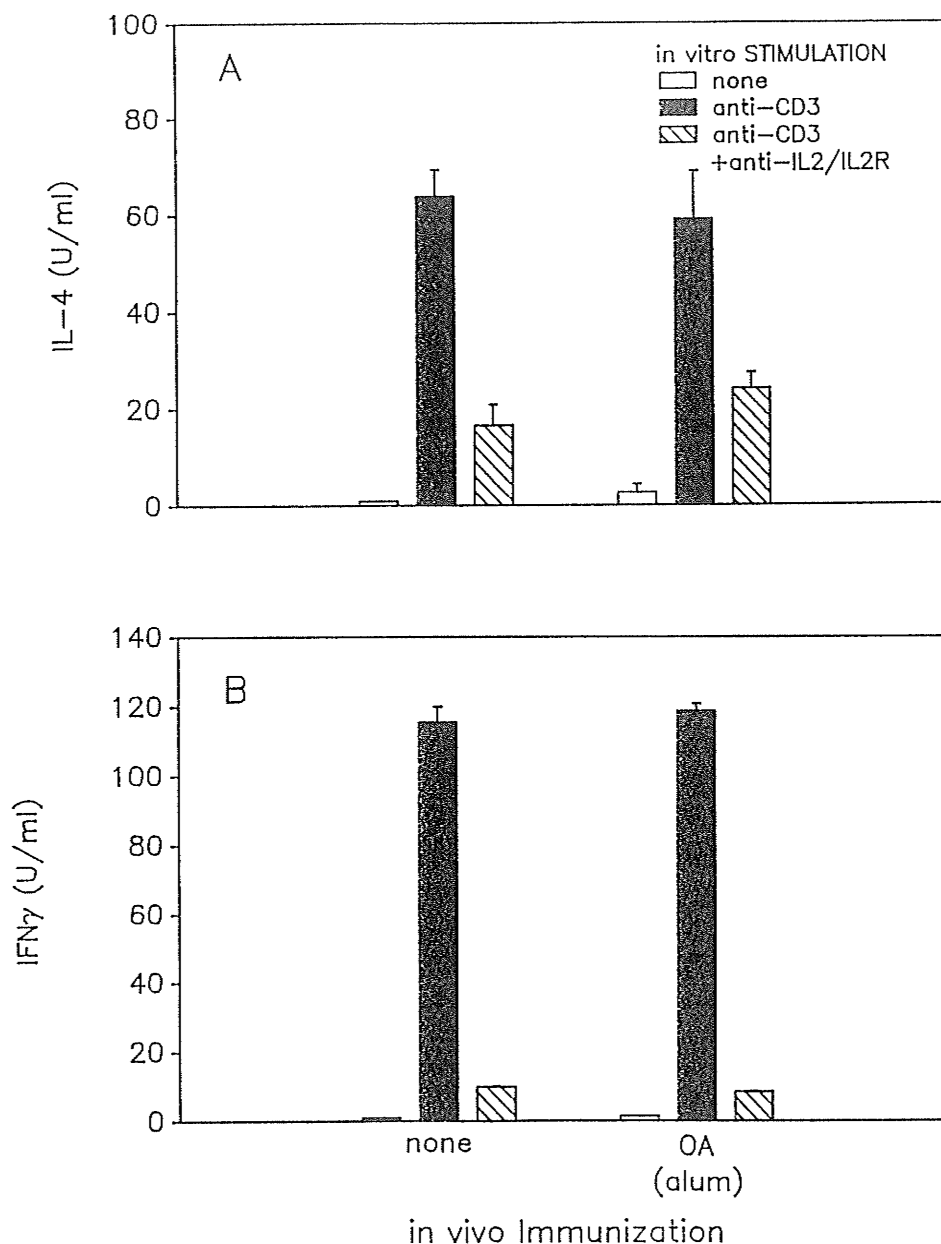

Fig 19: Addition of anti-IL-2/IL-2R mAb blocks most polyclonally stimulated IL-4 and IFNγ production by spleen cells of naive and antigen primed mice. Spleen cells were obtained from naive, OA (alum) immunized mice 5 days post-immunization and cultured for 24 hours alone, in the presence of immobilized anti-CD3 mAb or anti-CD3 and anti-IL2/IL2 receptor mAb cocktail S4B6/7D4.

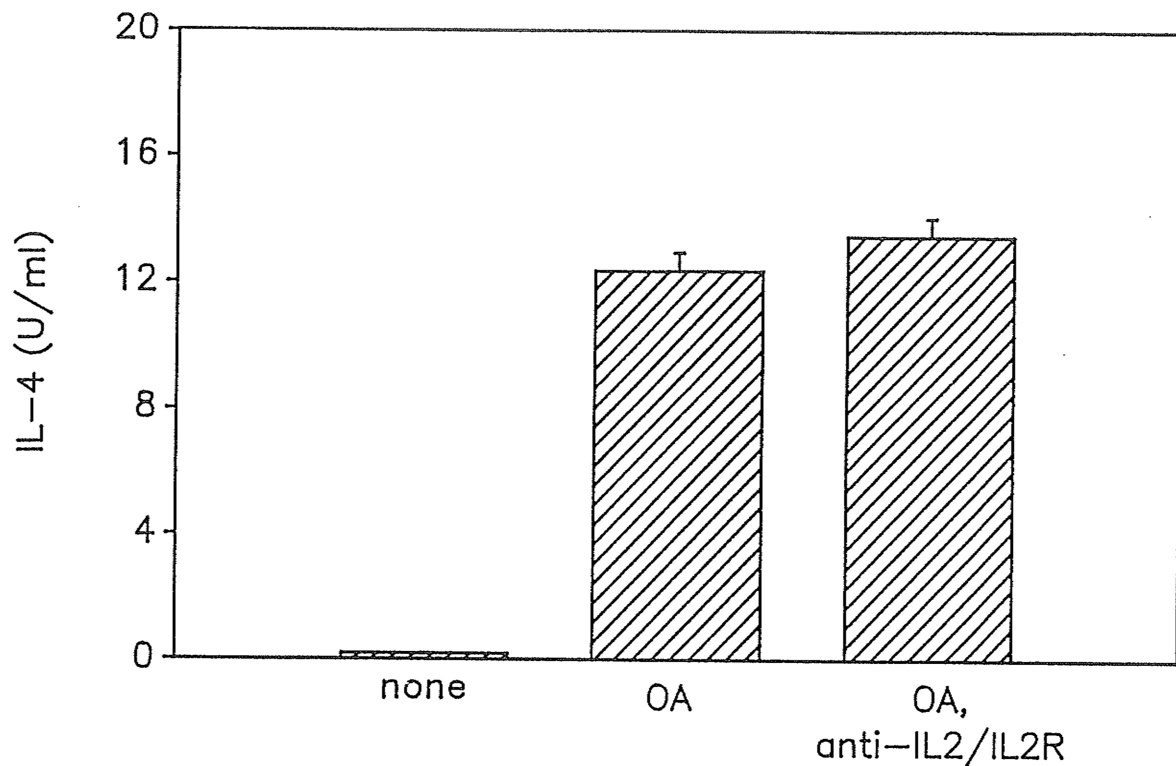

Fig 20: Antigen driven IL-4 synthesis of spleen cells from allergen immunized animals is IL-2 independent. Spleen cells from mice immunized with OVA (alum) five days prior to sacrifice exhibit equivalent IL-4 response in the presence or absence of anti-IL2/IL-2R mAbs in culture.

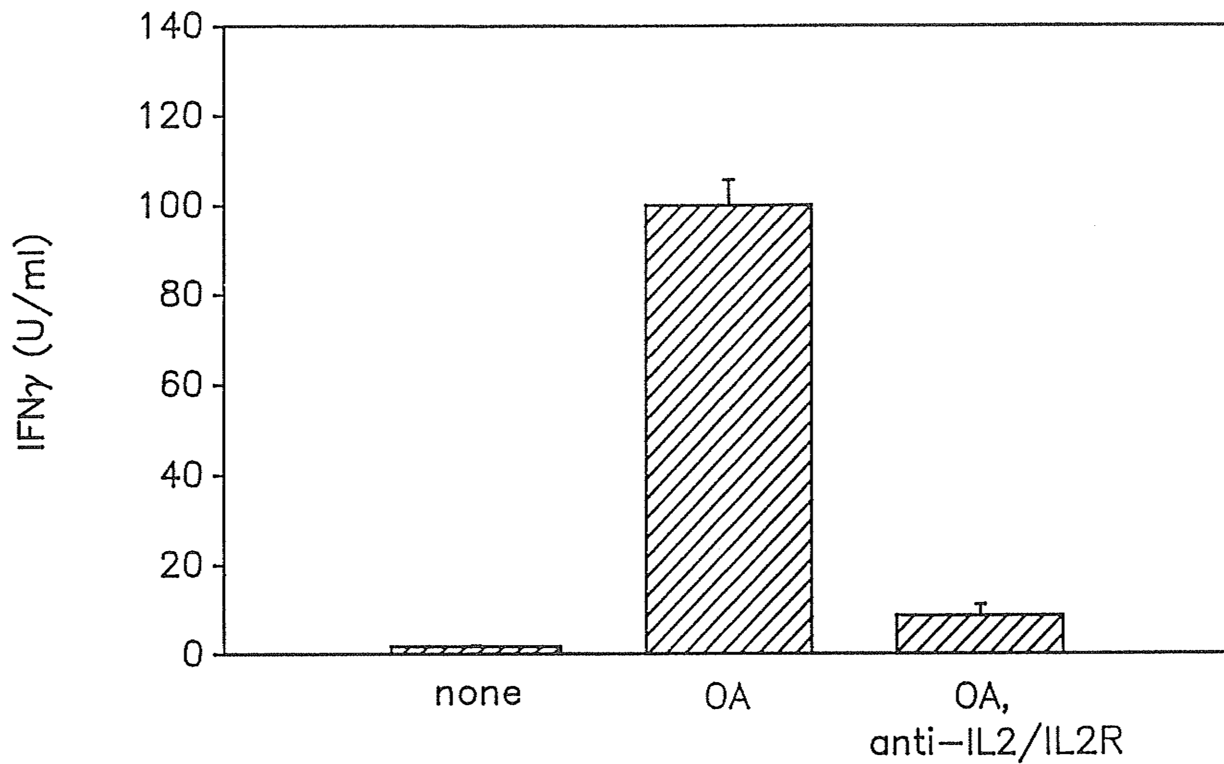

Fig. 21: IFNγ synthesis following antigen specific restimulation of antigen primed spleen cells is strictly IL-2 dependent ($p < 0.001$). Supernatants from cultures derived in Fig. 20 were used.

3.3 Effects of exogenous IL-2 on IL-4 and IFNγ synthesis following polyclonal and antigen-specific stimulation

An alternative approach used to examine the effects of IL-2 on IL-4 and IFNγ synthesis was to determine the effect of supplementing such cultures with rIL-2 (20 U/ml). Moderate increases in IFNγ production (20-30%) were observed following antigen-specific or polyclonal stimulation upon the addition of rIL-2 (Fig. 22). In contrast, addition of rIL-2 failed to alter IL-4 production (Fig. 22). These results support the dependence of IFNγ, and independence of IL-4 synthesis, on IL-2.

3.4. IL-2 independence of long established IL-4 synthesis

These studies were extended by examination of the relative IL-2 dependence of IL-4 production in long established, multiply immunized hypersensitivity responses. In contrast to peak primary OA-specific IgE responses of PCA titre 400 to 800 typically observed 10 days after initial immunization of C57BL/6 mice, OA-specific serum IgE production in multiply immunized mice under the conditions used in Table 7 ranged from 2560 to 4000 (PCA titre).

Using this model system, IL-4 and IL-2 synthesis by spleen cells were examined in the presence and absence of anti-IL-

2/IL-2R mAb (Table 7). As demonstrated earlier (Fig. 9), IL-4 and IFNγ gene expression are highly transient after antigenic stimulation. Cell culture $\geq$ 10 days after immunization (i.e., Table 7, group C) results in very low IL-4 production. Most important is the finding that IL-4 synthesis recalled by reexposure to specific Ag seven months after initial immunization is essentially independent of the presence of anti-IL-2/IL-2R mAb in vitro (group D).

The results in these studies suggest that IL-4 response could be divide into two stages in terms of IL-2 requirement, i.e. IL-2 dependent (initial activation, clonal expansion) and IL-2-independent stages (production and release of biologically active IL-4). However, IFNγ synthesis in de novo and established responses is strongly IL-2 dependent. The relative IL-2 independence of established IL-4 responses may be important for maintenance of hypersensitivity in individuals with ongoing IgE responses.

Table 6

IL-2 production after antigenic or polyclonal in vitro stimulation is neutralized by incorporation of an anti-IL-2/IL-2R mAb cocktail

In Vitro Stimulus[a]	HT-2 Proliferation (dpm × 10^{-3})[b]		
	Expt. 1	Expt. 2	Expt. 3
None	0.75	2.2	1.5
None + anti-IL-2/IL-2R	1.7	1.9	2.3
OVA	31.6	42.1	58.7
OVA + anti-IL-2/IL-2R	1.3	2.8	2.1
OVA + J11d.2	29.1	46.7	ND
OVA + NRIgG[c]	29.4	40.5	ND
Anti-CD3	71.0	94.8	81.5
Anti-CD3 + anti-IL-2/IL-2R	2.4	5.6	2.7
Anti-CD3 + J11d.2	75.9	81.2	ND
Anti-CD3 + NRIgG[c]	68.2	88.8	ND

[a] Tissue culture supernatants were harvested 14 to 16 h after establishing spleen cell cultures from OVA-immunized mice as described for Figure 2.

[b] HT-2 [^{3}H]TdR incorporation was determined as described in *Materials and Methods*. SEM, 5 to 15%, are omitted for clarity. Three experiments, representative of six carried out, are shown.

[c] Normal rat IgG.

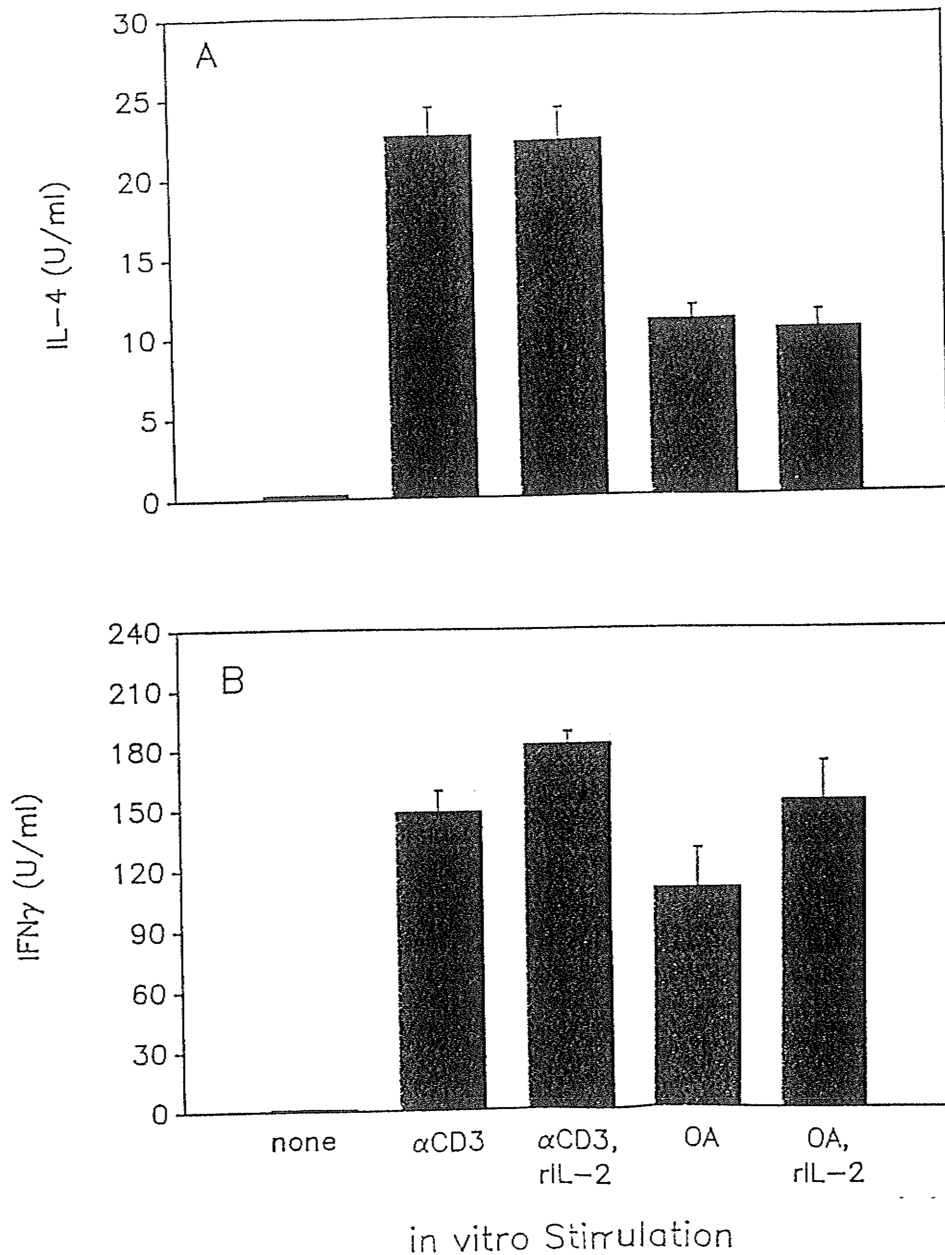

Fig. 22. addition of exogenous rIL-2 fails to increase antigen or polyclonally driven IL-4 synthesis but increases IFNγ synthesis. Spleen cells obtained from C57Bl/6 mice immunized with OA (alum) five days prior to sacrifice were restimulated in vitro by immobilized anti-CD3 mAb or OA (1 mg/ml) in the presence or absence of rIL-2 (20 U/ml).

Table 7

IL-2 independence of IL-4 gene expression in well established hypersensitivity responses

Group	Donor	Immunization[a] (Days)	Day Killed	IL-4 (U/ml)[c]: In Vitro Restimulation			IL-2 (dpm × 10^{-3})[c]: In Vitro Restimulation		
				None	OVA	OVA+a-IL-2/IL-2R	None	OVA	OVA+a-IL-2/IL-2R
A	Naive		5	<0.5	<0.5	ND	ND	ND	ND
B	OVA	0	5	0.7	13.0	11.9	2.7	58.9	3.0
C	OVA	0	21	1.3	2.2	2.0	3.0	9.6	2.6
D	OVA	0, 210	215	1.4	53.1	44.9	3.3	31.9	1.8

[a] Mice were immunized with 2 μg OVA (alum) at the indicated days, with group D given a booster immunization on day 210.
[b] Mice were killed and overnight in vitro cultures established at the days indicated.
[c] IL-4 and IL-2 cytokine levels in 14- to 16-h tissue culture supernatants were determined as described in *Materials and Methods*. Data obtained from one of two similar experiments is presented.

IV ALLERGEN-SPECIFIC MODULATION OF CYTOKINE SYNTHESIS PATTERNS AND IgE RESPONSES IN VIVO WITH CHEMICALLY MODIFIED ALLERGEN

Administration of chemically modified allergen (OA-POL) inhibits antigen-specific IgE responses in both de novo and established responses concurrently with IgG_{2a} elevation (HayGlass, 1991a, 1991b). The reciprocal change of IgE/IgG_{2a} responses induced by OA-POL, and the reversal of this change by systemic treatment with anti-IFNγ suggest a predominant Th1-like cell induction following OA-POL treatment. To determine the differences in cytokine producing patterns induced by native and modified allergen and their relationship with antibody isotype selection in vivo, we examined cytokine synthesis patterns following (1) initial exposure to native or modified allergen in the absence of any adjuvants; (2) native and modified allergen pretreatment and subsequent immunization with OA (alum) under conditions established as inductive for strong IL-4 and IgE responses, and (3) OA (alum) immunization followed by subsequent native or modified allergen adminstration.

4.1. Chemically modified, but not native allergen, elicits Th1-like cytokine synthesis patterns in vivo

As different adjuvants qualitatively influence the type of

response induced (Warren, 1992), we initially examined the pattern of cytokine synthesis in mice exposed to native and modified OA in the absence of adjuvants. Mice were given a course of OA or OA-POL treatment (three 80 μg i.p. injections at d0, 2, 4) and killed at d5, 8, 10, or 12. As shown in Fig. 23, IL-2, IL-4, IL-10 and IFNγ synthesis were readily detectable following short term culture of spleen cells in the presence of an optimal concentration of OA (1 mg/ml). Tissue culture supernatants from cultures without antigen added typically showed no detectable cytokine production. Cytokine synthesis kinetics following these treatments were similar to those of OA alum immunization, with peak at d8. Therefore all the cytokine synthesis profile examination in this section were carried out 8 days after first injection.

As can be seen in Fig. 24, in the absence of adjuvant, OA elicited responses not dominated by Th1-like or Th2-like cytokine gene expression pattern, as demonstrated by production of similar levels of IFNγ and IL-4/IL-10 and ratios of IFNγ:IL-4 and IFNγ:IL-10 synthesis of 1.89 ± 0.44 and 1.49 ± 0.53 respectively (Fig. 25).

In marked contrast, as shown in Fig. 24, cultures established from mice administered OA-POL under the same conditions exhibit reduced IL-4 and IL-10 synthesis (mean

of six and four experiments: 6.1-fold and 5.2-fold reductions respectively relative to levels
observed following in vivo administration of unmodified OA). Concomitantly, IFNγ production was elevated by an average 2.6 fold in the cultures established using cells from OA-POL treated groups (mean of six experiments).

Collectively, the ratio of antigen stimulated IFNγ:IL-4 or IFNγ:IL-10 synthesis in groups given OA-POL was increased some 20-fold relative to that seen in cultures derived from groups given unmodified antigen (Fig. 25), providing direct evidence of preferential induction of a Th1 dominated cytokine responses, at the population level, amongst OA reactive T cells following administration of appropriately modified allergen.

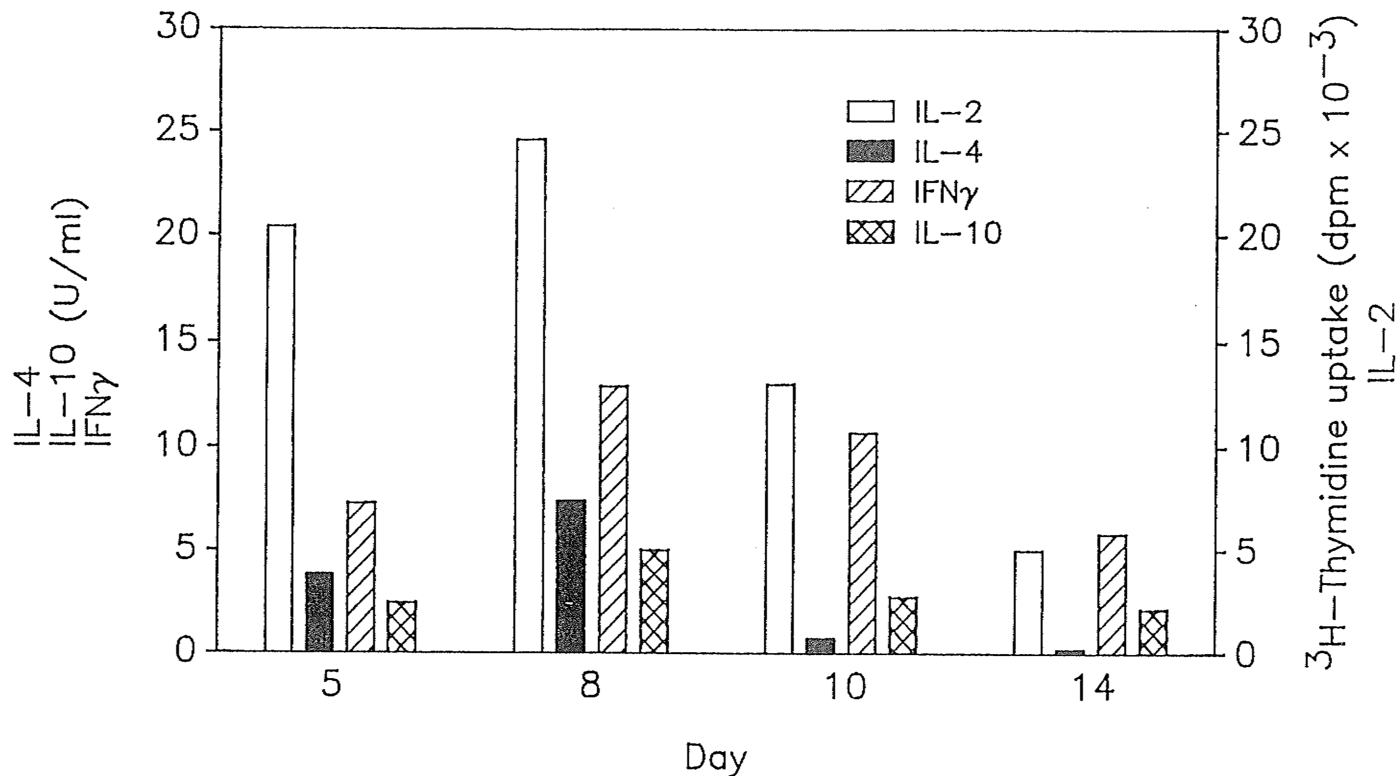

Fig 23: Kinetics of cytokine synthesis induction following native OA treatment without adjuvant in vivo. Mice were treated with three i.p. injections (d0, d2, d4) of 80 μg unmodified OA in the absence of adjuvant and killed at different times. Spleen cells were cultured with OA (1 mg/ml) for 24 - 48 hours. 24 h culture supernatants were tested for IL-2 (HT-2 assay), IL-4 (MTT CT.4S assay) and IFNγ (ELISA) and 48 h supernatants were measured for IL-10 production using ELISA. IL-2 is presented as ^{3}H-thymidine uptake, other cytokines as U/ml calibrated against standards described in Materials and Methods.

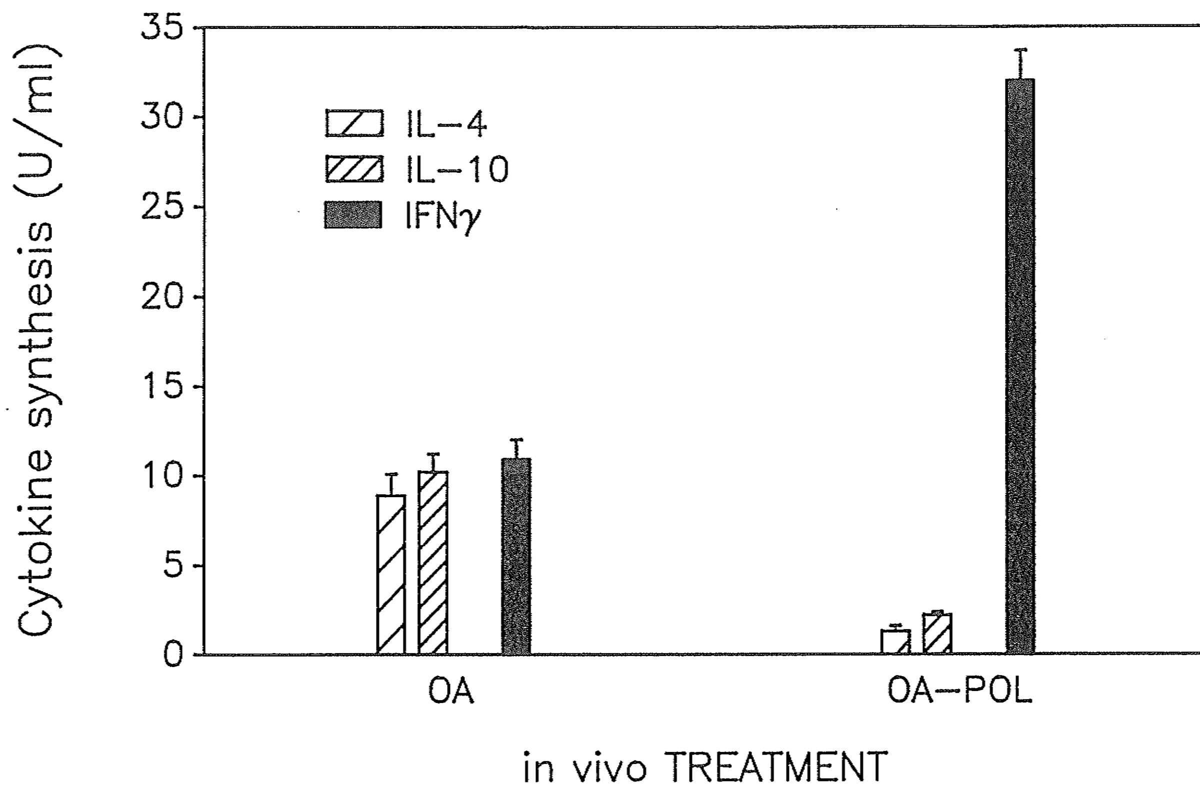

Fig 24: In vitro production of Th1:Th2 associated cytokines following in vivo administration of native or chemically modified OA. Mice were treated with one course of native or modified OA (three 80 μg i.p. injections at d0, 2, 4) and killed at d8. Spleen cells were stimulated in vitro with OA (1 mg/ml) and tissue culture supernatants were measured for cytokine synthesis using same method as Fig 23.

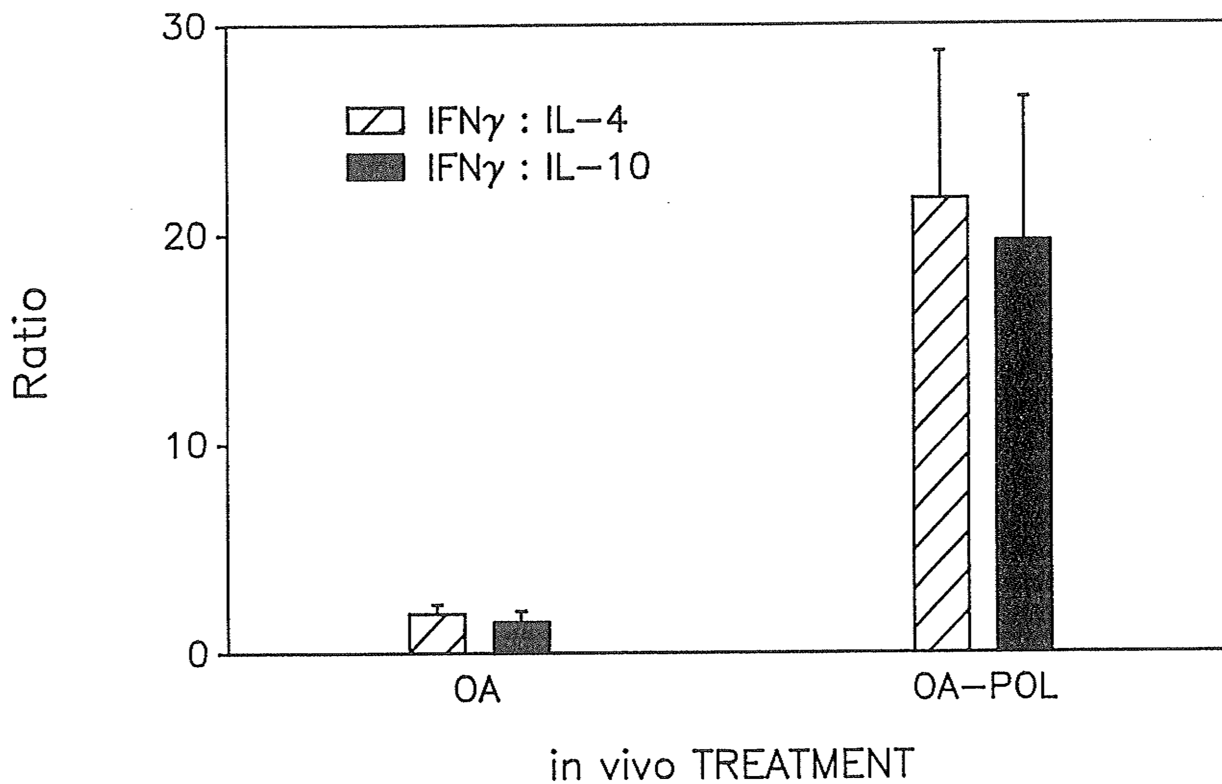

Fig 25: Ratio of Th1:Th2 associated cytokines following native or modified allergen treatment. Cytokines measured in Fig 24 are expressed as the ratio of IFNγ:IL-4 (U/ml:U/ml) and IFNγ:IL-10 (U/ml:U/ml).

The detection of similar levels of antigen-driven IL-2 synthesis in cultures derived from both unmodified and modified OA treated animals supports the role played by IL-2 in the initiation and maintenance of IFNγ synthesis (Table 8). Similarly, the levels of IL-2 production induced by administering either unmodified or modified OA (Fig. 18) may be attributed to the requirement for at least a small amount of IL-2 for IFNγ induction, and gene expression. Therefore, preferential Th1-like responses may not routinely be associated with increased IL-2 synthesis. Indeed, recent studies have demonstrated that IL-2, in many cases, was not a distinguishing cytokine for Th1 responses. Moreover, Th1 and Th2 cells may differentiate from a common precursor which produces IL-2 (Fitch, 1993).

4.2. Concurrent anti-IFNγ mAb and OA-POL administration reverse OA-POL induced Th1 dominant cytokine secretion pattern

Recent studies suggest that the nature of cytokines present in the local environment at the time of initial T cell activation strongly influences commitment to the particular pattern of cytokine synthesis (Swain, 1990; Le Gros, 1990; Gajewski, 1988). The preferential IFNγ synthesis by OA-POL treated mice suggested to us that early induction of IFNγ production may drive commitment of OA specific responses by

native T cells towards a Th1-like pattern of cytokine gene expression. To better characterize the dependency of this commitment on in vivo IFNγ synthesis elicited at the time of initial antigen exposure, mice were treated with unmodified OA or OA-POL (three 80 μg i.p. injections at d 0, 2, 4) concurrent with anti-IFNγ mAb. Animals were killed at d8 and cytokine synthesis patterns were evaluated via short term in vitro culture in the presence of OA (1 mg/ml).

As demonstrated in Fig. 26, concurrent in vivo administration of anti-IFNγ mAb and OA-POL resulted in marked increases in IL-4 and IL-10 production and decreased IFNγ production. This resulted in a reversal from a mean 8:1 ratio of IFNγ:IL-4/10 synthesis in OA-POL treated groups to a mean ratio of 0.26 following concurrent OA-POL/anti-IFNγ treatment.

Concurrent anti-IFNγ/OA administration resulted in a similar polarization of the response towards a Th2 dominated pattern of cytokine synthesis (Fig. 26). Of interest was the observation that in vivo treatment with anti-IFNγ treatment and OA or OA-POL led to similar increases in IL-4 and IL-10 synthesis even in the absence of antigen in vitro. Collectively, these data support the hypothesis that the balance of cytokine production which is

elicited in the very early stages of antigen stimulation in vivo can play a pivotal role in determining the nature of the T cell
response which ultimately comes to dominate the mature cytokine and effector responses to that antigen.

Table 8

IL-2 synthesis induced by native or modified OA exposure

in vivo Treatment	HT-2 Proliferation (dpm x 10^{-3})		
	Expt.1	Expt.2	Expt.3
OA	8.3	12.2	15.6
OA-POL	25.6	10.3	11.1

Legend: Mice were treated with one course of native or modified OA (three 80 µg i.p. injections at do, 2, 4) in the absence of adjuvants and killed at d8. Spleen cells were stimulated with OA (1 mg/ml) in in vitro cultures. Tissue culture supernatants harvested at 24 h were measured for IL-2 synthesis by HT-2 assay.

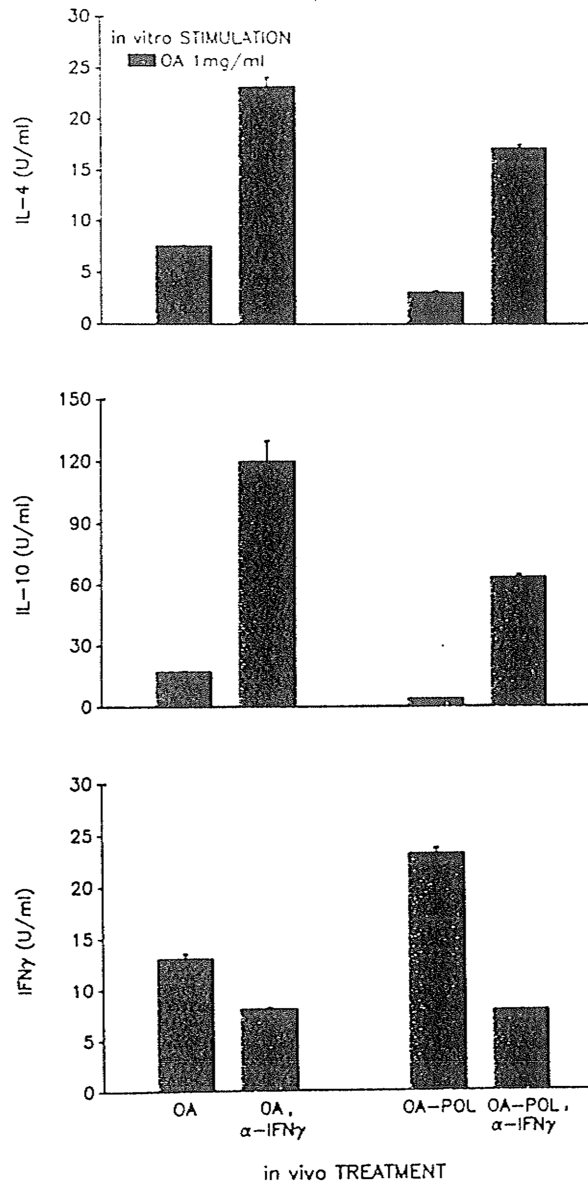

Fig 26: In vivo administration of anti-IFNγ mAb concurrent with OA or OA-POL treatment leads to induction of Th2 dominated responses. Mice were treated in the same manner as Fig 24 except that anti-IFNγ mAb was concurrently injected starting one day before antigen treatment and continuing until three days before mice were sacrificed. Spleen cell culture and cytokine measurements are the same as in Fig 24.

4.3. OA-POL induced predominant Th1 responses are not altered by subsequent OA-alum immunizations.

The demonstration of different cytokine producing patterns elicited by unmodified and modified allergen in the absence of adjuvant strongly supports the hypothesis that the nature of the antigen can determine the pattern of immune responses. It is encouraging that a Th1-like cytokine pattern could be intentionally selected by chemical modification of a potent allergen. However, whether this commitment to a given pattern is easily changeable or relatively "fixed" is a more challenging question in the evaluation of this achievement. As shown in section II, alum is a potent inducer for IgE responses, and OA-alum immunization induced readily detectable IL-4 synthesis and OA-specific IgE response in vivo. We, therefore, examined cytokine patterns induced by OA-alum immunization following native OA or OA-POL or saline pretreatment. The effect of OA-POL pretreatment on OA-alum induced cytokine and antibody responses was compared with that of native OA.

Mice were pretreated with a course of OA or OA-POL (three i.p. 80 μg injections in saline) 2 weeks before primary OA-alum immunization. Cytokine synthesis patterns and the antibody profile were examined. After OA-alum immunization,

some mice were killed at d5 for measuring cytokine production, the others were bled d7, d10, and d14 day.

As can be seen in Table 9, IL-4 synthesis following OA-alum immunization (as assessed by in vitro restimulation) was markedly decreased (3.9 vs 13.0, 70% decrease) in OA-POL pretreated mice compared to saline treated controls. In contrast, OA-POL pretreatment increased IFNγ synthesis in some cases following subsequent OA-alum immunization. Native OA pretreatment, which decreased IL-4 synthesis to some extent (6.2 vs 13.0, 50% decrease), also decreased (40%) IFNγ synthesis in most cases. This difference in cytokine synthesis following administration of native and modified antigen was more apparent when IFNγ:IL-4 ratios were compared (Fig. 27). Pretreatment with OA-POL increased the ratio compared with saline pretreated controls (48.1 $\pm$ 19.2 vs 10.1 $\pm$ 2.1). However, the ratio in native OA pretreated group was not significantly different from the control (8.5 $\pm$ 1.9 vs 10.1 $\pm$ 2.1).

The putative correlation between cytokine modulation and antibody production was then examined by comparison of levels of cytokine synthesis with antibody secretion. Fig. 28 demonstrates the reciprocal regulation of OA-alum induced antigen-specific IgE and IgG_{2a} synthesis in vivo following modified OA pretreatment in one of the

experiments shown in Table 9. OA-specific IgE responses in OA-POL pretreated animals were suppressed >90% while IgG_{2a} synthesis exhibited $>10^3$-fold increases. Adminstration of unmodified OA under the same conditions neither inhibited IgE nor enhanced IgG_{2a} synthesis but did induce accelerated primary IgE responses.

Table 9

Effect of Native And Modified OA Pretreatment on OA-alum Induced Cytokine Synthesis

Expt.	In vivo pretreatment	In vitro cytokine synthesis: IL-4 (SEM) U/ml	IFNγ (SEM) U/ml
1	Saline	6.60 (1.10)	13.50 (0.10)
	OA	5.65 (0.85)	16.70 (0.20)
	OA-POL	1.80 (0.30)	66.80 (2.30)
2	Saline	13.60 (0.01)	90.10 (0.70)
	OA	9.00 (0.05)	79.20 (0.70)
	OA-POL	4.20 (0.60)	61.05 (0.35)
3	Saline	18.90 (4.80)	3.30 (0.50)
	OA	3.95 (0.05)	0.55 (0.01)
	OA-POL	7.20 (0.70)	14.35 (1.35)
4	Saline	12.80 (0.80)	96.40 (1.10)
	OA	6.05 (1.25)	67.10 (1.70)
	OA-POL	2.65 (0.15)	96.40 (1.10)

Table 9 legend: C57Bl/6 mice were treated with one course of native OA or OA-POL (three 80 μg i.p. injections at d-14, -12, -10) or saline alone before given OA-alum immunization (2 μg at d0). Mice were killed on d5 and spleen cells were cultured at 7.5×10^6 cell/ml in the presence of OA (1 mg/ml). Culture supernatants were harvested at 24 h and IL-4 and IFNγ synthesis were measured.

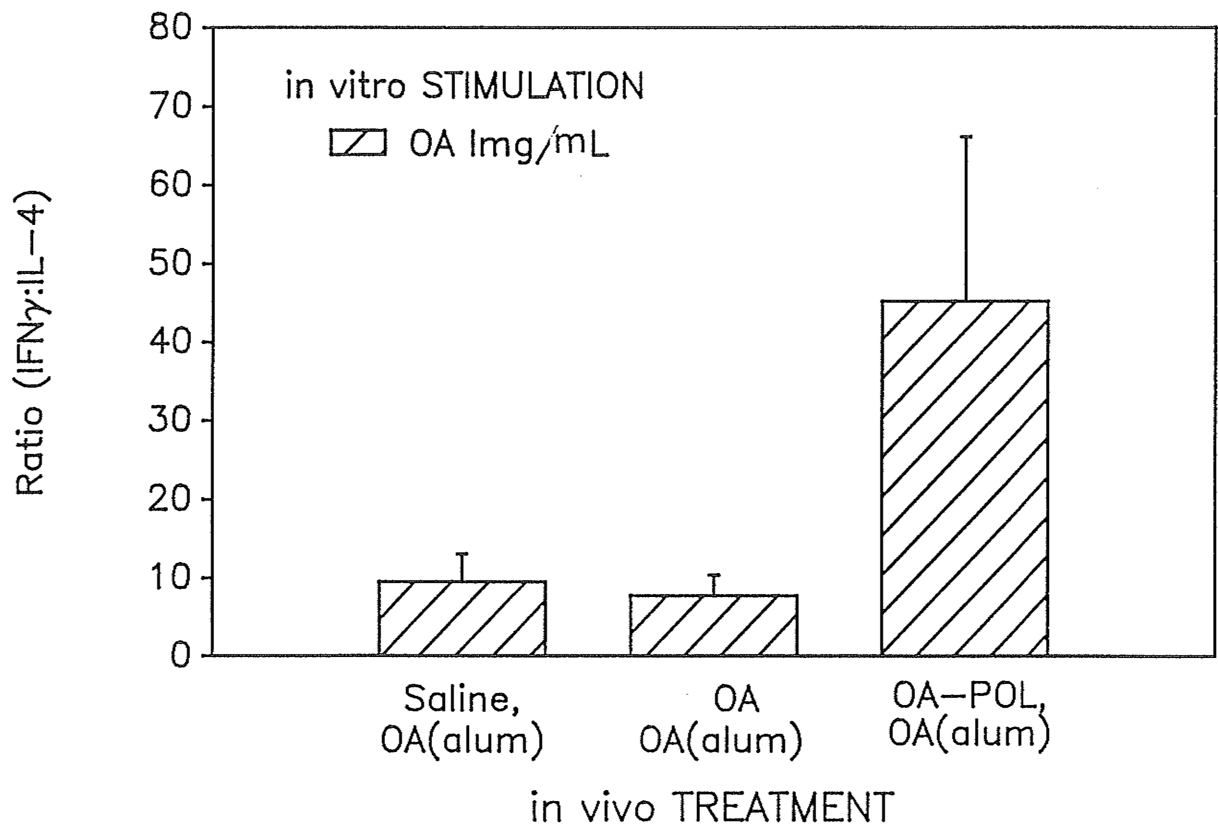

Fig 27: Modified OA pretreatment increases the ratio of IFNγ:IL-4 synthesis following OA-alum immunization. Data from Table 9 is presented as IFNγ:IL-4 ratio (mean of 4 experiments ± SEM).

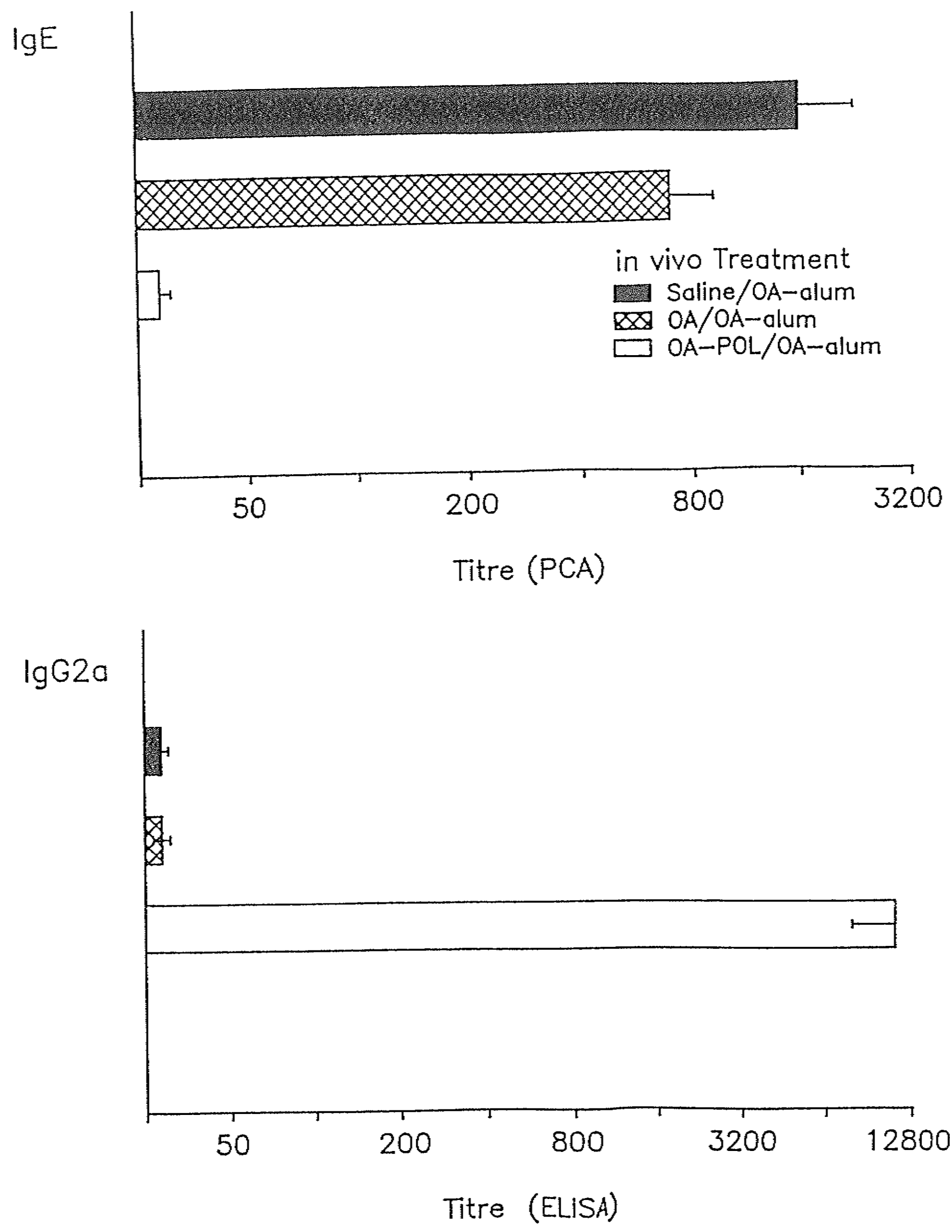

Fig 28: Reciprocal regulation of OA-specific IgE and IgG_{2a} responses by OA-POL pretreatment. Mice were treated and immunized as for Table 9. Antibody responses were measured 10 and 14 days after OA-alum immunization.

4.4. Chemically modified allergen modulates established cytokine patterns thereby abrogating ongoing IgE responses

Many modified allergens have been reported to inhibit induction of de novo IgE responses. However, most of them have been unsuccessful in abrogating pre-established IgE responses (Sehon, 1982; Ishizaka, 1985). The capacity to abrogate previously established responses is much more difficult, but better reflects a manoeuvre that would be invaluable clinically.

OA-POL treatment has been demonstrated to be capable of abrogating well-established IgE synthesis (HayGlass, 1990, 1991d). To characterize the mechanism for this activity, we examined the relationship between IgE production and alterations in cytokine patterns. As described in Materials and Methods, mice were first immunized with OA-alum (0.2 μg) and then given three courses of native OA or OA-POL treatment (three i.p. 80 μg injection each). 14 days after the last treatment, mice were boosted with 0.2 μg OA-alum. Cytokine synthesis and IgE secretion were measured 5 and 7 days after challenge.

The data in Table 10 demonstrate that administration of OA-POL *following* establishment of IgE responses markedly

increased IFNγ synthesis (2.5-3.2 fold). IL-4 synthesis decreased upon these treatment, but with higher variation between experiments (1.5-13 fold decreased). As in pretreatment experiments, administration of unmodified OA failed to enhance IFNγ synthesis markedly, or to inhibit IL-4 production. This Th1-like pattern shift induced by OA-POL treatment is most apparent by the comparing the ratio of antigen-stimulated IFNγ:IL-4 among different groups (Fig. 29).

In parallel with the cytokine pattern shift, previously established antigen-specific IgE responses were strongly suppressed (>90%, $p < 0.001$) by three courses of OA-POL treatment, although never to undetectable levels (Fig. 30). OA-alum induced IgE responses exhibited no significant change following native OA pretreatment, consistent with the minimal change of cytokine profile produced by such pretreatment (Fig. 30).

Taken as a whole, the results in this section show that the pattern of cytokine synthesis elicited after in vivo exposure to protein antigens, and the resulting immune response, is dependent upon the form of antigen to which the individual is exposed, and consequently are hence this cytokine pattern can be controlled by the appropriate form of in vivo immunization.

Table 10

Effect of Native and Modified OA Treatment
on Established Hypersensitivity

Expt.	In vivo treatment	In vitro cytokine synthesis	
		IL-4 (SEM) U/ml	IFNγ (SEM) U/ml
1.	Saline	4.75 (0.55)	32.15 (1.35)
	OA	2.65 (0.05)	46.05 (0.65)
	OA-POL	2.55 (0.35)	74.10 (0.50)
2.	Saline	6.55 (0.35)	26.75 (2.88)
	OA	8.50 (0.70)	21.90 (2.20)
	OA-POL	0.60 (0.02)	86.85 (2.75)

Legend: Mice were primed with OA-alum (0.2 μg i.p.) on d0. Three courses of native or modified OA (each course consisting of three 80 μg i.p. injections) or saline treatment were started at d28, d56 and d72 respectively. Mice were boosted with OA-alum (0.2 μg i.p.) on d100 and killed 5 days later. Spleen cells were cultured in vitro with OA (1 mg/ml) stimulation and the culture supernatants were measured for cytokine synthesis. Results are presented as mean of 2 tests ± SEM.

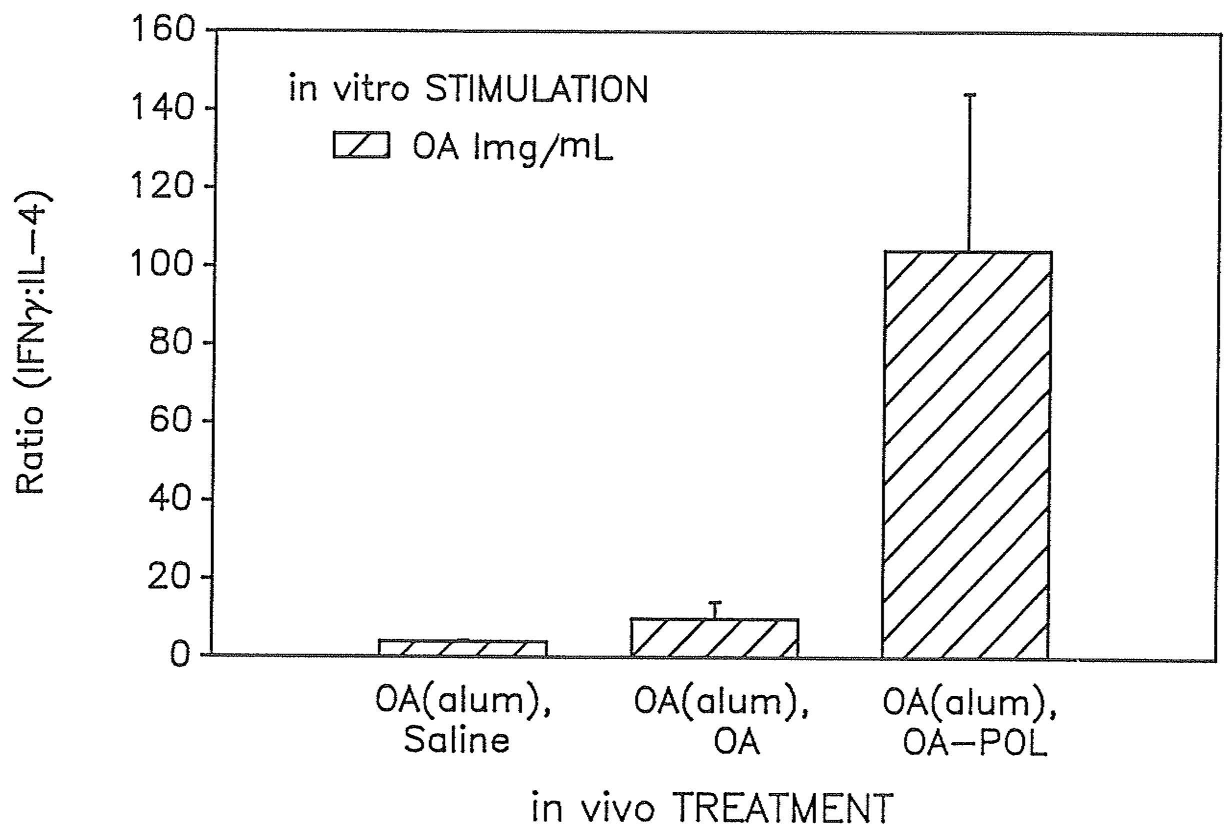

Fig 29: Alteration of the antigen-stimulated IFNγ:IL-4 ratio following post-immunization OA-POL treatment. Data from Table 10 is presented as IFNγ:IL-4 ratio (mean of 2 experiments ± SEM)

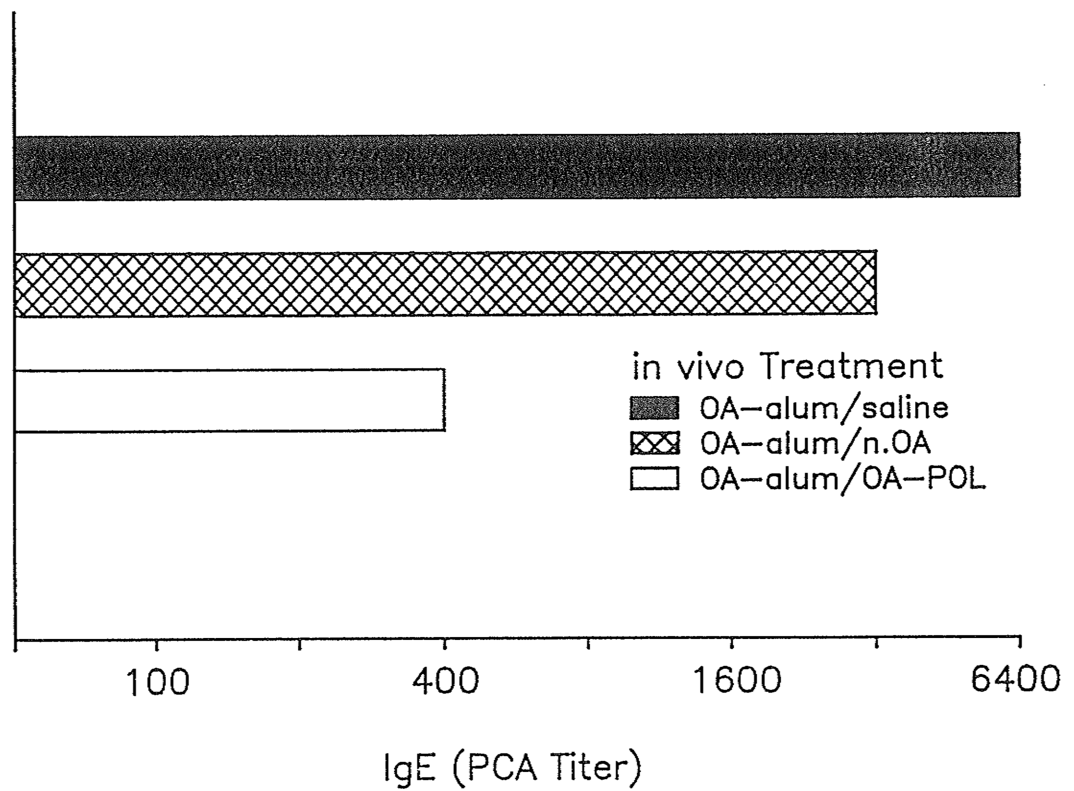

Fig 30: Abrogation of established IgE responses by post-immunization OA-POL treatment. Anti-OA IgE synthesis in one the experiments of Table 10.

The administration of the chemically modified antigen induced the Th1-like subset of helper T cells, thereby preferentially determining the antibody isotype in a primary response, and such administration after establishment of an IgE antigen-specific response could result in an alteration in both the subset of antigen specific helper T cell present and the isotype of antibody induced.

V. DISTINCT ANTIGEN-PRESENTING CELL POPULATIONS MAY BE RESPONSIBLE FOR PREFERENTIAL INDUCTION OF PARTICULAR PATTERNS OF T CELL ACTIVATION

The finding of distinct and discrete cytokine producing patterns induced by modified and native allergens provides framework for understanding the basis of the suppressive effect of administering modified allergen on de novo and established IgE responses. The critical question which follows is how in vivo exposure to modified allergen leads to different patterns of cytokine synthesis and production of antibody of different isotypes compared to those induced by unmodified allergen. Elucidation of the mechanism for distinct T cell subset selection is critical not only for understanding of this particular system, but important for developing general strategies of immunotherapy.

Several potential mechanisms could be responsible. First, functionally distinct CD4 T cell subsets might express different antigen receptor repertoires and be selectively activated by different epitopes expressed on modified or unmodified allergen. This hypothesis, however, is not supported by many recent studies which demonstrated that the same T cell epitope can induce different T cell subsets. Secondly, the density of MHC class II molecules on antigen-presenting cells may determine the cytokine pattern. Recent data argues against this possibility as well. Thirdly, the type of antigen-presenting cell, or costimulatory factors (cell membrane molecules, soluble factors) produced by it, may play an important role in differential activation of Th1-like or Th2-like patterns of cytokine synthesis.

In light of the pronounced differences of antigenicity between modified and unmodified allergen at the level of antibody recognition, and by implication at the level of recognition by antigen-specific B cells, we considered the hypothesis that the antigen-presenting cell which stimulates the responses in vivo plays a selective role in determining the type of committed T cell subset that is activated or in determining the differentiation path of activation of a common T cell precursor cell. Previous studies have demonstrated that chemically modified OA (OA-

POL) retains only 0.5-5% of its capacity to be bound by anti-OA antibodies (IgE or IgG). Consequently, we hypothesized the role played by B cells is expected to be markedly different in the presentation of modified and unmodified allergen, i.e. OA-specific B cells may be potent for native OA, but not for OA-POL presentation. At the same time, high M_r OA-POL may be more likely to be handled by antigen-presenting cell populations other than B lymphocytes such as macrophages. Using B cell deficient mice obtained via chronic anti-μ treatment, we directly examined the role played by B cells in priming of Th1-like and Th2-like cells.

5.1. Chronic anti-μ treatment depletes murine B lymphocytes in vivo.

Chronic anti-μ in vivo treatment of mice beginning within 24 h of birth has been reported to be able to deplete B cell populations. To assess the efficiency of anti-μ antibody treatment for murine B cell depletion, we determined the frequency of surface Ig-bearing (sIg+) cells in these mice by flow cytometry. Age-matched untreated mice as well as mice treated with NRIgG were used as controls. The percentage of sIg+ cells in each group was compared and the results are shown in Table 11. As can be seen, treatment of mice with anti-μ antibodies from the time of

birth, resulted in elimination of practically all sIg+ cells in mouse spleen.

In a complementary approach, mature B cell activity in anti-μ treated mice was functionally evaluated via LPS stimulation. As shown in Fig 31, spleen cells from anti-μ treated animals had no proliferative response while spleen cells from normal or NRIgG treated mice were highly responsive to in vitro stimulation with LPS.

Gross T cell function in anti-μ treated mice was examined by comparing the in vitro proliferation and cytokine production following polyclonal stimulation in vitro with Con A or immobilized anti-CD3 mAb. As shown in Fig. 32, T cell proliferation following polyclonal stimulation was not significantly different among anti-μ treated , NRIgG treated or normal mice. Similarly, polyclonally driven IL-2, IL-4, IL-10 and IFNγ synthesis by spleen cells from anti-μ treated groups was virtually identical to that in NRIgG treated and normal mice following the stimulation (Fig. 33). These data were taken as evidence that T cells in anti-μ treated mice have potential for proliferation and cytokine production similar to that in normal or NRIgG treated mice.

5.2 OA-alum immunization fails to prime CD4 T cells in B-cell deficient mice

To evaluate the role played by B lymphocytes in presentation of native OA to CD4 T cells, we examined the capability of adoptively transferred CD4 T cells from normal or B-cell deficient mice to accelerate primary IgE responses in recipients. Briefly, anti-μ treated mice (B cell deficiency was confirmed by testing one mouse each time from anti-μ treated group using flow cytometry), as well as NRIgG treated and untreated mice, were immunized with 2 μg OA (alum) 10 or 20 days before being killed. Anti-μ was continuously injected after OA-alum immunization until sacrifice. CD4 T cells were isolated from spleen cells by negative selection using murine CD4 enrichment immunocolumns, yielding populations >90% CD4+ with CD8+ cell contamination less than 2%. These CD4 enriched donor cells (2×10^7) were transferred to syngeneic recipients intravenously. The recipients, as well as mice given no cells, were OA (alum) immunized 4 hours later and bled 6, 7, and 14 days thereafter to assess CD4 T cell priming via evaluation of their capacity to elicit accelerated primary antibody responses.

As shown in Fig. 34, antigen-specific serum IgE responses were detected beginning d10-13 after OA-alum immunization

in normal mice (without transfered cells), indicating typical primary responses. However, recipients of CD4 donor cells from NRIgG treated and normal mice displayed accelerated primary IgE responses (d7) representative of CD4 T cell priming in donor mice. In contrast, IgE synthesis in recipients of CD4 cells from B cell deficient/OA (alum) immunized donors failed to generate accelerated primary IgE responses and went on to develop normal primary responses by d.13. The failure of CD4 T cells from OA (alum) immunized, B cell deficient mice to initiate accelerated primary IgE responses -- while CD4 T cells obtained from identically primed B cell containing donors were able to is consistent with markedly decreased Th2-like CD4 T cell priming in the absence of B lymphocytes. This result provides indirect evidence in favour of the importance of B lymphocytes in selective activation of Th2-like CD4 T cells in vivo.

Table 11

Effect of Chronic Anti-μ Treatment on

B cell Populations in vivo

Treatment	sIg+ cells (%)
None	35.32
NRIgG	37.56
Anti-μ	0.93

Legend: Mice were treated with rabbit anti-murine μ chain or normal rabbit IgG (NRIgG) starting within 24 h of birth until being killed. Spleen cells were stained with FITC conjugated goat anti-mouse IgG (H+L) and tested for sIg+ cells using flow cytometry.

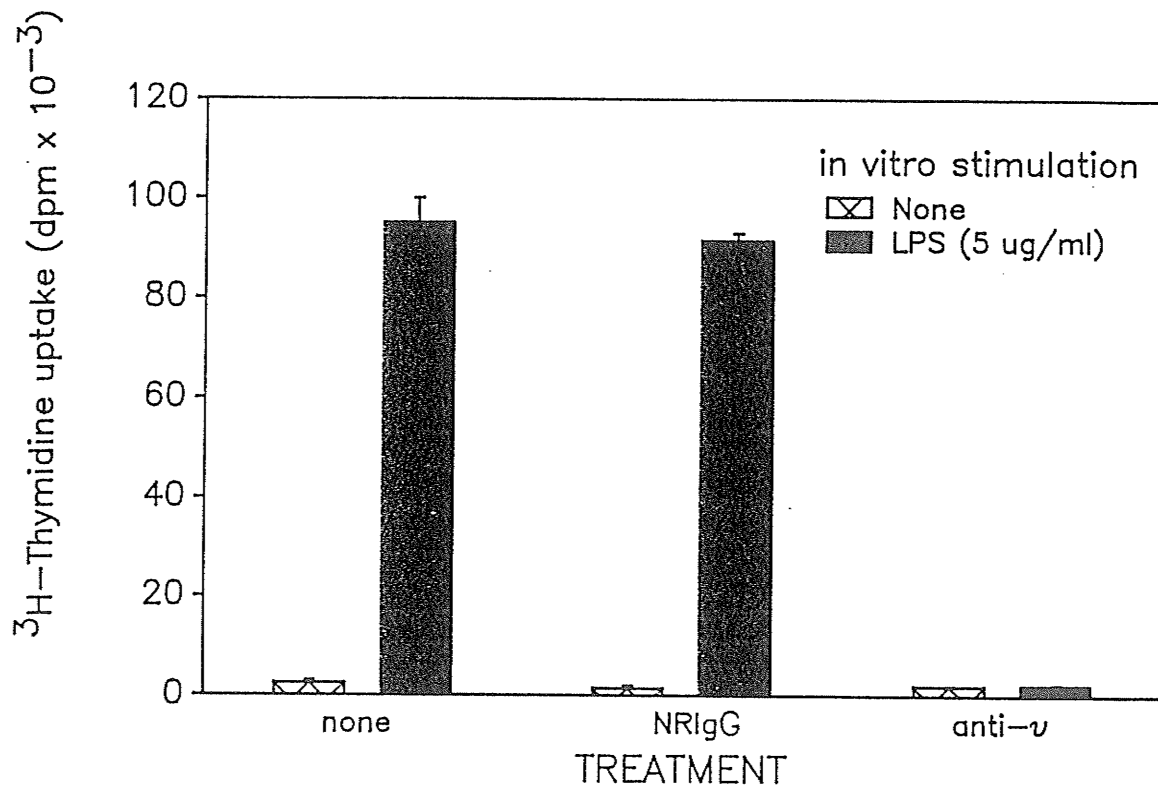

Fig 31: B cell proliferative responses to mitogen (LPS) in normal, normal rabbit Ig- treated, and anti-μ treated mice. Mice were treated as in Table 11, spleen cells were cultured in absence or presence of LPS (5 μg/ml), and ^{3}H-thymidine incorporation was measured by liquid scintillation counting.

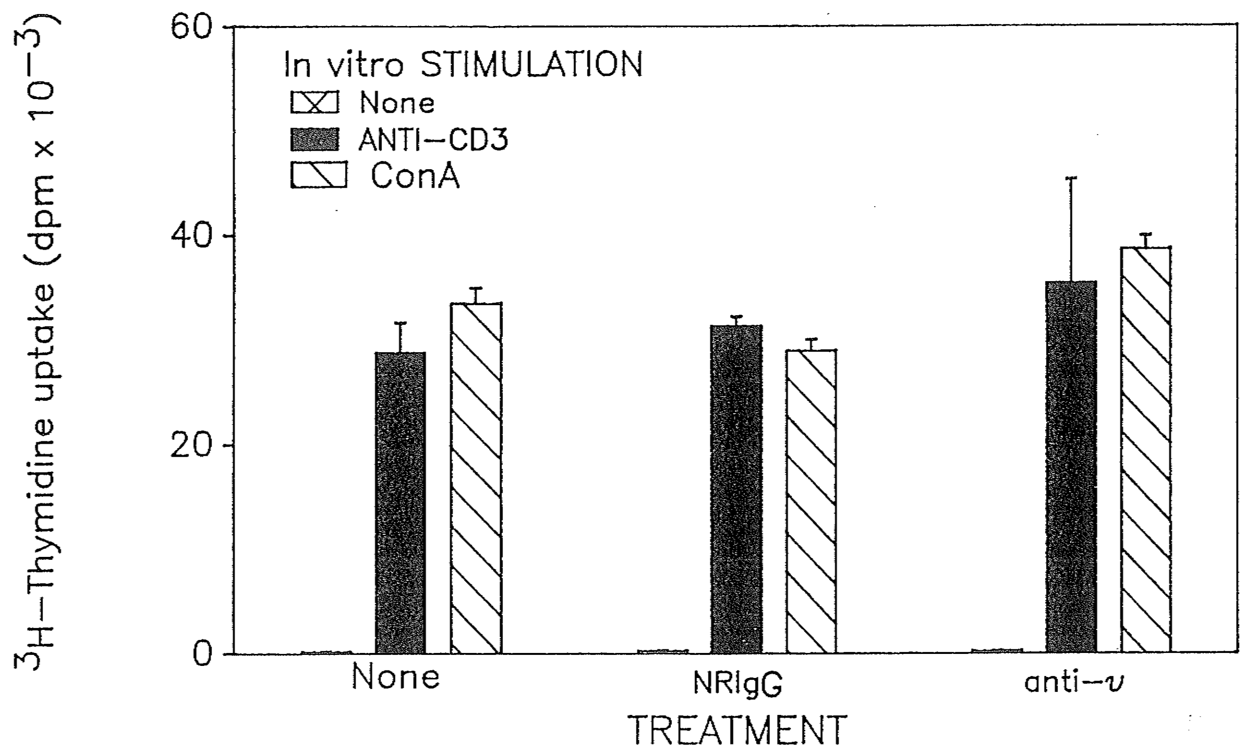

Fig 32: T cell proliferative responses to polyclonal stimulation in normal, normal rabbit IgG- treated (NRIgG), and anti-μ treated mice. Spleen cells obtained as in Fig 31 were stimulated in vitro with immobilized anti-CD3 or Con A, pulsed with ^{3}H-thymidine, and ^{3}H-thymidine uptake was measured by liquid scintillation counting.

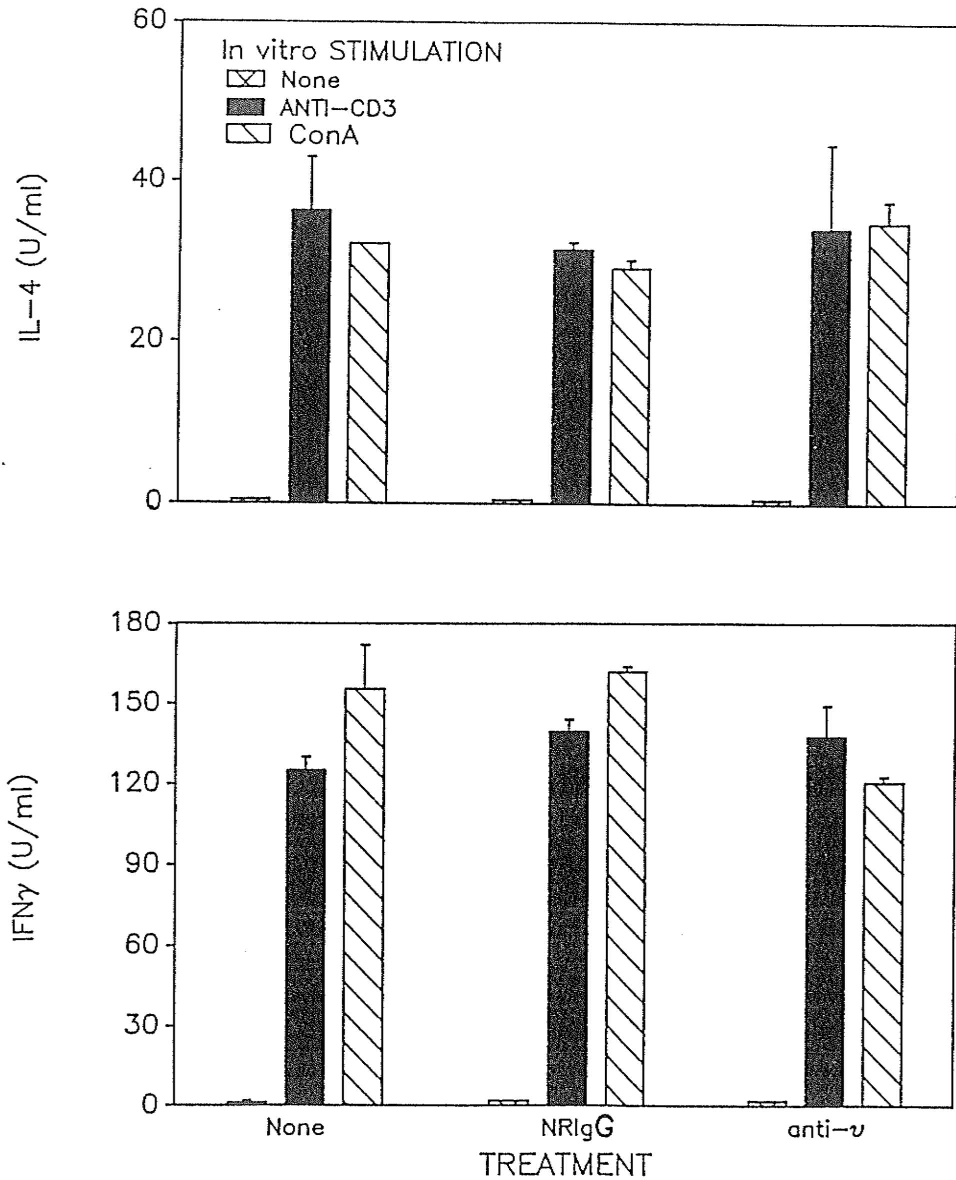

Fig 33: Cytokine synthesis following in vitro T cell polyclonal stimulation in normal, normal rabbit Ig-treated and anti-μ treated mice. IL-4 and IFNγ synthesis of anti-CD3 and Con A-stimulated spleen cells were tested by MTT CT.4S bioassay and ELISA respectively.

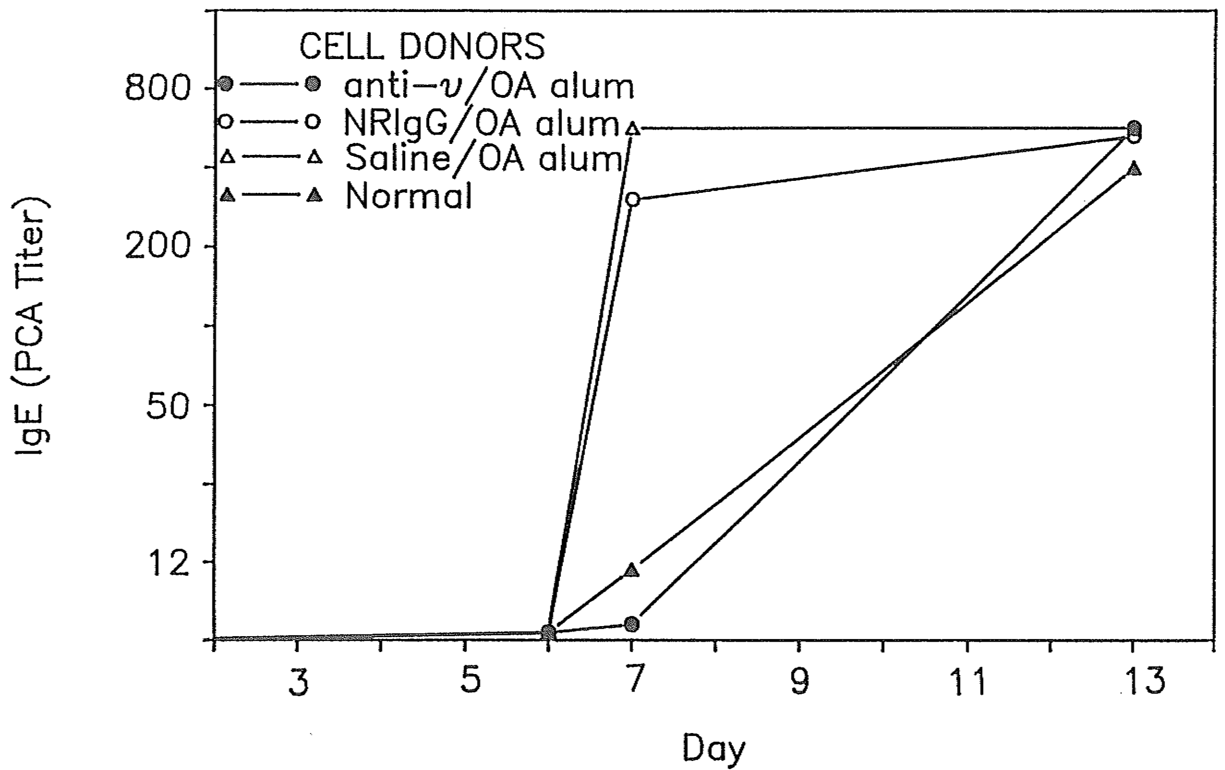

Fig 34: B cell deficient mice fail to prime Th2-like CD4 T cells in vivo. Mice treated with anti-μ or normal rabbit IgG (NRIgG) from birth were immunized with OA-alum (2 μg) at 5 weeks of age. Age-matched normal mice were immunized in the same way. CD4 T cells enriched by negative selection immunocolumns (see Materials and Methods) were transferred to normal syngeneic recipients which were then immunized with OA-alum (2 μg) and bled at d6, d7, and d13. The capacity of donor cells to accelerate primary IgE responses (defined as d7) of recipients were compared.

5.3. CD4 T cells from OA-POL treated, B-cell deficient donors inhibits antigen-specific IgE responses in recipients

In light of the two order of magnitude decreases in antibody binding (defined as the relative capacity of ^{125}I-OA-POL vs ^{125}I-OA to inhibit anti-OA IgG:OA binding in competitive inhibition assays), and the importance of B cell surface Ig in antigen handling (Pierce, 1988), we predicted that B cells may be of minimal importance in the presentation of modified allergen. This hypothesis was directly tested using the same B-cell deficient mouse model. Briefly, OA-POL was administered to anti-μ treated, NRIgG treated and normal mice (three i.p. 80 μg injections) 14 days before cell transfer. Anti-μ injections were continued until the donors were killed. 4 x 10^7 CD4 cells were transferred to syngeneic recipients intravenously. Recipients (and normal control mice which received no cells) were then primed with OA (alum) and bled 7, 10, and 14 days later, to determine the capacity of cells transfered from OA-POL treated normal and B cell deficient mice to inhibit the induction of the IgE response.

As seen in Fig. 35, OA specific IgE responses in recipients of CD4 T cells from OA-POL treated, normal or NRIgG donors were markedly suppressed (PCA: 1600 vs 80). This confirms

our previous finding that OA-POL induced (CD4 mediated) suppression of IgE response is adoptively transferable and demonstrates the commitment of Th1-like CD4 cells in donors follow OA-POL exposure. Most notably, CD4 enriched cells from OA-POL treated, B-cell deficient donors efficiently transfer suppressive activity, as demonstrated by their ability to inhibit OA-alum induced IgE responses (PCA: 1600 vs 50, <3% of normal controls). These results suggest that despite the absence of B lymphocytes as antigen-presenting cells at the time of initial CD4 T cell priming, OA-POL is capable of efficiently generating Th1-like cells in vivo, which inhibit IgE responses in cell transfer recipients.

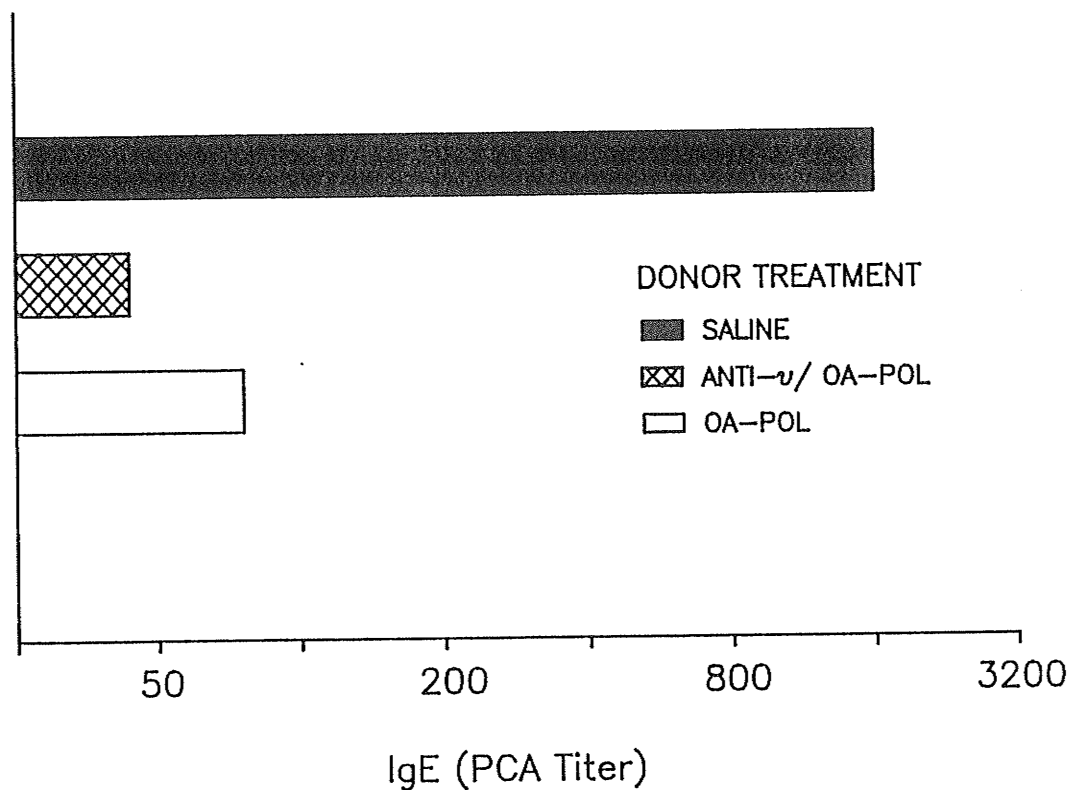

Fig 35: B cell deficient mice can induce capable of inducing Th1-like CD4 T cells. Anti-μ and normal rabbit IgG (NRIgG) treated and normal mice were administered one course of OA-POL (three 80 μg i.p. injections). CD4 enriched T cells, prepared as in Fig 34, were transferred to normal naive recipients which were then challenged with OA-alum (2 μg) and bled at d10. The capacity of donor CD4 T cells to inhibit OA-specific IgE responses was examined. CD4 T cells derived from OA-POL treated normal and B cell deficient mice exhibited equivalent capacities to inhibit IgE responses in OA (alum) immunized recipients.

DISCUSSION

CD4 T cell-derived cytokines play a pivotal role in determining the nature and intensity of immune responses. The mechanisms that control the induction and expression of different cytokine genes are poorly understood. From the initial characterization of different cytokine profiles produced by long term cultured CD4 T cell clones (Mosmann & Coffman, 1989), much attention has been given to identify their potential physiological relevance and the signals which are involved in initiating and sustaining the cytokine patterns in vitro and in vivo.

Therapeutic modulation of patterns of cytokine production requires an understanding of the influence of different cytokines and accessory cells involved in the initial induction and maintenance of Th1 or Th2-like responses. The present study, carried out with fresh T cells directly ex vivo, examines (1) cytokine synthesis by murine spleen cells in a model of human immediate hypersensitivity, (2) differential IL-2 requirements in the induction and maintenance of Th1-like vs Th2-like cytokine production patterns, (3) a strategy by which murine cytokine production patterns can be deliberately manipulated by in vivo treatment with chemically modified allergen and the consequent effects on antibody responses, and (4) preliminary investigation of a mechanism which may underlie the capacity of this modified allergen to preferentially

induce distinct cytokine gene expression patterns.

I. INITIATION AND MAINTENANCE OF ANTIGEN STIMULATED IL-4 AND IFNγ RESPONSES BY IN VIVO MURINE CD4 T CELLS IN VIVO.

1.1. Techniques for in vitro culture and cytokine determination.

Use of freshly derived CD4 T cells, though arguably more relevant to physiological T cell responses, has several technical disadvantages. Cytokine production is more transient, carried out by a smaller proportion of the cells in culture and is of markedly lower intensity than that observed following antigenic polyclonal stimulation of T cell clones or hybridomas. Consequently, the objective of the present study required the availability of highly sensitive cytokine measurement techniques. The modification of commonly used techniques notably increased the sensitivity of cytokine determination. The application of these modified assays allowed us to characterize cytokine production by normal "fresh" T cells directly ex vivo to antigen specific stimulation. The development of dual mAb IFNγ ELISA greatly decreased assay time as well as increased simplicity and sensitivity (Yang and HayGlass, 1993d). Using a highly sensitive MTT CT.4S bioassay, we successfully demonstrated antigen-driven IL-4 production by

CD4 T cells directly ex vivo even by cells obtained from mice exposed with allergen in the absence of adjuvant. Indeed, the MTT CT.4S assay is capable of measuring IL-4 synthesis by very few cells such as in limiting dilution analysis (LDA) (personal communication, R. Gieni).

It should be noted that although the bulk culture system used in the present study has several advantages, the interpretation of experimental results are restricted by certain inherent shortcomings. The major disadvantages of bulk culture systems in the analysis of cytokine production are (1) cytokine consumption occurs simultaneously with production, allowing us to measure net cytokine production rather than total production and (2) potential cross-regulatory activity of cytokines secreted by different cell subsets in the culture supernatants. In another words, secretion of certain cytokines in the culture system may inhibit/facilitate the synthesis of other cytokines. Although this study used very brief periods of restimulation in order to minimize this concern, with culture periods much shorter than most other studies (14-24 hours vs 4-5 days to 14 days), the influence of the cytokines produced on cells activity in the culture system is not completely avoidable. In this sense, the application of limiting dilution analysis (LDA) techniques can certainly provide a complementary approach by evaluating

the frequency of different T cell functions induced by different forms of allergen.

1.2. Allergen-dependent induction of IL-4 synthesis in vivo

The stated absence of detectable IL-4 production by freshly derived CD4 T cells was earlier attributed to inhibitory effects of Th1-derived cytokines on clonal expansion of IL-4 secreting cells in vitro (Powell, 1990), or more popularly at that time, the belief that IL-4 secreting cells require additional differentiative steps before detectable IL-4 is produced (Swain, 1988; Powers, 1988; Kayakawa, 1989; Powell, 1990). However, the results of the present study, obtained also from bulk culture system, demonstrated that antigenic stimulation of murine CD4 T cells in vivo leads to rapid induction of IL-4 synthesis, suggesting that such cells do in fact constitute a normal component of the T cell repertoire. We speculate that the failure of previous investigators to detect IL-4 production by freshly isolated T cells without repeated cycles of rest and restimulation over periods of up to two weeks may be mainly due to the IL-4 inhibitory activity of the adjuvant (CFA) used in some studies (Kishimoto, 1982). The suppressive effects of CFA on IgE synthesis have been well characterized. Here, direct comparison of the role played by alum vs CFA in induction of dominant Th1-like or Th2-

like responses demonstrates the selective effect of adjuvant on cytokine profiles induced by the same allergen, a concern with important clinical as well as theoretical implications given that alum is the sole adjuvant licensed for human use and is in fact incorporated in some allergen preparations currently prescribed for immunotherapy. Clearly, use of an adjuvant long known to promote IgE synthesis (Levine, 1970; Revotella, 1969; Vaz, 1971) and now directly demonstrated to preferentially elicit IL-4 synthesis (Yang & HayGlass, 1993) in mice, should be reevaluated.

Results obtained from studies of long term cultured CD4 T cell clones suggested a regulatory potential of the cytokines produced by distinct T cell subsets in immune responses. However, the conclusions drawn from these observations may not reflect the in vivo situation because (1) the culture conditions under which human or murine T cell clones are derived greatly influence the composition of the resulting populations of clones; (2) technical and logistical constraints make it possible to derive only a very limited number of clones (frequently 5 - 10) from each donor, thus representing 5-10 T cells from a potential allergen reactive repertoire which may be 10^2 - 10^4 fold greater in allergic individuals, leading to conclusions being based on an extremely small sampling of the reactive

cell population; and (3) long term in vitro culture frequently changes the characteristics of cells in both phenotype and function. Therefore, the conclusions obtained from such studies should be interpreted with caution. Indeed, many studies have demonstrated the influence of in vitro culture conditions in cytokine profiles of generated CD4 T cell clones (Seder, 1992; Le Gros, 1990; Swain, 1991; Coffman, 1991; Maggi, 1992; Fitch, 1993). Therefore, the utilization of cells directly ex vivo and short term (14-24h) culture periods may largely minimize these concerns.

The differences demonstrated in the cytokine profiles observed following antigen-specific vs polyclonal T-cell activation of the same cell population are particularly important (Fig. 12 and Fig. 13 vs Fig. 15). Polyclonal activators have been widely used in human and murine studies to assess the status of the immune system, in terms of cytokine synthesis, notwithstanding the evidence that in many disorders (i.e. allergy) the changes which occur are not global in nature. Our results, however, demonstrate very different patterns of cytokine synthesis following antigen-specific activation compared to polyclonal activation. When the cytokine patterns of freshly derived T cells are compared with serum antibody responses, only the cytokine patterns elicited by antigen-specific in vitro restimulation are correlated with antibody production,

suggesting its representativeness of the in vivo situation. We hypothesized that polyclonal stimulation, which activates virtually all T cells may reflect the potential of the overall T cell repertoire to secrete cytokines whereas antigen-driven stimulation is likely to be more representative of the component of T cell repertoire activated by antigen priming in vivo. The results in the present study support this concept and underline the importance of the experimental conditions selected for in vitro analysis of in vivo cytokine gene expression.

1.3. Differential IL-2 requirements for IL-4 and IFNγ synthesis in de novo and established hypersensitivity.

Identification of the parameters which are relevant to initiating and sustaining the generation of T cells committed to Th1 and Th2 patterns of cytokine synthesis is a prerequisite for intentional modulation of the cytokine synthesised and of the antibody responses elicited following in vivo antigen exposure. Although IL-4-mediated T cell proliferation has been reported as IL-2 independent (Lichtman, 1987; Hergen, 1987; Lorre, 1990), the weight of evidence clearly supports an IL-2 dependence in the early stages of developing IL-4 responses by small, resting, naive T cells (Powers, 1988; Weinberg, 1990; Seder, 1991). Because previous studies of IL-4 induction and gene

expression were carried out using multi-day in vitro stimulation of T cells, it was impossible to separate the role played by IL-2 in clonal expansion of IL-4 producing cells from that in IL-4 gene expression itself.

In the present study, we used a very short period (14 - 24 h) of in vitro stimulation, therefore largely avoiding clonal expansion (Yang & HayGlass, unpublished data), which allowed us to focus on the role played by IL-2 in Th1 and Th2 cytokine gene expression. The distinct results obtained for IL-2 requirements for IL-4 synthesis with polyclonal (where IL-2 is necessary) and antigen-specific (where IL-2 plays no detectable role) stimulation may be attributable to the nature of the T cell population targeted by antigen-specific vs polyclonal stimulation. A large percentage of T cells activated by anti-CD3 stimulation are likely resting, naive cells. The requirement for IL-2 in their "maturation" to IL-4 secretion is consistent with earlier demonstrations of a requirement for IL-2 in this process. In contrast, use of specific allergen for short term in vitro stimulation, targets only previously activated and memory CD4 T cells, bypassing the necessity for IL-2 which is likely to be associated with clonal expansion. Therefore, the utilization of these different approaches allowed us to dissociate the role played by IL-2 in cytokine synthesis in de novo and established responses

respectively. Based on the data of this study, we hypothesize that IL-4 responses may be divided into two stages in term of IL-2 requirements i.e. IL-2 dependent (initial activation, clonal expansion) and IL-2 independent (production and release of biologically active IL-4) stages.

In contrast to IL-4, IFNγ synthesis in both polyclonally stimulated de novo responses of spleen cells from naive donors and in allergen-restimulated responses from immunized donors is strongly IL-2 dependent.

This difference in the IL-2 dependence of IL-4 and IFNγ synthesis suggests a differential requirement for cytokine gene expression by distinct T cell subsets. Although IL-2 and IL-4 are important in the differentiation and /or clonal expansion of cells destined to produce IL-4, the role of IL-2 at the effector level of IL-4 gene expression in antigen-driven responses, and by extension in individuals with ongoing allergic responses, appears minor (Yang & HayGlass, 1993).

These findings have several implications for IL-4 regulation in allergic humans. The fact that cytokine synthesis is clearly dissociable from proliferation (Bloom, 1972; Evavold, 1991) implies that such cytokines could be

produced locally without further expansion of antigen-specific clones. Indeed, in the case of naturally hyperimmunized allergic individuals, one can envisage allergen-driven IL-4 synthesis being largely IL-2 independent. In contrast, both the induction and expression of IFNγ responses are strongly IL-2 dependent. This differential requirement for IL-2 in cytokine gene expression may be significant in the maintenance of IL-4-dominated responses in allergic individuals.

The relative IL-2 independence of established IL-4 responses may have an implication for maintenance of hypersensitivity in individuals with ongoing IgE responses. Under such circumstances, IFNγ synthesis may be actively inhibited as a consequence of coordinate regulation of IL-2 and IFNγ in IL-4 dominated, ongoing immune responses. In support of this hypothesis one may cite observations which indicate that IL-4 has the capacity to: inhibit expression of IL-2R (Martinez, 1990), suppress IL-2-dependent proliferation (Martinez, 1990; Karry, 1988) and IL-2-and/or IFNγ-induced cytokine gene expression (Gautam, 1992), and inhibit IL-2, IFNγ (Peleman, 1989; B-Fernandez, 1991), and PGE2 (Hart, 1989) synthesis directly. Consequently, not only would allergen-driven IL-4 response in such individuals be largely IL-2 independent, but ongoing IL-4 responses could serve to maintain commitment to a

phenotypically Th2 response.

II. MODULATION OF CYTOKINE SYNTHESIS PATTERNS WITH CHEMICALLY MODIFIED ALLERGEN

2.1. Chemically modified ovalbumin (OA-POL) preferentially induces Th1 cytokine synthesis in de novo IgE responses.

The demonstration of the critical role played by Th1 and Th2 subsets in the regulation of IgE responses indicates a key point for hypersensitivity manipulation, i.e. successful modulation of cytokine production patterns. In vivo administration of rIFNγ or mAb to IL-4 or its receptor strongly inhibit murine IgE production (Finkelman, 1990), suggesting potentially valuable strategies for controling human allergic disease. However, the therapeutic potential of these approaches may prove to be constrained by several limitations: (1) in animal studies large amounts of IFNγ or mAb against IL-4 are usually necessary; (2) IFNγ in particular, exhibits significant toxicity at the dose required in the animal studies; (3) formation of human anti-mouse antibodies is a likely possibility on continued administration of xenogenic mAb; moreover, (4) the longevity of the effects elicited by direct administration of cytokine or therapeutic mAb in human or murine systems is as yet undermined and may prove to be limited by the in

vivo t1/2 of the cytokine or mAb used.

Therefore, we tested whether specific cytokine production patterns could be intentionally elicited by antigen (native or modified) treatment. The approach used in the present study, administration of chemically modified allergen (OA-POL), led to suppression of antigen-specific murine IgE and increased IgG_{2a} responses, suggesting the alteration of cytokine production in vivo. Direct analysis of the cytokine synthesis patterns shows that chemically modified, but not native allergen, preferentially induced Th1-like cytokine responses as indicated by a higher ratio of antigen-driven IFNγ:IL-4 and IFNγ:IL-10 synthesis. This Th1 dominance was resistant to native allergen exposure even in the presence of alum adjuvant (Yang, 1993b; Gieni, 1993).

The ratio of Th1:Th2 cytokine synthesis elicited following allergen exposure 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 immunity) induced. Romagnani has suggested that the ratio of antigen-driven IFNγ:IL-4 synthesis is much more relevant to antibody profile and clinical status than absolute levels of IL-4 or IFNγ production (Romagnani, 1993). Although the level of IL-4 and IFNγ production elicited by native or chemically modified allergen exposure varied

among independent experiments, the *ratio* of IFNγ:IL-4 was constantly increased, in parallel with inhibition of IgE synthesis, upon OA-POL treatment. The correlation between the ratio of IFNγ:IL-4 production and antibody isotype selection strongly suggests that administration of modified allergen modulates in vivo antibody response via regulating cytokine gene expression.

While it remains unclear if commitment by normal T cells at the single cell level is as polarized in vivo as is observed in many long term Th1 and Th2 clones, or if commitment to a given pattern of cytokine synthesis by normal T cells is transitory or permanent, it is well established that such commitment of the responses can occur at the population level (Yamamura, 1991; Scott, 1989; Locksley, 1991; Gazzinelli, 1992). It is reported that certain antigens inherently induce dominant Th1 or Th2-like responses in vivo. Synthetic peptides corresponding to tandemly repeating regions of leishmanial peptides were demonstrated to induce Th2 dominated responses in vivo (Liew, 1990). An unrelated leishmanial antigen was found to elicit preferential Th1 activation, defined by detection of substantial IL-2 production in the absence of detectable IL-4 synthesis (IFNγ was not examined (Jardim, 1990)). Our data differ from previous studies in that we demonstrate a capacity to purposefully redirect cytokine responses from

one pattern, characterized in the model used by balanced production of cytokines indicative of Th1 and Th2 cell induction, to a response in which Th1 cell activity is dominant.

Other approaches may also allow deliberate selection of specific patterns of cytokine gene expression in response to antigen exposure. Recently, Bretscher et al reported an approach to achieve a stable, protective cell-mediated response to leprosy, tuberculosis and leishmaniasis. Encouragingly, using a strategy of low dose parasite injection, they successfully established a cell-mediated response in susceptible Balb/c mice which normally produce antibody to *L. major*, consequently making the susceptible mice resistant to a larger, normally pathogenic *L. major* infection (Bretscher, 1992a, 1992b). Taken together with our data of cytokine production, this suggests that the type of immune response and the correlated pathological processes occurring as a consequence of natural exposure to antigen, could be redirected in certain, if not all, responses.

2.2 Chemically modified allergen abrogates established IgE response via induction of antigen-specific Th1 cells

Abrogation of established IgE responses by chemically

modified allergen treatment highlights the potential significance of this system for developing immunotherapy. It has long been recognized that ongoing IgE responses are much more difficult to inhibit than primary IgE responses. This was originally attributed to the generation of long-lived Bϵ cells which were thought to be virtually refractory to regulatory signals once activated (Okudaira, 1981). However, more recent studies demonstrated that depletion of CD4 T cells in vivo leads to rapid decreases in polyclonally stimulated (*Nippostrongulus brasiliensis* or *Heligmosomoides polygyrus*) (Katona, 1988; Finkelman, 1990) IgE production, suggesting that ongoing IgE responses are, in fact, subject to external regulatory control. Similarly, administration of anti-IL-4 antibody reduced serum IgE, but not IgG1, levels by >95%, in spite of persistent *H. polygyrus* infection (i.e. ongoing polyclonal stimulation) (Finkelman, 1990, 1988). These results suggest that maintenance of established ongoing IgE responses is IL-4 dependent. A requirement for continued IL-4 production raises the possibility that such responses are also susceptible to negative regulation under conditions where one could achieve preferential stimulation of IFNγ gene expression in vivo. The cytokine data presented here indicate that the mechanism by which the ongoing IgE response is abrogated following modified allergen treatment may be similar to that identified in the inhibition of de

novo IgE responses, i.e. via preferential induction of Th1-like cytokine responses. This preferential Th1 induction thereby shifts the ratio of cytokine production elicited in the OA specific repertoire away from IL-4 and toward IFNγ. Indeed, OA-POL treatment given following OA-alum immunization dramatically increased the ratio of antigen driven IFNγ:IL-4 synthesis (25 fold, see Table 10). This switch of cytokine production patterns and inhibition of IgE synthesis required three courses of subsequent OA-POL treatment, suggesting the difficulties in redirecting established responses. It should be noted, however, that this experiment has only been carried out two times, so it is still too early to draw general conclusions.

It is still not clear whether the cytokine pattern shift observed in mice with ongoing IgE responses is attributed to de novo Th1 cell induction, primed Th1 cell proliferation or both, or alternatively, to changes in the cytokine profile of established Th2 cells. Although extensive studies have been carried out to elucidate the nature of initial activation and differentiation of Th subsets, few observations are pertinent to assessing the stability of established cells. Romagnani recently showed that culture of human Th2 clones in the presence of exogenous rIL-12 induces IFNγ gene expression, which suggests that it is possible to change the established Th2-

like cytokine profile to that of a Th0-like cell even at the single cell level (Romagnani, 1993). Obviously, this is a critical point because the redirection of established cytokine profile are much more valuable in clinical practice than the modulation of de novo cytokine production patterns. It is important to note that OA-POL initiated Th1 dominated cytokine patterns were resistant to subsequent unmodified OA (alum) exposures whereas native OA initiated patterns were subject to change upon subsequent OA-POL treatment. This discretion may reflect an inherent difference which has not yet been characterized between Th1 and Th2-like cells or the experimental conditions employed. It is possible that Th1 cells, thought to represent a "default" pathway of T cell activation, are easier to activate and expand to dominant populations in vivo, or alternatively, that Th1-like cytokine patterns may be more resistant to the challenge. The clear answer to these possibilities certainly needs further study.

It is important to note that the abrogation of an ongoing IgE response by OA-POL, although well over 90%, was not complete. Previous work in this laboratory has shown that increasing the amount of OA-POL given or prolonging the period of administration still fails to yield 100% abrogation of anti-OA IgE responses (HayGlass, 1991). This finding is consistent with the possibility that some IgE

production during in vivo secondary polyclonal responses is IL-4 independent (Finkelman, 1990). Thus, although the strong inhibition of allergen-specific IgE responses observed with glutaraldehyde-modified OA raises interesting possibilities with respect to potential therapeutic use, it is unlikely that a 90% decrease in allergen-specific IgE would result in a 90% reduction in clinical manifestations. Moreover, the amount of protein administered to the experimental animals, although consistent with related studies (Ishizaka, 1989; Sehon, 1986), is much greater than could be introduced to humans. Consequently, direct extrapolation of these data to the human system would be unwarranted at this stage . Our major objective in using this particular class of chemically modified allergen lies not in its direct clinical application but in its development as an experimental model to examine the role of cytokines in the regulation of immediate hypersensitivity. Such knowledge may allow for design of effective immunotherapeutic strategies based on knowledge of underlying control mechanisms.

III. MECHANISMS FOR INDUCTION OF DIFFERENTIAL CYTOKINE PRODUCTION PATTERNS BY NATIVE AND CHEMICALLY MODIFIED ALLERGENS --ROLE OF ANTIGEN-PRESENTING CELLS

Conventional T cells recognize antigen fragments only in

association with MHC molecules on the surface of other cells. CD4 T cell responses to exogenous protein antigens are initiated by the interaction between T cell receptor (TCR) and class II MHC-associated peptides derived following antigen processing. A large number of cell types have been demonstrated to be capable of processing and presenting antigen to murine CD4 T cells, including dendritic cells (Bujdoso, 1989; Crowley, 1990), B cells (Pierce, 1988), Langerhans cells (Picut, 1988; Hauser, 1989), macrophages (Unanue, 1987), and eosinophils (Del Pozo, 1992).

One hypothesis to explain why native and chemically modified allergens preferentially induce the expansion of functionally different CD4 T cell subsets is that these subsets respond optimally to antigens presented by different accessory cells. Given that OA-POL exhibits 95 - 99% decreased antibody binding (measured as the ability to inhibit binding of anti-OA antibody to modified OA in competitive inhibition immunoassay) and is of very large M_r, we hypothesized that the properties of these two forms of the allergen might lead to their preferential processing by phagocytic (OA-POL) and B (OA) cells respectively. In order to test this possibility, we examined the CD4 T cell function following priming in anti-μ treated (B-cell deficient) mice. CD4 T cells obtained from anti-μ

treated/OA-alum immunized mice failed to mediate accelerated primary responses following transfer to syngeneic recipients upon challenge. In contrast, cells from OA-POL treated B-cell deficient mice successfully transferred IgE suppressive activity to recipients, consistent with effective priming of Th1 cells by OA-POL even in a B cell deficient milieu. These results argue for a capacity of B cells in the presentation of native but not chemically modified antigen.

It has been known for a long time that B cells can function as APCs (Rock, 1984; Tony, 1985, HayGlass, 1986; Schultz, 1990; Pierce, 1988). B cells which express antibody specific for a particular antigen are highly efficient for the presentation of that antigen, capable of activating specific T cells when provided with 1/1000 to 1/10,000 the antigen level required by nonspecific APC such as macrophages. Under conditions of limiting antigen concentrations, as may occur in vivo, antigen-specific B cells may play a critical role as APC, attracting helper T cells to their surface for activation. If the cytokines or other factors produced by stimulated T cells function only in the immediate vicinity of the T cell, then only the antigen-specific B cell would be activated to grow and differentiate into antibody-producing cells. Thus, the APC function of B cells may play a critical early role in the

B cell-T cell interaction, ultimately ensuring a specific immune response (Pierce, 1988).

However, the capacity of B cells as APCs in primary immune responses is still very controversial. There are reports that resting B cells are ineffective (Metlay, 1989; Lechler, 1982; Lassila, 1988) or inferior (Webb, 1985; Crowing, 1983; Lombard, 1989) APCs for primary responses to alloantigens and foreign antigens. Moreover, in some systems, presentation of antigen by small, resting B cells to small resting T cells is tolerogenic and results in loss of T cell activity due to the lack of costimulatory signals (such costimulatory signals are unnecessary in previously activated cells (Ryan, 1984; Hori, 1989; Elizabeth, 1992)). However, much evidence suggests a role of B lymphocytes in antigen presentation during primary responses (Janeway, 1987; Ron, 1987; Kurt-Jones, 1988).

One of the critical functions of APCs is the provision of accessory signals that are necessary for eliciting the complete set of functional and proliferative responses of T cells (Weaver & Unanue, 1990). The recognition of MHC plus antigen in the absence of accessory signals may result in an abortive program of T-cell activation that leads to T-cell unresponsiveness. The inventory of accessory molecules that form the basis of APC-T-cell interactions

is, as yet, incomplete. Generally speaking, they include those that primarily facilitate physical interactions between APC and T cell (adhesion molecules), and those required for T-cell activation and division (costimulatory factors), although the function of these kinds of molecules may not be mutually exclusive.

The accessory signals are delivered independently of T-cell receptor occupancy. Although not yet defined, it is likely that it is differences in the ability to provide these signals that distinguish, both quantitatively and qualitatively, different APC populations. In light of the failure in identifying distinctions in the antigenic specificity of Th1 and Th2 cells (i.e. TCR usage) the determination of Th cell subset selection may mainly depend on the utilization of a certain APC type for antigen presentation due to its capacity to generate certain accessory signals. IL-1 is one of the costimulatory factors which have been extensively examined (Lichtman, 1988; Greenbaum, 1988). Some studies demonstrated that IL-1 was necessary for Th2 cell proliferation but not for cytokine production, while Th1 cells could proliferate in the absence of IL-1. However, surveys of large panels of cell lines indicate that Th2 clones vary greatly in their IL-1 dependence for proliferation. It should be noted that when the T cell response being assayed is lymphokine secretion,

rather than proliferation, B cells stimulate Th2 clones more efficiently than Th1 clones (Abbas, 1992; Gajewski, 1991). The reason may be that B cells, especially resting cells, lack the costimulators that are necessary for activating Th1 clones (Schwartz, 1990). Alternatively, Th1 cells may lyse B lymphocytes thus reducing their ability to function as APC (Tite & Janeway, 1984). The results of the present study which focused on the cytokine production pattern elicited by native or chemically modified allergens, are consistent with observations of T cell clones in vitro: B lymphocytes preferentially induce Th2-like activity.

Neonatal exposure to antibodies against the μ chain, the predominant membrane associated Ig class of the early stages of B cell differentiation, resulted in the depletion and blockage of maturation of the B cell population (Cooper, 1980). Impairment of certain T cell functions in these mice have been demonstrated for nearly two decades. While the capacity of the anti-μ-treated mice to generate delayed-type hypersensitivity (DTH), MLR, allograft rejection and T cell lectin responses were unimpaired, such mice failed to generate helper T cells for high affinity antibodies or T cells responding by proliferation to antigen stimulus (Ron, 1981, 1983; HayGlass, 1986). On the basis of the results in the present study it may be

concluded that B cells are critical for the generation of Th2 cells (facilitation of antibody responses) but not for Th1 cells (facilitation of DTH responses).

The advantage in the present study of using anti-μ treated mice is the opportunity to examine the role played by APCs in the priming rather than reactivation of Th cell subsets. Most previous studies used purified APCs to restimulate long-term cultured CD4 T cell clones (Th1 or Th2) in the presence of specific antigen. Although the information obtained from those studies are helpful for the understanding of the relationship between certain APCs and T cell subsets, the interpretation of their results is restricted by the fact that (1) the clones have already become committed to certain cytokine patterns, and therefore in vitro stimulation by each APC type may induce the same cytokine response; (2) antigen concentrations in vitro cultures may be highly different from that in the local environments where cell interactions take place in vivo; (3) different APCs have equal chance to contact T cell subsets in vitro, but possibly not in vivo. Since normal routes of antigen exposure and in vivo cell interaction in the initiation stage of immune response are not changed in anti-μ treated model, the difference of distinct APCs in the induction of CD4 T cell subsets may be more representative of in vivo responses. Certainly, the

possibility of the compensatory effect of other APC types due to the absence of a B cell population can not be ruled out in this model system.

The division of different APCs in the presentation of native and chemically modified allergens is speculated to be quantitative rather than absolute, because both Th1 and Th2 cytokines are induced following either native or chemical modified allergen treatment of normal mice. Numerous in vitro studies have demonstrated that both Th1 and Th2 clones can be activated by B cells, dendritic cells and macrophages. The outcome of cytokine patterns induced by different forms of allergen may be determined by the dominant APCs encountered in vivo. Even though the decrease of antigenicity of chemically modified allergen makes it more difficult be picked up by antigen-specific B cell, the possibility that it is processed and presented non-specifically by B cell can not be ruled out. On the other hand, the possibility that native OA is presented by cells other than B cell which passively adsorb allergen reactive antibody via Fc receptors is also need to be considered.

The division of APCs in the presentation of native and chemically modified allergens and consequent induction of different Th subsets is characterized by a rather indirect pathway (i.e. antibody responses) in the present study.

Further investigation of the cytokine patterns induced in B-cell deficient mice following different forms of allergen exposure will provide more direct information regarding this distinction and is currently underway.

Collectively, our study suggests that IL-4 producing CD4 T cells form a component of the normal T cell repertoire and that the mode of immunization is critical to the pattern of cytokine production which is elicited following antigen exposure. IFNγ, but not IL-4, synthesis by antigen-primed murine T cells is IL-2 dependent. IL-2 is necessary for IL-4 priming but not for gene expression. Moreover, cytokine patterns induced by protein antigens are subject to manipulation through proper modification of the antigen. This modulation is efficient not only in the inductive phase of the primary response but also in established hypersensitivity responses. Furthermore, the switch of cytokine producing patterns may be due to the change of APCs which preferentially process and present the antigen.

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