LONG-LIVED INHIBITION OF IgE RESPONSES AND INDUCTION OF IFNγ DOMINATED CYTOKINE SYNTHESIS PATTERNS BY CHEMICALLY MODIFIED ALLERGEN.

RANDALL S. GIENI

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

DOCTOR OF PHILOSOPHY

DEPARTMENT OF IMMUNOLOGY
FACULTY OF MEDICINE
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ABBREVIATIONS

$Al(OH)_3$	- aluminum hydroxide
APC	- antigen presenting cells
CFA	- Freund's complete adjuvant
Con A	- Concanavalin A
DNA	- deoxyribonucleic acid
DTH	- delayed type hypersensitivity
ELISA	- enzyme-linked immunosorbent assay
FACS	- fluorescence activated cell sorter
FCS	- fetal calf serum
FITC	- fluorescein isothiocyanate
$>$	- greater than
HIgG	- human myeloma IgG
IFNα	- interferon-α
IFNγ	- interferon-γ
Ig	- immunoglobulin
IL	- interleukin
i.p.	- intraperitoneal
i.v.	- Intravenous
KLH	- keyhole limpet hemocyanin
LPS	- lipopolysaccharide
log	- logarithm
mAb	- monoclonal antibody
mg	- milligram
MHC	- major histocompatibility complex
ml	- millilitre
MLR	- mixed lymphocyte reaction
mPEG	- monomethoxypolyethylene glycol

M_r	- Relative Molecular mass
mRNA	- messenger RNA
MTT	- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NK	- natural killer
NRIg	- normal rat immunoglobulin
OA	- ovalbumin
OA-POL	- glutaraldehyde-polymerized ovalbumin of M_r. 3.5×10^7
O.D.	- optical density
PBMC	- peripheral blood mononuclear cells
PBS	- phosphate buffer saline
PCA	- passive cutaneous anaphylaxis
PSF	- penicillin, streptomycin sulfate and fungizone
rIL-	- recombinant interleukin
S.D.	- standard deviation
SEM	- standard error of the mean
Th	- helper T cell
Ts	- suppressor T cell
U	- unit
ug	- microgram

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Abstract

Allergy is a common disease dependent upon the production of IgE. In both human and murine systems induction and continued synthesis of IgE is dependent on the cytokine interleukin-4 (IL-4) and is inhibited by Interferon-γ. Utilizing a murine model of immediate hypersensitivity to ovalbumin (OA), these studies are intended to develop approaches for the therapeutic manipulation of cytokine production *in vivo*, in order to specifically and permanently inhibit the IgE isotype.

Glutaraldehyde polymerization of OA near its isoelectric point yields a polymer of M_r 3.5 x 10^7, termed OA-POL. Our previous findings indicated that three i.p. injections of OA-POL (80 ug each) given approximately two weeks prior to OA[$Al(OH)_3$] immunization, induced antigen specific, isotype selective inhibition of primary and secondary anti-OA IgE responses while leading to concomitant 250-1,000 fold increases in OA specific IgG_{2a} synthesis in each of 15 mouse strains tested. Most importantly, and unlike the results obtained following *in vivo* administration of other types of modified allergens OA-POL treatment effectively inhibits even well established IgE responses.

A single course of OA-POL treatment induced 90-98 % inhibition of anti-OA IgE responses in C57Bl/6 mice for longer than one year. This state

of virtual IgE unresponsiveness was maintained despite at least five booster immunizations with OA in $Al(OH)_3$ adjuvant and was paralleled by 100-800 fold increases in anti-OA IgG_{2a} levels. In contrast to the longevity and efficacy of the IgE inhibition induced by OA-POL in C57Bl/6 mice, BALB/c display strongly inhibited antigen specific IgE levels when immunized soon after OA-POL treatment, but normal anti-OA IgE responses if immunized ≥ 10 weeks after treatment.

Studies investigating the cellular mechanisms involved in the successful application of this form of immunotherapy in C57Bl/6 mice demonstrated that the inhibition of IgE was antigen specific and CD4 T cell dependent. We hypothesized that *in vivo* administration of OA-POL, but not unmodified allergen, results in preferential induction of IFNγ synthesis by the OA specific CD4 T cell repertoire. We tested this hypothesis via three independent approaches.

In the first, *in vivo* administration of purified anti-IFNγ mAb during OA-POL treatment, or at OA[$Al(OH)_3$] immunization more than 10 weeks later, blocked the effects of OA-POL treatment, leading to restoration of normal anti-OA IgE levels.

In the second approach, we characterized the pattern of cytokine

production in murine strains that demonstrated successful versus failed induction of long-lived IgE inhibition. IL-4 and IFNγ synthesis by spleen cells of OA-POL/OA[Al(OH)$_3$] versus saline/OA[Al(OH)$_3$] immunized C57Bl/6 and BALB/c mice was determined following overnight bulk culture. These experiments established that there was an increase in IFNγ production and a decrease in IL-4 secretion in cultures from OA-POL treated C57Bl/6 mice. In marked contrast, in BALB/c mice, which consistently failed to demonstrate long-lived IgE inhibition, the production of IL-4 and IFNγ was not affected by OA-POL treatment when the bulk culture was performed 10 weeks or longer post-treatment. These findings provide direct support for the hypothesis that administration of this modified allergen induces a virtually permanent shift in the ratio of allergen induced lymphokine production to one dominated by IFNγ, in strains that demonstrate long-lived inhibition of IgE.

I extended these studies and further tested this hypothesis by utilizing a limiting dilution analysis assay system capable of detecting lymphokine secretion by the progeny of single allergen responsive CD4 T cells. This approach was chosen to determine if the change in the balance of IFNγ:IL-4 synthesis noted in OA-POL treated C57Bl/6 mice was the result of direct changes in the balance of CD4 T cells secreting IFNγ versus IL-4/IL-10 or ,alternatively, resulted from of cross-inhibitory influences known to occur between Th1-like and Th2-like responses. Limiting dilution analysis has the

advantage that the frequency of cells secreting a particular cytokine can be determined in the absence of the cellular interactions which occur *in vitro* in bulk culture.

We directly compared the induction of IFNγ, IL-4, and IL-10 by OA-POL compared to native OA treatment utilizing limiting dilution analysis of CD4 T cells directly *ex vivo*. We demonstrated that this modified allergen induces on average a 10 fold higher frequency of IFNγ synthesis and a 6.4 fold lower frequency of IL-10 synthesis while not significantly altering the frequency of CD4 T cells capable of IL-4 production. This reciprocal regulation of the frequency of CD4 T cells producing IFNγ and IL-10 directly paralleled the change in isotype pattern from IgE to IgG_{2a}.

Administration of anti-IFNγ during OA or OA-POL treatment verified a role for IFNγ in the regulation of IL-4 and IL-10 secreting CD4 T cell frequency *in vivo*, in responses directed against either native or chemically modified allergen. Anti-IFNγ treatment consistently resulted in increased frequencies of allergen specific CD4 T cells capable of IL-4 and IL-10 synthesis. Under these conditions there was no significant difference in the frequency of IL-10 secretion between OA and OA-POL treated mice. Anti-IFNγ treatment converted the cytokine balance induced during an OA-POL specific immune response from that associated with highly diminished IgE and elevated IgG_{2a}

levels to a balance associated with a strong IgE response with negligible production of IgG_{2a}.

These results demonstrate that selection of a desirable antigen specific immune response may be achieved via appropriate chemical modification of protein antigens. Studies are now underway to identify the factors that bias the commitment of CD4 T cells toward an OA-POL induced Th1-like pattern, versus the Th2- like pattern elicited by native OA.

Analysis of the frequency of CD4 T cells capable of IFNγ, IL-4 or IL-10 synthesis when mice were immunized between 18 and 22 *weeks* after a single course of OA-POL treatment, showed that successful long-lived inhibition of IgE responsiveness is associated with the permanent maintenance of strong increases in the balance of CD4 T cells capable of IFNγ versus IL-4 and IFNγ versus IL-10 production. In BALB/c mice the failure to demonstrate long-lived OA-POL induced IgE inhibition or IgG_{2a} enhancement is associated with a modest increase in the frequency of CD4 T cells secreting IL-4 and IL-10 in the absence of any significant enhancement in the frequency of IFNγ producing CD4 T cells, resulting in a balance shifted toward IL-4/IL-10 (Th2-like) cells. Therefore, in this model system, the ratio between the frequency of IFNγ and IL-4 (IFNγ:IL-4) and especially between IFNγ and IL-10 (IFNγ:IL-10) secreting CD4 T cells is increased in cases of successful experimental immunotherapy.

These findings demonstrate that successful immunotherapy is associated with a virtually permanent shift in the pattern of cytokines produced toward a dominant Th1-like response *in vivo*, while in unsuccessful cases the balance is tilted toward a dominant IL-4/10 Th2-like response. Further studies in this system will resolve the mechanism(s) responsible for the distinct cytokine responses induced by OA-POL treatment in C57Bl/6 compared to BALB/c mice.

Literature Review

1.1 Allergy and IgE.

Allergy is the clinical manifestation of immediate hypersensitivity reactions directed against allergen. Various forms of allergy affect between 10 and 20 % of western populations. Specific IgE antibody is central to the induction and maintenance of the capacity of allergens to elicit immediate hypersensitivity reactions. The fact that this isotype is found in only relatively minute levels in normal individuals suggests that its synthesis is under stringent regulation. The focus of this research project has been to develop methods that result in antigen specific, permanent inhibition of IgE responsiveness in an animal model of immediate hypersensitivity to ovalbumin. By accomplishing this goal we wish to better understand the mechanisms involved in the regulation of the IgE isotype *in vivo*. Only by better understanding the parameters involved in the regulation of IgE can therapeutic methods of improved efficacy be developed.

Allergic disease has been described clinically for centuries. Common forms of human allergy include asthma, hay fever (rhinitis), and in extreme cases, systemic anaphylaxis. Common allergens include animal dander, dust, drugs, food components, grass pollens and insect venoms. In

1966 "reaginic" antibodies in the serum of hay fever patients were found to belong to a novel class of immunoglobulin (Ishizaka, 1966). This unique isotype was named IgE three years later (Bennich, 1969).

The central role of IgE in immediate hypersensitivity reactions is dependent upon the ability of this isotype to be bound by high affinity IgE Fc receptors ($Fc_{\epsilon}R$ 1), expressed at high levels on mast cells and basophils. Once sensitized in this way, mast cells acquire the ability to react specifically and immediately in response to secondary exposure to the allergen. Cross-linking of surface bound IgE antibodies by allergen causes mast cell degranulation, releasing a variety of preformed and newly synthesized substances, which together mediate the clinical symptoms of allergy. Briefly, major preformed mediators include histamine, serotonin, chemotactic factors, heparin, platelet-activating factor and a variety of proteolytic enzymes. Activation of phospholipase A_2 releases arachidonic acid from the plasma membrane from which prostaglandin A_2 and D_2, thromboxane A_2 and leukotrienes LTC_4, LTD_4 and LTB_4 are synthesized. These mediators induce clinical symptoms including bronchoconstriction, mucosal edema and hypersecretion.

1.2 Allergen Immunotherapy.

Allergic disease is commonly managed by some combination of avoidance of allergen, pharmacological treatment and, in appropriate cases, allergen immunotherapy. Avoidance of the allergen responsible for disease is most effective but often impossible to accomplish. Pharmacological approaches to the treatment of allergy offer significant relief from symptoms and represent the most widely used and effective means of patient management. However, this therapy does nothing to correct the underlying disregulation of the IgE isotype responsible for disease manifestation. The attraction of allergen immunotherapy is its potential to provide long-term relief from symptoms in appropriately selected patients. Although initially developed on an empirical basis, modern attempts to improve allergen immunotherapy have as a major goal the specific and permanent inhibition of allergen specific IgE, thus correcting the defect responsible for allergic pathology.

Attempts to alter patient sensitivity to allergen through immunotherapy were first introduced in 1909. In this study of rhinitis patients, clinical symptoms were reduced after patients were given serial injections of crude pollen extracts (Noon, 1911). This approach continues to be practised and currently involves multiple injections of increasing doses of allergen extracts over time. The success of therapy is measured by the degree of reduction in

sensitivity to the allergen, as manifested by the ability of the patient to tolerate higher allergen doses, a decrease in clinical symptoms and reduced need for medication.

In cases of allergy to a defined substance, that do not respond well to pharmacologic intervention, immunotherapy offers an alternative, widely utilized, form of treatment. The ability of this therapy to reduce symptoms has been demonstrated in controlled clinical trials for asthma (Creticos, 1989), allergic rhinitis (Norman, 1968; Norman, 1978), and insect venom allergy (Hunt, 1978). The major liability in the administration of allergen is the rare induction of severe systemic anaphylactic reactions, which may result in death (C.S.M., 1986; Lockey, 1987). Moreover, the efficacy of allergen immunotherapy varies widely in different individuals, thereby decreasing the attractiveness of this approach.

Because allergic patients have mast cells sensitized with allergen specific IgE effectively acting as a trigger for mast cell activation, administration of native allergen can cross-link the mast cell bound IgE causing immediate local or systemic reactions. This problem is amplified by the demonstration that clinical benefit from allergen immunotherapy is related to the dose of allergen employed in therapy. Higher doses were better at reducing clinical symptoms but were more likely to induce anaphylaxis (Creticos, 1987). Because small

allergen doses are required for safety, effective treatment often requires years of therapy. An additional complication is the return of symptoms in up to 50% of patients after stopping therapy (Norman, 1988).

Various strategies of chemical modification of allergens have been investigated in order to reduce the antigenicity of allergens and consequently increase the safety of treatment, while retaining therapeutic efficacy. These include reaction with formaldehyde (Marsh, 1970), urea denaturation (Ishizaka, 1975), coupling to monomethoxypolyethylene glycol (mPEG) (Lee, 1977), and polymerization by glutaraldehyde (Patterson, 1973, Johansson, 1974) among others. Under the appropriate reaction conditions such chemical modification results in products displaying markedly reduced antigenicity that retain immunogenicity. Pretreatment studies in animal models demonstrated the ability of some forms of modified antigen to inhibit induction of allergen specific IgE (Marsh, 1971; Bach, 1974; Usiu, 1979; HayGlass, 1983) and, in some cases, also IgG responses (Takatsu, 1975; Sehon, 1982; Wilkinson, 1987).

Clinical studies of immunotherapy utilizing glutaraldehyde polymerized crude ragweed allergen extract or polymerized purified ragweed antigen E established that the modified preparation was essentially equal to unmodified allergen in reducing patient symptom scores (Patterson, 1981; Grammer, 1983). Notably, the reduction in antigenicity displayed by these products resulted in

40 fold lower erythema and 15 fold less induration at the injection site compared to crude extract treatment. Furthermore, while there were seven systemic reactions when crude allergen was utilized none occurred with the polymerized allergen preparation (Bacal, 1978). To date, the main advantage of this approach has been the reduction in local and systemic reactions allowing higher doses of the polymerized product to be used, thereby reducing the number of treatments required to gain clinical benefits. Treatment with this form of chemically modified allergen failed to inhibit the continued production of allergen specific IgE, instead causing a small increase in serum IgE levels in some cases. As with other forms of immunotherapy, allergen specific IgG levels were markedly elevated after treatment (Hendrix, 1980).

The mechanism(s) responsible for the clinical improvement observed after immunotherapy remain controversial. Although reductions in allergen specific IgE after prolonged immunotherapy have been reported (Van Meter, 1979; Reid, 1986) IgE levels are not consistently reduced by either allergen or chemically modified allergen immunotherapy. In fact, in some studies, IgE antibody levels have been shown to be elevated at the time clinical improvement was noted (Cockroft, 1977; Hendrix, 1980; Bousquet, 1988). The most dependable outcome derived from immunotherapy of human allergy patients is the induction of high levels of allergen specific IgG. This "blocking IgG antibody" was postulated to interact with allergen before it enters the

vicinity of mast cells, in effect competing with mast cell bound IgE for the allergen. While this mechanism may play a role in the benefits derived from insect venom immunotherapy, blocking antibody levels correlate weakly with clinical improvement in inhalant allergies (Sadan, 1969; Bousquet, 1990), and are therefore unlikely to be of significant protective valuein inhalent allergy (Bousquet, 1989; Ewan, 1989).

A survey of the literature indicates that various forms of allergen immunotherapy can result in the reduction, but not elimination, of allergic symptoms. It should be noted that most studies employed relatively crude allergen extracts containing multiple components, many of which are not related to the allergic disease being treated. Moreover, the potency of these extracts vary from lot to lot, a factor thought to contribute to serious systemic reactions. Chemical modification of such extracts usually results in a wide range of reaction products each with potentially distinct antigenic and immunogenic properties. This may partially explain the difficulty in evaluating the immunological mechanisms involved in clinical improvement, and may account for the differences in IgE regulation observed in animal pretreatment models (using defined homogeneous chemically modified antigens) compared to clinical application.

The slow progress in elucidation of the mechanism involved in allergen

immunotherapy, the variable clinical benefit often derived and the danger of anaphylactic shock have resulted in restrictions on the use of such therapy in some countries (Committee, 1986), and a reexamination of this practise by the World Health Organization (WHO) and the International Union of Immunological Societies (Thompson, 1989). These reports conclude that immunotherapeutic methods of increased safety and efficacy are required. To meet these objectives, models of immunotherapy which utilize homogeneous allergen preparation are needed to define the mechanisms involved in successful therapy.

The increasing availability of pure recombinant allergens may lead to significant improvements in allergen immunotherapy. Recombinant allergens are much easier to standardized, increasing the safety of immunotherapy. Chemically modified derivatives of recombinant allergens should display reduced heterogeneity which should also improve safety. At the same time, in the absence of an understanding of the immunological mechanisms responsible for decreased responsiveness following allergen immunotherapy, administration of recombinant allergens is unlikely to lead to a substantial improvement in this form of patient management.

1.3 Regulation of the IgE isotype.

The mechanisms regulating IgE production remain controversial despite intense investigation. Understanding the factors involved in the induction and maintenance of IgE responses are critical to development of an effective strategy for the management of allergy. Evidence has been provided by several laboratories to support the existence of a wide range of IgE regulatory molecules produced by CD4 and/or CD8 T cells. These include suppressor T cells and their factors, IgE binding factors, and the reciprocal activities of IL-4 and IFNγ on IgE synthesis. Of these three major schools of thought, the role of T cell subsets and their cytokines is the most intensely studied at present.

1.4 Inhibition of IgE responses by suppressor T cells.

A large body of evidence is consistent with the existence of suppressor or suppressor/inducer derived molecules (TsFs) capable of regulating antibody responses, including IgE. Features common to many suppressor T cell systems include the involvement of a Lyt-1^{+}, 2^{-} suppressor inducer population that activates a Lyt-1^{-}, 2^{+} suppressor effector population. In addition, some systems include an intermediate population between inducer and effector. Communication between inducer and effector populations and the suppressive

effect itself is mediated by soluble T suppressor factors (TsFs) that often act in an I-J and\or IgH restricted manner (Dorf, 1984).

The study of these cells and their factors is often hampered by the relative complexity of the systems in which such activity is observed, an inability to define the I-J molecule thought to act as a restriction element, the lack of identification of Ts cell receptors capable of directly binding antigen, and/or an inability to define the nature of the regulatory TsF molecules at the molecular level. However, advances in the understanding of Ts cells, their receptors and the nature of TsF have recently accumulated (Murphy, 1993).

The first demonstration of a T cell derived antigen specific suppressor factor was made in a system where hapten specific IgE responses of recipient rats were suppressed following the transfer of spleen cells, or the cell free extracts derived from these cells, from donor rats hyper-immunized with carrier antigen and *Ascaris suum* extract (Tada, 1979). The factor was characterized as a protein of M_r 35-60. This study suggested that unique isotype specific factors and cells were involved in the regulation of IgE, however, these results could not be duplicated in mice. Although a number of other studies have demonstrated IgE specific suppressor T cells, this research suffers from a lack of progress in defining the active factors at the molecular level.

A well studied model of IgE regulation utilizes conjugates of protein antigen and monomethoxypolyethylene glycol. This form of chemical modification converts the antigen into a tolerogenic conjugate exhibiting greatly reduced antigenicity. Treatment of mice with this modified antigen inhibits immunoglobulin production through induction of antigen specific CD8 T cells that actively mediate suppression. Clones representing this type of regulatory T cell have been isolated from mice treated with a human myeloma IgG (HIgG) mPEG conjugate and, independently, from mice treated with OA mPEG conjugates (Takata, 1990; Chen, 1992). Both Ts clones expressed an $\alpha\beta$ TCR/CD3 complex and produced factors that strongly suppressed *in vitro* antibody production in an antigen specific and MHC class I restricted manner. The factor from the HIgG specific clone was found to be serologically related to the β chain of the TCR, while the factor derived from OA specific Ts clones reacted with antibodies directed against both the α and β chains. These studies revealed that the suppressive activity of these cells was dependent upon the production of factors related to, but at least in one case, not identical to the $\alpha\beta$ TCR. Molecular definition of the TCR utilized by these clones has been accomplished (Mohapatra, 1993) and characterization of the TsF at the molecular level is an active area of research.

1.5 IgE Binding factors.

A model proposed by Ishizaka and colleagues (Ishizaka, 1985), argues that the enhancement or inhibition of an IgE response depends upon the state of N-linked glycosylation of a cDNA characterized IgE binding factor produced by $FC\epsilon R^+$, Lyt 1^+ T cells. In a humoral immune response where IgE is not prominent, a glycosylation inhibiting factor (GIF) is produced by Lyt 2^+ T cells. This factor acts on the $Lyt1^+$ T cell to inhibit the addition of an N-linked oligosaccharide moiety to the IgE binding factor, resulting in an IgE-suppressive factor, which inhibits IgE synthesis. In contrast, in a situation where IgE responses are induced a glycosylation enhancing factor (GEF) is thought to be produced by Lyt 1^+, FcR^+ T cells. This factor promotes N-linked glycosylation of the same IgE binding factor, resulting in enhancement of IgE responsiveness.

A monoclonal antibody specific for lipomodulin was found to recognize GIF and T cell suppressor factors from two types of hapten specific Ts hybridomas. In *in vitro* assays, these Ts factors were found to have IgE binding factor glycosylation inhibiting activity similar to GIF and lipomodulin (Steele, 1989). Both antigen binding and non-binding forms of GIF and GEF have been described. The antigen binding factors are produced by hybridomas that express the CD3 $\alpha\beta$ T cell receptor complex and the factors themselves were

found to be serologically related to this TCR (Iwata, 1989). Thus there appears to be a relationship between the cells and factors active in this system and those found in hapten specific suppressor cell circuits.

The weakness of this model is that it is based primarily on *in vitro* observations. To date persuasive evidence supporting of the importance of GIF, GEF, and IgE binding factors in the *in vivo* regulation of IgE responses is lacking.

1.6 The role of helper CD4 T cell subsets and their cytokines in immunoregulation.

The discovery that murine CD4 T cell clones could be divided into groups designated Th1 and Th2 based on their ability to produce partially exclusive cytokine synthesis patterns, has led to a new concept of immunoregulation. Th1 clones produce IL-2, IFNγ and LT, while Th2 clones produce IL-4, IL-5, IL-6, IL-10 and IL-13, in response to activation by antigen or conconavalin. IL-3, GM-CSF, TNF and other cytokines are produced by both subsets (Mosmann, 1986, 1989). Th1 and Th2 clones appear to represent polar extremes of helper T cell cytokine synthesis differentiation. Mixed patterns of cytokine production (Th0) are commonly observed in clones isolated soon after *in vivo* antigen exposure (Kelso, 1988; Firestein, 1989; Sreet, 1990), and are

commonly seen among human T cell clones (Pene, 1988a).

Prior studies of *in vitro* polyclonal B cell activation in response to LPS demonstrated that IL-4 is strictly required for the induction of IgE class switching (Coffman, 1986). In this system, addition of rIFNγ was found to inhibit the IgE isotype in a dose dependent manner, instead promoting the production of IgG_{2a} (Snapper, 1987, 1988). At high doses IFNγ inhibits B cell activation/proliferation, thereby suppressing antibody production (Reynolds, 1987).

These findings led to the demonstration that differences in the activities of Th1 and Th2 derived cytokines could cause these cells to carry out distinct effector functions. Th1 cytokines had the capacity to: 1. inhibit IgE synthesis, 2. enhance IgG_{2a} production, 3. increase macrophage MHC class II, and Fc receptor expression, 4. activate macrophage killing pathways (Murray, 1985), and 5. participate in delayed hypersensitivity responses (Cher, 1987). Each of these activities are characteristic of responses dominated by cell mediated immunity .

In contrast, Th2 cytokines were found to: 1. be efficient at inducing B cell activation and differentiation, 2. provide potent help for Ig production especially IgE and IgG_1 (Coffman, 1988), and 3. induce the expansion of

eosinophils and mast cells (Sanderson, 1986). Each of these activities are thought to be important parameters in the induction and maintenance of allergy.

The accumulation of evidence that Th1 and Th2 cytokine synthesis patterns allow these helper subsets to mediate distinct effector functions helped explain the ability of helper T cells to participate in both humoral and cell mediated immune responses. This model has now been shown to be involved in many aspects of immunoregulation and is generally believed to be an important component in the regulation of IgE responses in humans (Plaut, 1990; Romagnani, 1990). Definition of the parameters involved in the induction of Th1 versus Th2 responses may be central to understanding the genesis of allergy.

1.7 Regulation of immunoglobulin isotypes by cytokines.

In vivo studies have confirmed the ability of IL-4 and IFNγ to reciprocally regulate IgE synthesis. Neutralization of IL-4 activity in vivo by anti-IL-4 or anti-IL-4 receptor mAb inhibited 95% of primary and secondary IgE responses induced by goat anti-mouse IgD, parasite infection or antigen adsorbed to $Al(OH)_3$ (Finkelman, 1986, 1988a, 1990, 1991). Goat anti-mouse IgD

treatment induces large T cell dependent polyclonal increases in serum IgE and IgG_1 and smaller increases in IgG_{2a} and IgG_3. *In vivo* administration of high doses of recombinant IFNγ or IFNα strongly inhibited IgE production and simultaneously induced increases in IgG_{2a}. Furthermore the high levels of IgG_{2a} induced in mice by injection of fixed *Brucella abortus* was largely suppressed by administration of anti-IFNγ mAb (Finkelman, 1988b, 1991b). These findings added support for the notion that IgE responses could be regulated by the reciprocal antagonistic activities of IFNγ and IL-4 on B cell differentiation *in vivo*.

Many of the findings in the mouse system were confirmed to operate in humans. The induction and maintenance of human IgE production in cultures of peripheral blood mononuclear cells (PBMC), or by purified B cells cultured with human T cell clones, is highly dependent on IL-4. IL-4 induced IgE synthesis in these cultures is enhanced by IL-5, IL-6, IL-9 (Dugas, 1993) and sCD23 and strongly inhibited by IFNγ (Del Prete, 1988; Pene, 1988a, 1988b; Vercelli, 1989). IFNα, IL-8 (Kimata, 1992), IL-10 (Punnonen, 1993) and prostaglandin E2 also appear to have the capacity to inhibit *in vitro* induced IgE. In B cell cultures containing Th2 clone supernatants, the addition of anti-IL-4 antibodies specifically inhibited IgE production, with little effect on other isotypes (Pene, 1988c).

IgE synthesis was found to be similar in cultures derived from either atopic or normal donors when stimulated with recombinant IL-4, suggesting that the defect resulting in the over production of IgE in atopics does not reside in the B cell compartment (Claassen, 1990). A related study of IgE production in IgE nonresponder SJA/9 and nude mice, found that these mice do not produce detectible IgE in response to *Nippostrongylus brasiliensis* infection. However, cultures of purified B cells in the presence of IL-4 and LPS produced quantities of IgE very similar to that induced in IgE responder mice (Azuma, 1987). This data supports the finding in humans that differential production of IgE was not the result of defective B cell class switching or differences in sensitivity to the effects of IL-4.

1.8 The requirement for CD40/CD40L interaction in isotype switching.

In addition to IL-4, a contact dependent signal between B and T cells is required to elicit IgE synthesis (Vercelli, 1989). The finding, by a number of laboratories, that purified plasma membranes from activated but not resting Th clones could induce resting B cells into cell cycle resulted in the hypothesis that activated T cells expressed a membrane associated protein that could induce the activation of resting B cells. The discovery of CD40 expression by human and mouse B cells and the ability of anti-CD40 mAb to substitute for

T cells in IL-4 induced IgE production suggested that interaction of this molecule with its ligand expressed on activated T cells was sufficient to provide the required second signal for IgE synthesis.

Molecular cloning of the CD40 ligand demonstrated its identity to gp39, a type II membrane protein expressed on all subsets of human helper cells after activation with anti-CD3. The gp39 gene shares homology with TNF-α and TNF-β. *In vivo* administration of anti-gp39 inhibited both primary and secondary humoral immune responses arguing in favor of a role for this interaction in B cell activation (reviewed, Noelle, 1992).

Further evidence of the importance of this interaction *in vivo* is derived from a study of X-linked hyper-IgM syndrome. This disease is characterized by very low levels of serum immunoglobulin other than IgM. In three patients with this disease the CD40L gene contained distinct point mutations resulting in a protein unable to bind to CD40. The B cells derived from these patients responded normally to CD40L. The profound inhibition of immunoglobulin class switching observed in this disease highlights the importance of the interaction between B cell CD40 and activated T cell gp39 in initiating B cell activation/differentiation (Allen, 1993).

1.9 Factors involved in the determination of cytokine synthesis patterns.

2.0 Cross-regulation between Th1 and Th2 subsets.

The idea that Th1 and Th2 induce distinct immune effector functions was supported by earlier work that suggested that delayed hypersensitivity reactions (DTH) and antibody responses were "opposing immunological processes" with mutually exclusive properties. Although immune responses are usually found to be a mixture of humoral and cell mediated immunity, cases of immune deviation (Asherson, 1965) and split tolerance (Crowle, 1966) were described where DTH responses were suppressed in the absence of any inhibition of the antibody response. In a reciprocal fashion, antibody tolerance in the presence of normal levels of DTH had also been described (Genic, 1970).

Parish established that the degree of chemical modification (acetoacetylation) of flagellin dictated whether the product induced an antibody or DTH response (Parish, 1971). Most notable in these studies was the fact that as a DTH response was induced the antibody response was inhibited and vice versa. More recent studies have confirmed that not only do Th1 and Th2 induce distinct immune effector mechanisms, they also have mutual inhibitory activities on the induction/expansion or activity of clones producing the opposite cytokine pattern.

In vitro studies with T cell clones in the laboratory of Frank Fitch demonstrated that proliferation, but not cytokine synthesis, of a panel of Th2 clones was inhibited by relatively low levels of IFNγ. This effect occurred regardless of the mode of activation (antigen, mitogen, or TCR ligation by mAb) even in the presence of exogenous IL-2 and IL-4 (Gajewski, 1988; Fitch, 1993). Furthermore, derivation of clones in the presence of IFNγ resulted in the predominant isolation of Th1 (Gajewski, 1989). Thus, IFNγ has the capacity to inhibit the clonal expansion of Th2.

The fact that antibody responses occurred in the absence of DTH implied that Th2 clones also have the capacity to inhibit Th1 activity. This notion guided the discovery of IL-10 (Fiorentino, 1989). IL-10 is produced by murine Th2, but not Th1 clones, B cells and LPS stimulated macrophages. This cytokine acts indirectly by inhibiting the ability of macrophages to support Th1 production of IFNγ, IL-2 and IL-3. The synthesis of IFNγ by CTL clones is also inhibited. Decreased production of IFNγ would minimize the antagonistic effect of this cytokine on Th2 clones. Furthermore, by decreasing the secretion of IL-2, IL-10 may function to regulate the clonal expansion of IFNγ producing T cells.

Rapid advances in the study of IL-10 function have determined that this cytokine decreases macrophage cytokine synthesis, inhibits macrophage killing pathways activated by IFNγ, increases the expression of MHC class II by B cells, augments activated B cell proliferation/differentiation, and has a synergistic effect with IL-4 or IL-3 to stimulate mast cell growth (reviewed, Mosmann, 1991; Howard, 1992; de Waal Malefyt, 1992; Moore, 1993). Collectively, these activities are consistent with the enhancement of humoral immune responses and allergy, while simultaneously inhibiting cell mediated immune responses. The immunosuppressive properties of this cytokine on the expansion and activity of Th1 clones is thought to be central to the inhibition of cell mediated immune responses observed after parasite or retrovirus infection (reviewed, Sher, 1992a).

A potentially significant discrepancy between the mouse and human systems is the fact that IL-10 is produced by Th0, Th1 and Th2 human CD4 clones, although it is only produced by murine Th2. In mice this cytokine appears to have the function of inhibiting Th1 activity and macrophage activation while promoting B cell function and humoral responses. In humans the activity of this cytokine appears to be more complex, inhibiting the proliferation of all Th subsets, IFNγ production by Th1 and IL-4/IL-5 production by Th2 (Del Prete, 1993a). This activity may reflect a dampening mechanism designed to regulate the intensity of immune responses. Alternatively, a role for

IL-10 in the initial differentiation of naive human T cells toward distinct patterns of cytokine synthesis has not yet been thoroughly investigated.

2.1 IL-4 and IFNγ/IL-12 reciprocally regulate the differentiation of helper T cell cytokine synthesis patterns

Cytokines present in the local environment at the time of immunization have the potential to bias helper T cell cytokine synthesis patterns. The addition of exogenous IL-4 to cultures of naive CD4 T cells stimulated with polyclonal activators resulted in the development of effector cells capable of producing high levels of IL-4 and IL-5, while inhibiting the outgrowth of cells producing IFNγ (Le Gros, 1990). In this system, the presence of IFNγ at the time of activation drove naive cells toward a Th1-like response (Swain, 1990). Subcutaneous implantation of diffusion chambers containing IL-4 producing (IL-4 cDNA transfected) plasmacytoma cells prior to KLH/CFA immunization significantly enhanced the ability of KLH specific CD4 T cells primed *in vivo* to produce IL-4 upon *in vitro* restimulation (Yoshimoto, 1993).

Three independent studies of cytokine synthesis by PBMC demonstrated that addition of IL-4 to these cultures inhibited the spontaneous synthesis of IFNγ, and IFNγ induced in mixed lymphocyte cultures or by mitogen. The

degree to which IFNγ synthesis was inhibited correlated well with the level of IgE induced. Addition of anti-IFNγ to these cultures resulted in strongly enhanced IgE synthesis (Peleman, 1989; Vercelli, 1989; Chretien, 1990) These studies confirmed that IL-4 supports the development of increased IL-4 production and inhibits IFNγ synthesis *in vitro*.

IL-4 has the capacity to bias cytokine synthesis patterns when present during priming *in vitro*, however, the potential *in vivo* sources of IL-4 prior to T cell activation remain undefined. Both mast cells (Plaut, 1989) and non-B non-T cells (Ben-Sasson, 1990) secrete IL-4 in response to $Fc_{\epsilon}R$ crosslinking, however, this activity would depend upon the presence of IgE. IL-4 produced in this way may contribute to the maintenance of allergic responses and could potentially influence the outcome of T cell priming during a concurrent immune response. However, the absence allergen specific of IgE prior to sensitization makes it unlikely that this mechanism would contribute significantly to Th2 development during the primary immune response.

Although suppressive IL-4 producing CD8 T cells have been described in leprosy (Sieling, 1993), and a number of restimulation procedures are capable of inducing de novo IL-4 synthesis from CD8 T cells (Seder, 1992; Erard, 1993), CD8 T cells generally produce IFNγ at levels similar to Th1 (Fong, 1990). NK cells also posses the ability to secrete large amounts of IFNγ

(Michael, 1989). The production of IFNγ by these cells before or during the activation of helper T cells is suggested to prejudice the response toward Th1 development.

Recent characterization of IL-12 activities has found that this cytokine may elicit the activation of such IFNγ producing cells early during an immune response. IL-12 is a heterodimeric cytokine produced by macrophage and B cells. It was first characterized by its involvement in the regulation of NK and T cell cytotoxicity, proliferation and IFNγ production (D'Andrea, 1992; Perussia, 1992). The ability of this cytokine to induce the synthesis of IFNγ by NK and CTL may be an important mechanism that provides a source of IFNγ early during the genesis of an immune response.

In mice injected with goat anti-mouse IgD, *in vivo* administration of recombinant IL-12 strongly augmented the expression of IFNγ and IL-10 mRNA, while almost completely inhibiting the induction of IL-4 message (Morris, 1993). This treatment inhibited IgG1 and IgE by more than 95% and IgG_{2a} and IgG_3 by 70%. Anti-IFNγ mAb (± anti-IFNα/β mAb) greatly abolished the suppressive effect of IL-12 on IgG1 synthesis, but only slightly decreased the inhibition of IgE. Surprisingly IgG_{2a} and IgG_3 responses were enhanced in these mice. This study suggests that the strong induction of IFNγ by IL-12 is responsible for much of the inhibition of Ig caused by this cytokine, in a manner similar to high levels of IFNγ inhibiting production of all isotypes in the

in vitro LPS B cell activation model. However, it also implies that IL-12 may have the ability to inhibit IgE and enhance IgG_{2a} and IgG_3 responses independent of IFNα or IFNγ. The ability of IL-12 to inhibit IgE by an IFNγ independent mechanism was also reported in an *in vitro* human mononuclear cell culture system (Vessio, 1993).

In summary, cytokines in the local environment during T cell activation may influence the development of T helper cell cytokine synthesis patterns. The presence of IL-4 strongly directs the development of Th2-like responses. This activity is inhibited by IFNγ, which directs the development of Th1. The potential source of IL-4 *in vivo* during a primary immune response remains undefined. IL-12 may provide an indirect mechanism inducing the synthesis of IFNγ by CTL and NK cells early during the induction of an immune response.

2.2 The role of antigen presenting cells and co-stimulation in the expansion of Th1 and Th2 subsets.

The antigen dose and route of immunization profoundly influences the nature of the resulting immune response. One explanation for this is that antigen entering the body through different pathways is predominantly picked up and presented by distinct APC populations. A number of cell types are

capable of MHC class II expression and have antigen presentation abilities. These include B cells, dendritic cells, endothelial cells, eosinophils, keratinocytes, Langerhans cells, murine hepatic accessory cells and macrophage. The *in vivo* function of these distinct APC populations during the initiation of immune responses has been studied, but to date is not well understood. Naive T cells require initial interaction with dendritic cells in order to induce proliferation (Croft, 1992). Once committed to a cytokine synthesis pattern, Th1 cells proliferate optimally in response to antigen presented by macrophage, while resting B cells optimally stimulate Th2 expansion (Gajewski, 1991). Once activated, B cells efficiently stimulate both Th1 and Th2 proliferation (Stack, 1993). Similarly, adherent murine hepatic accessory cells stimulate proliferation of Th1 but not Th2 (Magilavy, 1989). Equivalent production of cytokine from both Th1 and Th2 occurs regardless of the APC employed. This effect is independent of antigen processing differences between APC types. These results suggest that preferential presentation of antigen by macrophage/liver adherent cells or by resting B cells during the initiation of an immune response or during restimulation may lead to the predominant expansion of Th1 or Th2 clones respectively.

The primary APC of the epidermis is the Langerhans cell. The use of FITC or TNP haptenated Langerhans cells as APC during the generation of T cell lines resulted in the exclusive isolation of lines producing IL-4 but no IL-2 (IFNγ

was not determined) that were efficient at inducing IgE production, characteristic of a Th2 phenotype (Hauser, 1989). In an independent report, Langerhans cells (Ia^+ epidermal cells) were found to induce proliferation of both Th1 and Th2 KLH specific clones. However, after low dose ultraviolet B-irradiation these APC lost the ability to stimulate Th1 but retained their ability to support proliferation of Th2. UV Irradiated splenic adherent cells lost their ability to induce proliferation of either subset (Simon, 1990).

The recent demonstration of IL-10 production by keratinocytes offers a reinterpretation of these studies. Keratinocytes express MHC class II upon exposure to pro-inflammatory cytokines and have the ability to produce IL-1, IL-6 and a wide range of other cytokines, making this cell type a potential APC. Injection (i.p.) of mice with hapten modified Ia^+ keratinocytes induces the selective inhibition of DTH but not antibody responses (Gaspari, 1991). A similar phenomena also occurs after total body UV irradiation and can be mimicked by the injection of supernatants from *in vitro* UV irradiated keratinocytes (Rivas, 1992). It was recently established that both haptens with contact allergen activity and UV irradiation induce high level IL-10 production by keratinocytes (Enk, 1992). Thus contamination of the Langerhans cell preparations by keratinocytes in both the study by Hauser and Simon could have been responsible for IL-10 production. This IL-10 may have acted on the Langerhans cells selectively inhibiting their ability to support Th1 but not Th2

cytokine production and as a result, proliferation. This would account for the isolation of only Th2-like clones in this study.

Although the activity of IL-10 on Langerhans cells has not been reported, IL-10 acts on dendritic cells to inhibit their ability to induce IFNγ production by Th1 clones or naive T cells (Macatonia, 1993). It is therefore reasonable to assume that IL-10 may affect the APC function of Langerhans cells also. The release of IL-10 in response to haptenation may represent a dampening mechanism by which sensitization to contact allergens may be specifically inhibited. The ability of UV B-irradiation or haptenation to induce keratinocyte IL-10 production could be an important nonspecific regulatory mechanism capable of altering APC function during the initiation of a concurrent immune response, biasing immune reactivity in the skin toward a Th2 response. Further studies on the factors capable of inducing keratinocyte IL-10 secretion will determine whether this mechanism has a general role in cutaneous immunity.

The capacity of APC to differentially stimulate Th1 and Th2 proliferation may depend upon their ability to express co-stimulator molecules. It is clear that both specific and nonspecific signals are required to induce helper T cell proliferation. Ligation of the T cell receptor by APC MHC class II/peptide complexes delivers the specific signal which must be accompanied by

costimulation, delivered by factors such as IL-1. In some studies, Th2 clones appear to require APC expression of IL-1 in order to induce optimal proliferation (Weaver, 1988), whereas Th1 fail to express the IL-1 receptor (Lichtman, 1988). The molecule responsible for Th1 costimulation is present on freshly isolated macrophage and can be induced on B cells following activation by anti-Ig and IFNγ (reviewed, Weaver, 1990). This mechanism may be important to the ability of activated but not resting B cells to induce vigorous proliferation of both Th1 and Th2 clones (Stack, 1993). Collectively, these findings suggest that distinct APC populations may predominantly express factors that provide optimal costimulation for only Th1 or Th2 proliferation, selectively expanding one or the other phenotype. Thus, the state of co-stimulator expression may be an important factor regulating the ability of distinct APC populations to induce proliferation by clones already committed to Th1 or Th2 cytokine synthesis patterns.

Prostaglandin synthesis by APC is another factor which may have a significant role in the induction of distinct cytokine synthesis patterns. PGE_2 is produced by several APC after activation of the cyclo-oxygenase pathway, and inhibits the production of IFNγ and IL-2 *in vitro*, without inhibiting Th2 cytokines (Snijdewint, 1993). Thus, presentation of antigen in the presence of PGE_2 may direct the development of Th2 responses. Insect venom contains mediators with phospholipase activity that induce prostaglandin production by

APC (Hoffman, 1990) and human clones specific for phospholipase A2 produce high levels of IL-4 (Carballido, 1992). One report suggests that APC from allergic patients produce higher levels of prostaglandins in both the resting and activated state (Jakob, 1990). Therefore, at least in some cases, differential induction of PGE_2 in APC populations may represent an additional mechanism capable of disrupting the early balance between cytokines that support the development of Th1 versus Th2 dominated responses.

2.3 Transgenic T cell models of naive CD4 T cell priming.

A weakness of studies focusing on the *in vitro* characteristics of helper T cell clones is that such cells are already committed to a particular cytokine pattern and thus can not be used as a model of naive T cell priming. The extremely low frequency of antigen specific precursor T cells in unimmunized mice has necessitated the use of *in vitro* polyclonal activation in models of naive T cell differentiation, which may not accurately represent T cell differentiation induced by antigen *in vivo* (Yang, 1993). One approach to address the factors involved in the differentiation of naive T cells into cells capable of high level IFNγ or IL-4 production is to employ mice transgenic for a single T cell receptor as a source of pure "naive" T cells of a single antigenic specificity. Although not fully equivalent to normal T cell priming, activation

of these naive cells under defined conditions *in vitro* has been utilized as a useful model of *in vivo* T cell priming.

One model employed mice transgenic for an $\alpha\beta$ T cell receptor specific for peptide 88-104 of pigeon cytochrome C in the context of I-E^k (Seder, 1992). CD4 cells from these mice express a naive phenotype, and produce high levels of IL-2 and detectible, but relatively low levels of IL-4 or IFNγ after primary antigen stimulation. In this model, naive cells were primed in cultures containing dendritic cells, IL-2 and peptide ± cytokines or anti-cytokine mAb. After secondary stimulation, high levels of IFNγ, but relatively little IL-4, was produced. Addition of IL-4 to the priming culture resulted in cells able to produce high levels of IL-4 and little IFNγ. The addition of IL-10 or anti-IL-10 to the priming culture in the absence of IL-4 did not significantly alter the development of IFNγ production. Addition of IL-10 or anti-IL-10 to priming cultures containing IL-4 did not increase the amount of IL-4 produced in secondary culture, nor did it affect the decrease in the amount of IFNγ produced, implying that the inhibition of IFNγ production in secondary cultures, caused by priming in the presence of IL-4, was not mediated by IL-10.

Addition of moderate to high levels of exogenous IFNγ to priming cultures containing suboptimal levels of IL-4, completely inhibited IL-4 production in the secondary culture and instead led to production of high levels of IFNγ. In this

model, IL-4 directed the development of IL-4 production while the addition of anti-IL-4 mAb led to IFNγ production, irrespective of which APC was used for priming. The addition of IL-1 could not replace IL-4 in promoting Th2 differentiation, and the production of this phenotype was not inhibited by anti-IL-1 mAb or IL-1 antagonist. These results suggest that the balance in which IL-4 and IFNγ are present during priming or the early production of predominantly one or the other cytokine, may be central to the commitment of naive CD4 T cells toward a Th1 or Th2 cytokine synthesis pattern. The lack of an effect of IL-1 suggested that this cytokine is not required as a cofactor for helper T cell differentiation into Th2.

A second similar model utilized a transgenic mouse line expressing the $\alpha\beta$ T cell receptor from a T cell hybridoma reactive to an ovalbumin peptide (Hsieh, 1992). In this system, transgenic T cells were primed in the presence of irradiated whole spleen cells as APC and the specific peptide, with no addition of IL-2 for two rounds of stimulation. Secondary cytokine levels were measured after a third stimulation of washed cells with APC and antigen alone. During primary culture of these cells IL-2 was predominantly produced with low or undetectable levels of IL-4 and IFNγ. If no additional cytokine was added these cells produced a mixture of IFNγ, IL-4 and IL-2 after secondary stimulation. The addition of anti-IL-4 to the priming culture abrogated IL-4 production and enhanced IFNγ and IL-2 levels. Addition of IL-4 to the priming

culture increased the amount of this cytokine produced in secondary culture and decreased IFNγ and IL-2 levels.

Addition of exogenous IL-10 did not increase IL-4 or IL-5 production or inhibit IL-2 or IFNγ, however, neutralization of endogenous IL-10 during priming strongly increased IFNγ and decreased IL-4 and IL-5 production in secondary cultures. Anti-IL-10 increased IFNγ and decreased IL-4 and IL-5 production only when spleen cells from normal or SCID mice were used as APC. Neutralization of IL-10 allowed non-B spleen cells to induce Th1 responses, where as, a Th2 phenotype developed when IL-10 was neutralized in cultures containing TA3 as APC. In contrast, IL-4 was able to mediate its effects when normal spleen cells, SCID spleen cells, or the B cell line TA3 were used as APC.

Many reports indicate that dendritic cells are extremely efficient at activating naive T cells compared to the relative inability found among all other APC tested. Naive T cells derived from $\alpha\beta$ TCR transgenic mice strictly require dendritic cells to induce initial clonal expansion, but these APC do not strongly bias the development of cytokine synthesis patterns (Croft, 1992). However, the addition of peritoneal macrophage to transgenic CD4 T cell cultures stimulated by dendritic cells, resulted in a strongly IFNγ dominated secondary response (Macatonia, 1993a). This finding demonstrates the potential for macrophage to participate in directing naive CD4 T cells toward a Th1

phenotype.

Addition of IL-12 to these cultures also resulted in predominant IFNγ production, while neutralization of endogenous IL-12 blocked differentiation towards Th1 cytokine production regardless of the APC used. In the presence of anti-IFNγ, IL-12 was inefficient at directing Th1 development. The addition of heat killed *Listeria monocytogenes* to cultures containing macrophage also directed Th1 development, through the induction of IL-12 synthesis (Hsieh, in press).

Consistent among these reports is the ability of IL-4 in the primary culture to markedly enhance the development of IL-4 producing cells, and the lack of effect of addition of exogenous IL-10. While anti-IL-10 appeared to have no impact in the cytochrome c model, neutralization of IL-10 markedly enhanced IFNγ and decreased IL-4/5 synthesis when non-B cell APC were used in the OA model.

The demonstration that IL-10 inhibits the ability of dendritic cells to induce IFNγ synthesis by naive CD4 T and CD8 T cells may be a mechanism by which IL-10 can cause the preferential development of a Th2 phenotype (Macatonia, 1993b). In a primary MLR, purified dendritic cells were able to induce detectible IFNγ synthesis when mixed with mis-matched CD4 T cells.

In CD8 T cell primary MLR, 13 fold higher levels of IFNγ were obtained. Since the early presence of IFNγ directs Th1, and inhibits Th2 development, the function of IL-10 to inhibit the production of IFNγ by both CD4 and CD8 T cells when stimulated by either dendritic cells or macrophage, may be essential to the Th2 promoting activity of this cytokine during helper T cell priming.

Peritoneal macrophage directed the development of Th1 in this model. This activity may be based on the ability of this APC to stimulate optimal expansion of Th1, but may also relate to the capacity of this APC to produce IL-12. In this model, IL-12 was also found to direct the synthesis of Th1 cytokines by inducing IFNγ production. The ability of *Listeria* to induce IL-12 production by macrophage may be an important mechanism governing the general induction of cell mediated immunity against intracellular pathogens *in vivo*. The function of IL-12 in this model confirmed the notion that early production of this cytokine by macrophage may induce IFNγ, that then acts to inhibit Th2 development and directs the preferential expansion of Th1.

Collectively, results from these transgenic systems indicate that IL-4/IL-10 and IL-12/IFNγ may reciprocally regulate the development of helper T cell cytokine synthesis patterns. Differences among the findings in these two systems may relate to the use of IL-2 as an exogenous growth factor during priming in the pigeon cytochrome c transgenic T cell receptor model, while it

was absent in the OA mode. The length and number of stimulations used for "priming" may have also been an important variable. Whether relatively pure populations of transgenic T cells primed in bulk cultures *in vitro* reflect the mechanism of priming *in vivo* remains to be determined.

These studies also address whether expression of a particular T cell receptor dictates the cytokine pattern which will develop. Both Th1 and Th2 cytokine patterns can be established using different priming conditions, clearly demonstrating that T cells expressing a single receptor can differentiate into either Th1 or Th2 depending on the activation conditions used. This was confirmed in the *Leishmania major* infection model were both Th1 and Th2 clones derived from normal, non-transgenic mice where commonly found to use the same T cell receptor genes (Reiner, 1993).

The question of whether Thp (naive) T cells must progress through a Th0 stage is also addressed by the TCR transgenic model. Activation of cytochrome c transgenic T cells in the absence of IL-4 leads very quickly to a population of cells that resemble Th1 while the addition of IL-4 leads to the prompt appearance of cells producing the Th2 cytokine pattern. The ability of naive transgenic T cells in either model to quickly progress to a restricted cytokine synthesis pattern argues against the requisite progression of Thp through Th0 to become Th1 or Th2. If this does occur, the Th0 phenotype is very

transitory in this model. Although this area remains unclear the possibility is raised that the Th0, Th1 and Th2 phenotypes may be independent lineages that differentiate from naive T cells under distinct regulatory signals.

2.4 Differentiation pathways of CD4 T cell cytokine synthesis patterns.

Characterization of CD4 T cell clones early after isolation demonstrated the existence of a Th0 cytokine pattern consisting of a mixture of Th1 and Th2 cytokines. In contrast, naive cells had the ability to produce only IL-2 and low levels of IFNγ (Swain, 1988). This phenotype was designated Thp (precursor). In this model, naive T cells required exposure to antigen and differentiation for 4-12 days *in vivo* or *in vitro* before gaining the ability to produce high titers of other cytokines including IL-4. The studies indicating that naive cells can express only IL-2 along with the finding that a Th0 phenotype is isolated shortly after immunization, led to the proposal that Thp cells, once activated, differentiate into Th0 and then under the influence of repeated stimulation, become Th1 or Th2. It is important to note that a number of studies that did not detect IL-4 production employed complete Freund's adjuvant (Powers, 1988; Swain, 1988; DeKruyff, 1992), an immunization method that induces strong IgG_1 and IgG_{2a} responses, but very little or no detectible IgE (personal observation). This type of response is indicative of Th1 activity. In contrast, the use of $Al(OH)_3$ as adjuvant induces strong IgG_1 and IgE responses and

virtually no IgG_{2a}, an immune response indicative of Th2 activity. In our hands, immunization with OA adsorbed to $Al(OH)_3$ results in readily measurable, antigen specific, IL-4 production within 8 hours after restimulation in spleen cell bulk cultures, set up two to four days after immunization (Yang, 1993; Gieni, 1993). Additionally, IL-4 is an important growth and differentiation factor for a variety of cell types and is quickly consumed in culture (personal observation). This may have been a contributing factor to the inability to detect the low levels of this cytokine produced during immune responses where IgE is not a prominent isotype. Hence, the inability to detect IL-4 during the primary response induced by antigen in CFA is not surprising.

In further support of the notion that CD4 T cells capable of IL-4 synthesis reside in the spleen of normal mice is the fact that IL-4 and IL-2 production become detectible the same length of time after OA[$Al(OH)_3$] immunization (2-3 days, Xi Yang personal communication) and both cytokines are induced very rapidly after *in vivo* administration of anti-CD3 mAb (Flamand, 1990; Scott, 1990; Wang, 1992). The lack of detectible IL-4 producing CD4 T cells shortly after immunizations with antigen in CFA may reflect the preferential induction of Th1 and subsequent inhibition of IL-4 producing cells by this form of immunization, or the induction of a low frequency of IL-4 competent cells which require expansion, *in vivo* or *in vitro*, to attain a clonal size that produces detectible IL-4 levels.

2.5 Parasite models: A valuable tool for the study of helper T cell function and differentiation.

A number of models, particularly those involving parasite infection, have established that dominant Th1-like and Th2-like responses can be distinguished *in vivo*, demonstrating that induction of distinct patterns of cytokine synthesis have an important role in the regulation of immune effector function. Parasite infection is a convenient model for the study of Th1 versus Th2 activity *in vivo*, due to the strong immune response elicited against such an infection and the fact that the response is commonly biased toward Th1 or Th2. The best studied example of this being the immune response elicited upon infection with the intracellular protozoa *Leishmania major*. Leishmania species infect humans through injection of the promastigote into the skin via the bite of sandflies of the species *Phlebotomus*. The parasite then enters mononuclear phagocytes where it becomes an amastigote and proliferates.

A strong DTH response often controls infection and induces healing without medical treatment. These patients retain strong immunity to reinfection. In contrast, in patients that mount a strong humoral response instead, the disease proceeds rapidly and often results in death unless intervened by

treatment with toxic drugs. Thus, a protective response in humans depends on the induction of cell mediated immunity, resulting in the activation of macrophage killing mechanisms required to destroy the intracellular parasites.

The immune response elicited upon infection of mice with *Leishmania major* has been extensively studied and reviewed (Scott, 1989; Locksley, 1991; Sher, 1992b). Susceptibility or resistance to infection depends on the nature of the parasite induced immune response. In resistant strains such as C57Bl/6 and B10.D2, infection causes the preferential induction of *Leishmania* specific, IFNγ secreting, CD4 T cells, as determined by bulk culture cytokine production (Sadick, 1986), isolation of clones (Scott, 1988), limiting dilution analysis (Moll, 1990; Morris, 1992) of cytokine synthesis patterns within the CD4 T cell repertoire, and cytokine mRNA analysis (Heinzel, 1991) The Th1 dominated response is characterized by strong parasite specific DTH, no detectible IgE, little IgG with disproportionate levels of IgG_{2a} and destruction of intracellular parasites. This protective response results in a limited infection that remains local and is self healing.

In susceptible strains such as BALB/c, identical infection causes preferential induction of *Leishmania* specific, IL-4/IL-10 producing CD4 T cells. This Th2-like response induces high levels of antibody including prominent IgE, but little IFNγ and DTH activity. This response appears to be non-protective,

does little to slow parasite growth, and results in systemic disease and death. The role of Th1 and Th2 in resistance versus susceptibility was firmly established in studies that found that transfer of parasite specific Th1 clones reversed the susceptibility of BALB/c mice and induced prolonged protection against reinfection while the transfer of Th2 clones exacerbated the infection (Scott, 1988).

In the murine model, susceptibility and resistance was independent of MHC haplotype. BALB/c (H-2^d), BALB/b (H-2^b) and BALB/k (H-2^k) mice were equally susceptible to *Leishmania* infection (Liew, 1985). Moreover, B10.D2 mice were highly resistant despite the presence of the H-2^d haplotype (Boom, 1990). These studies illustrate that non-MHC genetic differences between resistant and susceptible mice are important in the differentiation/commitment of CD4 T cells toward a Th1 or Th2 cytokine synthesis pattern in this parasite model.

Studies characterizing the immune response of resistant and susceptible mice to other intracellular parasites such as *Trypanosoma cruzi* (Silva, 1992) and *Trichinella spiralis* (Pond, 1989) have shown that resistance to these intracellular parasites also depends on the induction of Th1-like responses. In schistosomiasis, protective immunization with irradiated parasites induces a Th1 response (Scott, 1989), while egg deposition is the major stimulus for the

induction of non-protective Th2 responses (Grzych, 1991). In general, Th1 activity is critical for activation of macrophage killing mechanisms required for the effective destruction of intracellular pathogens. In contrast, the induction of a Th2-like response correlates with the ability of mice to expel the nematode *Trichuris muris*, while a Th1 dominated response correlates with chronic infection (Else, 1992). However, in murine malaria infection both Th1 and Th2 responses mediate protection through the induction of distinct effector mechanisms (Taylor-Robinson, 1993). Therefore, depending on the nature of the pathogen either Th1, Th2, or a combination of these responses offer protective immunity.

These studies and others have confirmed the existence of Th1 and Th2-like responses *in vivo*, and attest to the importance of distinct Th1 and Th2 effector functions in regulating immune function. Parasite models clearly demonstrate the potential of Th1 and Th2 to act as reciprocal regulators of cell mediated versus humoral immunity.

The effect of preferential presentation of antigen by macrophage was investigated in the L. *major* model. The capacity of macrophage to initiate Th1 responses *in vivo* was demonstrated by the ability of GM-CSF derived bonemarrow macrophage preincubated with *Leishmania major* antigens to induce a protective Th1 response when injected into susceptible BALB/c mice

(Doherty, 1993). Initial macrophage mediated antigen presentation induced high levels of IFNγ and DTH activity that provided protection against infection, and provided long-term resistance to reinfection. The induction of a protective Th1 response in mice which normally elicit a nonprotective Th2 response revealed the ability of macrophage to participate not only in the preferential expansion of Th1 clones, but also in the initial differentiation of Th1 cytokine synthesis patterns.

The murine leishmaniasis model has proven valuable in determining the role of cytokines in the differentiation of T helper cytokine synthesis patterns *in vivo*. A single injection of anti-IFNγ mAb at the time of *Leishmania major* infection reversed the dominant Th1 response normally produced by resistant C3H/HeN mice to a nonprotective Th2 response (Belosevic, 1989; Scott, 1991). Conversely, susceptible BALB/c mice given IFNγ directly, or a single dose of anti-IL-4 mAb at the time of infection generate a dominant protective Th1 response (Sadick, 1990; Chatelain, 1992). The ability of anti-IFNγ to reverse a protective Th1 dominated response was also demonstrated in a *Trypanasoma cruzi* model, wherein resistant B10.D2 mice were converted to susceptible by administration of anti-IFNγ at the time of infection (Silva, 1992). In these studies, anti-cytokine antibodies were found to mediate their effects primarily through the modulation of Th1 versus Th2 activation and/or expansion, by altering the relative balance of IFNγ versus IL-4 present during

the initiation of the anti-parasite immune response.

The potential role of IL-12 in the *in vivo* induction of protective Th1 responses to *Leishmania major* has been recently investigated. IL-12 levels were higher in resistant C57Bl/6 and CH3/HeN mice than in BALB/c after infection, and correlated well with IFNγ production (Scott, 1993a). Two reports have shown that treatment of BALB/c mice with rIL-12 results in durable resistance and a redirection of the T cell response, as indicated by reduced IL-4 and enhanced IFNγ synthesis. If treatment was withheld for 1 week, little effect on disease outcome was noted. Coincident administration of rIL-12 and anti-IFNγ mAb blocked the induction of a protective response and lead to increased synthesis of IL-4 (Heinzel, 1993). Polyclonal rabbit anti-mouse IL-12 administration at the time of infection rendered C57Bl/6 mice susceptible to infection, and markedly decreased the production of IFNγ (Sypek, 1993). Therefore, early induction of IL-12 in C57Bl/6 mice is central to the ability of this strain to develop protective Th1 immunity. The finding that administration of rIL-12 to BALB/c mice elicited protective immunity argues that the inability of *Leishmania major* to induce sufficient IL-12 synthesis in this strain may be the defect responsible for the failure to develop protective Th1 immunity. The study also demonstrates that IL-12 mediates its protective function through IFNγ.

The protective effect of IL-12 *in vivo* likely depends on its function of inducing IFNγ production by NK cells. A very recent report demonstrated that NK cell activity was much higher in resistant C3H/HeN mice compared to BALB/c in the first week of infection (Scharton, 1993). *In vivo* depletion of NK cells decreased IFNγ production and led to IL-4 synthesis in resistant mice, resulting in enhanced disease. A comparison of a number of murine strains found that the level of NK activity correlated well with parasite burden, illustrating the capacity of NK cells to mediate early resistance to infection and to modulate the differentiation of Th1 responses.

A number of intracellular protozoa and bacteria have now been shown to induce IL-12 production. Initial studies found that *Staphylococcus aureus* induced IL-12 production from both murine and human macrophage (D'Andrea, 1993). It was subsequently demonstrated that T*oxoplasma gondii* induced the production of a macrophage derived molecule capable of inducing NK derived IFNγ, that was essential to the induction of a protective Th1 response (Sher, 1993 in press). This factor has also been identified as IL-12 (Gazzinelli, 1993). Additionally, *Listeria monocytogenes* biases the differentiation of naive, transgenic TCR-bearing, CD4 T cells toward a Th1 phenotype, through the capacity of this bacteria to directly induce IL-12 synthesis upon interacting with macrophage (Hsieh, 1993). Based on these studies, it is concluded that the ability of intracellular pathogens to induce early IL-12 production is a key

parameter directing the development of protective cell mediated immunity. This natural immune mechanism likely provided a selective survival advantage, evolving as a mechanism to provide protection against such intracellular pathogens.

The ability to manipulate the dominant pattern of cytokine synthesis, and consequently disease outcome *in vivo*, by altering the level of IL-12 or the early balance between IFNγ and IL-4, underscores the importance of these cytokines in the early differentiation of a dominant cytokine synthesis pattern *in vivo*. Agents that induce any of these cytokines have the potential to influence the nature of an immune response. In general the studies to date indicate that the balance between IL-12/IFNγ and IL-4/IL-10 present at the time of immunization, or induced first during the genesis of immunity, can skew the differentiation of CD4 cytokine synthesis toward Th1 or Th2 patterns respectively.

2.6 Evaluation of IL-4 and IFNγ function in IL-4 and IFNγ knockout mice.

The creation of IL-4 and IFNγ (and IFNγ receptor) knockout mice has provided an alternative approach to evaluate the function of these cytokines *in vivo*. IL-4 knockout mice were engineered to be homozygous for a disrupted

nonfunctional IL-4 gene (Kopf, 1993). The use of these mice has confirmed the requirement for IL-4 to amplify Th2 development and the ability of this cytokine to inhibit the development of Th1 responses. IL-4 knockout mice failed to support detectible IgE production after administration of goat anti-mouse IgD or infection with *N. brasiliensis,* while high levels of this isotype were produced by normal littermates, confirming the requisite role of IL-4 for IgE synthesis *in vivo*. Specific antibody responses to DNP-OA demonstrated that total Ig levels were unchanged in IL-4 knockout mice. However, the response shifted from being dominated by IgG1 with prominent IgE to antibody production characterized by undetectable IgE, 10 fold reduced IgG_1 and 100-500 fold increased IgG_2 and IgG_3. Thus, in this *in vivo* model IL-4 is not required to induce the production of immunoglobulin, but this cytokine has a profound effect on the pattern of isotypes induced.

Conversely, in transgenic mice engineered to over-express IL-4, serum IgE levels are dramatically increased (Tapper, 1990). These mice develop an intense chronic conjunctivitis distinguished by infiltration by mast cells, eosinophils and mononuclear cells similar to that observed in human allergic disease.

The evaluation of immune function in IFNγ knockout (Dalton, 1993) and IFNγ receptor knockout mice (Huang, 1993) confirmed an essential role for IFNγ in the induction of cell mediated immune responses. In knockout mice infected with *Mycobacterium bovis*, macrophage nitric oxide production was absent and superoxide production and MHC class II expression markedly reduced, compared to IFNγ +/+ littermates. These knockout mice were unable to contain infection by intracellular bacteria or vaccinia virus, leading to lethal pathology not seen in normal mice. The anti-proliferative activity of IFNγ was also confirmed by the ability of spleen cell cultures from knockout mice to remain viable and strongly proliferate for extended time periods, compared to cultures with added exogenous IFNγ, or those from normal littermates.

The role of IFNγ in the regulation of IgE could not be determined due to the use of complete Freund's adjuvant in these experiments. Mice immunized in this way failed to produce detectible IgE. Antigen specific IgG_{2a} responses were reduced, but not absent, in IFNγ knockout mice, confirming a role for other IgG_{2a} inducing cytokines such as IFNα and possibly IL-12. The similarities between the findings in these two types of IFNγ knockout mice strengthens the conclusion that IFNγ is required to activate macrophage killing mechanisms *in vivo*. The ability of normal mice to quickly contain an identical infection demonstrates the necessity for macrophage activation in controlling such infections. Further studies defining helper T cell development and IgE

synthesis in these knockout mice will add to our understanding of the function of IFNγ *in vivo*.

2.7 Human helper T cell subsets in allergy and disease.

Initial evaluation of cytokine synthesis patterns in human alloantigen specific T cell clones suggested that the vast majority were of a Th0 phenotype, where IFNγ, IL-2, and IL-4 along with other cytokines were produced by individual cells (Pene, 1988c). Subsequently, It became apparent that in many cases of strong or continual immunization the majority of clones fell into a Th1 or Th2 phenotype.

CD4 T cell clones specific for dust mite allergens produced IL-4, IL-5 and IL-2, but no IFNγ (Th2-like), when derived from the peripheral blood of atopic patients. In contrast, dust mite specific clones isolated from a nonatopic patient produced IFNγ and IL-2 with at most small quantities of IL-4 (Th1-like). Clones specific for tetanus toxoid or *Candida albicans*, derived from either atopic or nonatopic donors, produced IFNγ and IL-2. These studies suggested that the CD4 T cell repertoire of allergenic patients was compartmentalized, wherein clones specific for dust mite allergens were Th2-like, while clones specific for nonallergens derived from these same individuals, were Th1-like

(Wierenga, 1990a). Th2-like house dust mite specific clones from atopics could induce IgE production by human B cells *in vitro* and this activity was inhibited by dust mite specific Th1-like clones from nonatopics (Wierenga, 1990b).

In independent studies, clones specific for excretory-secretory antigen of *Toxocara canis* (a helminth), derived from normal individuals, were Th2-like, whereas those specific for purified protein derivative of *Mycobacterium tuberculosis* (PPD) had a Th1-like cytokine pattern (Del Prete, 1991). Examination of PPD specific clones from atopics demonstrated that the majority were Th1-like, while clones derived from the same donors specific for dust mite or pollen allergens were Th2-like (Parronchi, 1991).

The ability of Th1 to act as helper cells was limited by their ability to kill B cells expressing the specific epitope, implicating this activity as a mode that may down-regulate humoral immune responses after expansion of Th1 clones (Del Prete, 1992). These studies imply that the defect responsible for allergy involves the disregulation of helper T cell phenotype development within the allergen specific CD4 T cell repertoire of atopic patients, resulting in the predominance of clones that produce IL-4 and IL-5, but not IFNγ.

Evidence that Th2 are involved in allergic disease pathology is provided by the predominant isolation of Th2-like clones from chronic skin lesions of house dust mite or grass pollen allergic dermatitis patients (Ramb-Lindhauer, 1992), or from lesions induced by the topical application of these allergens (Van dert Heijden, 1991; Sager, 1992). Predominantly Th2 clones are also isolated from the conjunctiva of patients with Vernal conjunctivitis (Maggi, 1991). Furthermore, in situ hybridization demonstrated the selective upregulation of Th2 cytokine mRNA expression after allergen challenge in the skin (Kay, 1991), the bronchoalveolar space (Robinson, 1992), and the nasal mucosa (Durham, 1992), suggesting that, in allergic patients, dominant Th2 cytokine synthesis patterns are found among allergen specific lymphocytes *in vivo*. Mostly Th2 clones were also isolated from the bronchi, or nasal mucosa, after similar allergen challenge (Del Prete, 1993b), confirming the importance of Th2 in allergen induced inflammation in allergic patients.

The list of disease states where clones (Haanen, 1991; Yssel, 1991; Salgame, 1991; Schlaak, 1992), or freshly obtained CD4 T cells (Yamamura, 1991) are found to demonstrate distinct Th1 or Th2 cytokine synthesis patterns continues to grow. Thus, it is now established that differential synthesis of distinct cytokine patterns also occurs in humans and may have a prominent role in human immunoregulation.

2.8 *In vitro* restimulation conditions modulate the cytokine synthesis patterns of human helper T cells.

The role of cytokines in the induction of human Th1 and Th2 phenotypes was studied in a model where the effect of cytokine or anti-cytokine mAb addition to PBMC bulk cultures, prior to cloning, was assessed. As described, dust mite specific clones derived from atopics are predominantly Th2-like, whereas PPD specific clones isolated from the same donors secrete a Th1 cytokine pattern. Addition of IFNγ or anti-IL-4 mAb, or particularly the addition of both together to PBMC cultures from atopic patients prior to allergen specific cloning resulted in the isolation of clones able to produce large amounts of IFNγ and little or no IL-4 and IL-5. The inhibitory action of IFNγ on the isolation of human Th2 clones was found to be at least partially mediated by inhibiting the proliferation of human clones producing IL-4. The addition of IFNα had a similar effect. In comparison, addition of IL-4 to bulk culture prior to PPD specific cloning converted the Th1 phenotype normally isolated to predominantly a Th0 or Th2 pattern (Maggi, 1992). Thus, the inhibition of Th2 expansion by IFNγ and the Th2 promoting activity of IL-4 are also operative upon *in vitro* restimulation of already committed human PBMC.

The role of IL-12 was also assessed in this model. The addition of exogenous IL-12 to PBMC cultures from atopics resulted in the isolation of dust

mite allergen specific clones displaying a Th0 or even Th1 phenotype. Conversely, PPD specific clones isolated from cultures in the presence of anti-IL-12 generally had a Th0 instead of Th1 cytokine production pattern. In this system the ability of IL-12 to alter the phenotype of house dust mite specific clones was not affected by the addition of anti-IFNγ mAb but could be partially inhibited by the removal of cells expressing the NK marker CD16 (Manetti, 1993). These results indicate an IFNγ independent, NK cell related mechanism, potentially involved in the inhibition of Th2 and preferential induction of Th1 responses.

A role for a NK cell dependent mechanism is supported by the ability of poly I-C or influenza virus to direct the development of allergen specific Th0 or Th1 instead of Th2, in cultures of PBMC from atopic donors. This alteration was associated with increased numbers of $CD16^+$ NK cells in the bulk PBMC cultures prior to cloning. In order to counteract the Th1 promoting effect of poly I-C exogenous IL-4 along with antibodies against IFNα, IFNγ and IL-12 were required at the initiation of culture. From these results it is suggested that viruses and intracellular bacteria induce Th1-like responses because they stimulate IFNα and IL-12 production by macrophage. Simultaneous presentation of specific antigen along with cytokines that promote Th1 differentiation is thought to drive the resulting response toward Th1 (Romagnani, 1992). The fact that clones specific for a variety of intracellular bacteria demonstrate the

Th1 cytokine pattern when derived from either atopic or normal patients, suggests that the natural immune response toward these pathogens has become fixed during evolution. It is reasonable to suggest that those individuals in which such pathogens elicited strong IL-12/IFNα levels developed protective Th1 immunity. This may have provided a selective advantage over individuals that failed to produce IL-12/IFNα and instead generated a nonprotective Th2 response. In this model, a defective natural IL-12/IFNα response or defective induction of NK activity would result in differentiation toward Th2.

In a number of patients with severe atopic dermatitis, addition of poly I-C to PBMC cultures, prior to cloning, was unable to change the allergen specific Th2 pattern to Th0 or Th1. In comparison to normal controls or patients with mild allergy, the proportion of $CD16^+$ cells in lines exposed to poly I-C did not significantly increase in these patients. This study suggests that, at least in cases of severe allergy, a defective IL-12/IFNα or NK system might contribute to the induction and maintenance of allergy (Romagnani, 1992). These results, along with the studies of naive transgenic T cell priming, and the effect of IL-12/anti-IL-12 in the *Leishmania major* model combine to provide solid evidence that the early induction of IL-12 and/or IFNα, and the subsequent activation of IFNγ synthesis by NK cells, can combine to bias helper T cell differentiation toward Th1 development.

The ability to manipulate the cytokine pattern of PBMCs that are already committed to producing a particular cytokine pattern (when restimulated in the absence of added cytokines, anti-cytokine mAb, or cytokine inducing substances *in vitro*) suggests that cytokine synthesis patterns can be altered even in cases of ongoing committed responses. Whether this represents conversion between cytokine synthesis patterns or the outgrowth of rare clones producing the opposite cytokine pattern remains unclear. However, if therapies can be devised to mimic this type of restimulation *in vivo*, it may be possible to redirect precommitted inappropriate cytokine patterns.

2.9 Summary.

In summary, overwhelming evidence has accumulated that T cell derived factors are central to the regulation of IgE responses. To date IL-4 is the only cytokine found to induce IgE class switching and continued production of this cytokine is necessary for the maintenance of IgE responses. In a number of model systems IFNγ, or IFNγ inducing cytokines such as IL-12 and/or IFNα, promote Th1 differentiation, whereas IL-4 and IL-10 favour the development of Th2. The balance in which IL-12/IFNα/IFNγ and IL-4/IL-10 are produced during the early phases of immune induction is a critical parameter influencing the direction of the response. Mechanisms that introduce any of these factors

during this critical phase may skew the subsequent differentiation of Th1 versus Th2 responses, and consequently the balance between cell mediated and humoral immunity.

Early production of Th1 inducing factors may occur via preferential presentation of antigen on macrophage or liver adherent cells, the initiation of IL-12/IFNα synthesis by bacterial components, or other agents, and the production of IFNγ by NK cells and perhaps CD8 T cells.

The early elaboration of cytokines promoting Th2 activation may occur by the preferential presentation of antigen by resting B cells or Langerhans cells, the nonspecific induction of IL-10 production by keratinocytes, a defect in the IL-12/NK cell system, and perhaps the production of high PGE_2 levels by APC. After the initiation of an IgE response, release of IL-4 by mast cells or non-B non-T cells may contribute to the maintenance of Th2 responses and the inhibition of Th1 activation. The induction of a Th2 dominated response through any of these mechanisms may cause aberrant synthesis of IL-4, resulting in allergy.

3.0 A murine model of immediate hypersensitivity to ovalbumin.

Egg allergies are quite common in humans, and exclusion of egg products from the diet is difficult. Of the numerous proteins found in eggs, only ovalbumin (OA), ovomucoid and ovotransferrin are major human allergens. Of these three, ovalbumin is the most abundant accounting for 60% of the egg white (Langeland, 1982, 1983).

The structure of OA is well characterized at both the level of amino acid sequence (Nisbet, 1981), and crystal structure (Stein, 1990). In addition, epitopes having allergenic and antigenic properties have been identified (Elsayed, 1986). Therefore this allergen is a good candidate for the study of IgE regulation in animal models.

HayGlass and Strejan utilized a murine model of immediate hypersensitivity to OA to investigate the regulation of IgE responses. They found that glutaraldehyde polymerization of ovalbumin at neutral pH, as previously performed with grass pollen extracts, results in a product exhibiting great variation with respect to molecular weight and immunological characteristics. Pretreatment of mice with this heterogeneous mixture failed to inhibit IgE responses, but did induce high levels of allergen specific IgG. In contrast, polymerization of OA 0.5 pH units above its isoelectric point yielded a

relatively homogeneous, soluble polymer of M_r. 3.5 x 10^6, termed OA-POL (HayGlass, 1983, 1984a). Pretreatment of mice with this product induced long-lived, antigen and isotype specific, T cell dependent inhibition of IgE responses in CBA mice (HayGlass, 1984b). The relative lack of heterogeneity within each OA-POL preparation and between independently prepared lots suggested that this product could be useful as an experimental tool to investigate the regulation of IgE responses *in vivo*.

3.1 An overview of the present study.

The focus of this research project has been to develop therapeutic strategies for the modulation of immunoglobulin isotype patterns *in vivo*, with particular emphasis on the inhibition of IgE responses. Using a murine model of human immediate hypersensitivity to ovalbumin, we developed a high M_r. glutaraldehyde polymerized antigen, denoted OA-POL, and characterized its effects on the regulation of antibody and cytokine gene expression *in vivo*.

Our previous findings indicated that three i.p. injections of OA-POL induce antigen specific, isotype selective inhibition of primary and secondary anti-OA IgE responses while leading to concomitant 250-1000 fold increases in OA specific IgG_{2a} synthesis in each of 14 mouse strains tested, if the mice were

immunized 10 days after the last OA-POL injection (HayGlass, 1991a). Administration of unmodified OA was without effect on IgE and IgG_{2a} synthesis. Most importantly, and unlike the results obtained following in vivo administration of other types of modified allergens, OA-POL treatment inhibits even well established IgE responses (HayGlass, 1990, 1991b). In these studies the ongoing IgE response of C57Bl/6 mice previously primed with $OA[Al(OH)_3]$ could be inhibited by 97-99% by three courses of OA-POL treatment. Importantly this inhibition was maintained for at least 260 days despite six additional booster immunizations with $OA[Al(OH)_3]$ in the absence of further treatment. The inhibition of IgE was antigen specific, isotype selective, and T cell dependent. As in the pretreatment model, IgG_{2a} responses were strikingly elevated after OA-POL treatment, suggesting the involvement of IFNγ.

The failure of many modified allergens to inhibit IgE responses in humans as observed in animal models may have been due in part to the short term nature of most animal studies. The ability to extrapolate findings from these studies to clinical situations is difficult and often proved disappointing. Hence, the <u>longevity</u> and <u>resilience</u> of OA-POL's effects on allergen specific antibody responses *in vivo* and the <u>mechanism</u> by which OA-POL mediates these effects were the primary goals of this research project.

In this study, OA-POL pretreatment of C57Bl/6 mice was found to elicit virtually permanent, CD4 T cell dependent, antigen specific, long-lived inhibition of IgE and enhancement of IgG_{2a} responses. This >90% inhibition could be observed when mice were immunized greater than one year after a single course of OA-POL treatment and was maintained despite at least 5 booster $OA[Al(OH)_3]$ immunizations over more than 140 days. In contrast, BALB\c mice, which produce inhibited IgE and enhanced IgG_{2a} responses if immunized shortly after OA-POL treatment, demonstrate IgE and IgG_{2a} responses not different from normal control mice when immunized 10 weeks or longer after treatment (Gieni, 1991). We utilized three independent approaches to investigate the role of IFNγ in the inhibition of IgE and enhancement of IgG_{2a} responses in this model, and compared the cytokine pattern produced in a case of successful induction of long-term IgE inhibition (C57Bl/6) and a case in which this treatment failed to permanently inhibit IgE responsiveness (BALB/c). These included studying the effect of anti-IFNγ mAb administration on the pattern of isotypes produced, the evaluation of IL-4 and IFNγ synthesis in antigen-stimulated bulk spleen cell cultures, and the use of limiting dilution analysis to characterize the frequency of CD4 T cells producing IFNγ, IL-4, or IL-10 in C57Bl/6 and BALB/c mice.

Evidence that the long-lived reciprocal regulation of IgE and IgG_{2a} induced by successful OA-POL immunotherapy was the result of increases in the balance of IFNγ to IL4/10 synthesis within the allergen specific CD4 T cell repertoire was provided by: 1. The ability of anti-IFNγ to block OA-POL induced IgE inhibition. 2. Increased synthesis of IFNγ and reduced production of IL-4 in bulk spleen cell cultures derived from OA-POL treated C57Bl/6, but not BALB/c mice (Gieni, 1993). 3. An enhanced frequency of IFNγ and decreased frequency of IL-10 secreting CD4 T cells soon after OA-POL compared to OA treatment of C57Bl/6 mice. 4. Maintenance of increased numbers of IFNγ producing CD4 T cells and a reduction in those producing IL-4 and IL-10 in OA[$Al(OH)_3$] immunized C57Bl/6 mice for at least 24 weeks after OA-POL treatment, compared to saline treated OA[$Al(OH)_3$] immunized mice. In contrast, OA-POL treated BALB/c mice demonstrated no increase in the frequency of IFNγ producing CD4 T cells at this time, and in fact, demonstrated mildly increased frequencies of CD4 T cells producing IL-4 and IL-10.

These studies demonstrated that successful long-term inhibition of IgE responses *in vivo* is associated with the induction and maintenance of an enhanced ratio of IFNγ to IL-4 (IFNγ:IL-4) and IFNγ to IL-10 (IFNγ:IL-10) producing CD4 T cells after OA-POL treatment, indicative of a shift from a Th2-like response to one dominated by IFNγ. This shift parallels the change in isotype pattern from IgE to IgG_{2a}. Further studies in this system will resolve the

mechanism(s) responsible for the differential cytokine response induced by OA-POL treatment of C57Bl/6 compared to BALB/c mice. By defining the immunoregulatory mechanisms involved in successful immunotherapy improved methods may be devised to modulate *in vivo* cytokine synthesis patterns.

Materials and Methods.

All data presented in Results are from one experiment representative of three performed, unless otherwise indicated.

3.2 Animals.

BALB/c, C.B-20, C57Bl/6, C57Bl/6-Igh^n, BALB/c x C57Bl/6 F1 and Swiss mice were bred at the University of Manitoba breeding facility. BDF$_1$ mice were purchased from Charles River, Canada (St. Constant, PQ). B10.BR, B10.A, B10.D2 and DBA/2 were purchased from Jackson Labs, Bar Harbor, Maine. BALB/b mice were a kind gift from Dr. K. Schultz, Fred Hutchinson Cancer Research Center, Seattle, Washington. Female Sprague-Dawley rats were bred at the University of Manitoba. All animals were maintained and used in strict accordance with the guidelines issued by the Canada Council on Animal Care.

3.3 Preparation of chemically modified ovalbumin.

Ovalbumin (Grade VI, Sigma Chemical Co, St. Louis, MO or 5 times recrystallized OA, ICN Biomedicals, Montreal, PQ) was treated with glutaraldehyde (Eastman Kodak Co, Rochester, NY) as follows: Glutaraldehyde (6%) was added dropwise over a period of several minutes to a 25 mg/ml solution of OA in 0.1 M acetate buffer (pH 5.3) to obtain a final

molar ratio of 200:1 GA:OA. The reaction was carried out over 5 hours in order to obtain high molecular weight OA polymers. The product was extensively dialysed and subsequently applied to a gel filtration column [Biogel A-50m (2.5 x 90 cm in borate buffered saline, pH 8.3) Biorad Laboratories, Mississauga, ON] for characterization and purification purposes. The product was recovered as a single symmetric peak eluting at an average M_r of 3.5 x 10^7. The product, designated OA-POL, was stored at 4° C until used.

<u>3.4 Treatment and immunization of mice.</u>

OA-POL treatment consisted of 80 ug intraperitoneal (i.p.) injections of OA-POL given on three alternate days (d. 0, 2, 4) prior to immunization unless otherwise stated. Mice were immunized i.p. with 2 ug unmodified OA (or in antigen specificity experiments, with 10 ug DNP_{22}-KLH) absorbed onto 2 mg of $Al[(OH)_3]$ adjuvant in all experiments presented. Mice were tested for the ability of OA-POL to induce IgE inhibition by immunizing ten days after the last injection of OA-POL (Table 1). To test for long-lived decreases in allergen specific IgE synthesis, OA-POL treated mice along with saline treated age matched normal controls were immunized between 10 to 72 weeks after treatment and boosted at 28 day intervals. Mice were bled by cardiac puncture 14 days after primary immunization and 7 days after each booster immunization. The sera collected were stored at -20 °C until analyzed by PCA and ELISA.

3.5 Determination of antigen-specific antibody levels.

OA-specific murine IgG, IgG_1, IgG_{2a}, IgA and IgM levels were determined in an alkaline phosphatase based ELISA calibrated against a murine polyclonal anti-OA standard. Briefly, ELISA plates (Corning 25805, Corning Science Products, Corning, NY) were coated overnight with antigen (OA or DNP-BSA) at 200 ug/ml in bicarbonate buffer (0.05M, pH 9.6). After 90 minutes blocking with a 1% BSA, 0.05% Tween 20 solution and extensive washing, serial dilutions of serum samples were incubated for 4 hours at 37°C. The plates were washed and an excess of alkaline phosphatase conjugated rabbit anti-mouse IgG, IgG_1, IgG_{2a}, IgA or IgM (Southern Biotechnology Associates, Birmingham, AL) was added overnight at 4 °C. After washing the plates extensively, p-nitrophenyl phosphate (Sigma Chemical Co.) was added as directed by the manufacturer and the reaction was allowed to proceed for 100 minutes. Background control values for wells missing one component in turn did not exceed 0.07 absorbance units at 405 nm. OA specific IgG levels were determined by comparison to a standard titration of a murine polyclonal anti-OA IgG standard. OA specific IgG concentrations are expressed in ug/ml. Results for IgG_1, IgG_{2a}, IgA and IgM are expressed as ELISA titers using the midpoint of the titration curves obtained as compared to a constant internal standard run in each assay. Each serum sample was assayed at least twice. The isotypic specificity of each of the antibody-enzyme conjugates used was confirmed prior to use.

IgE anti-OA levels were determined by 48 hour passive cutaneous anaphylaxis (PCA) in female S-D rats (Ovary, 1986). Briefly, serial dilutions of sera were carried out in saline. Rats were given intradermal injections of 0.1 ml from each dilution. Forty eight hours later the rats were injected i.v. with 2 mg OA in Evan's blue dye. The PCA titer was recorded as the reciprocal of the last dilution that resulted in a definite blueing reaction. A number of control experiments were carried out in which a group of six OA-POL or saline treated mice were immunized 10 weeks after treatment and bled individually 10 days later to determine the variability in IgE titer between individual mice within a group. In these experiments the titer determined for individual mice rarely differed by more than 4 fold. The data shown in results was determined with sera pooled from three mice per group. Triplicate analyses of these pooled sera, which rarely differed by more than one two-fold dilution, were conducted for each sample.

3.6 Cell transfers (T cell depletion).

Mice pretreated with OA-POL as described above were sacrificed at the specified time after treatment. Spleen cells were isolated and, where specified, T cells depleted prior to transfer by treatment with anti-Thy 1.2 mAb H013.4 (kindly provided by Dr. K. Rock, Harvard Medical School, Boston, MA) and complement (Cedarlane Laboratories Ltd, Grimsby, ONT). Under these conditions 40-45% of lymphocytes were lysed while complement

treated controls exhibited approximately 10% killing. T depleted spleen cells failed to proliferate significantly in response to various doses of concanavalin A (Sigma Chemical Co., St. Louis, MO) in comparison to a complement treated control, indicating the lack of T cells. T cell depleted or complement treated spleen cell populations were enumerated and 40 to 80 x 10^6 cells were injected i.v. into normal recipients. All mice were immunized the same day with 2 ug OA[$Al(OH)_3$] and bled 10, 12 and 14 days later.

3.7 CD4/CD8 T cell depletion in vivo - Cell Transfer

OA-POL treated mice (d. 0, 2, 4) were depleted of $CD4^+$ or $CD8^+$ cells by i.p. injection of the appropriate mAb (purified anti-CD4 mAb YTS 191.1 or anti-CD8 mAb YTS 169.4 tissue culture supernatant [Waldmann, 1991]) on days 89 and 90. On day 91 flow cytometric analysis with anti-CD4 mAb GK1.5 or anti-CD8 mAb HO 2.2 and second reagent goat anti-rat Ig-FITC conjugate indicated <3% of control $CD4^+$ or $CD8^+$ splenic T cells remained. The same day 90 x 10^6 spleen cells from each group were injected i.v. into normal recipients. All mice were immunized and bled 10 (shown), 12 and 14 days later.

3.8 CD4 T cell depletion in vivo.

OA-POL and saline treated mice (d. 0, 2, 4) were injected i.p. with purified anti-CD4 mAb YTS 191.1 on day 123 and 124. On day 126 these mice were found to contain <2% of control CD4+ splenic T cells as determined by flow cytometry using the unrelated mAb GK1.5 and a second FITC labelled goat anti-mouse/rat Ig. Treatment of mice with equivalent amounts of FCS containing culture media had no effect on CD4 splenic T cell frequency. By day 151 anti-CD4 treated mice were found to express splenic CD4 T cell frequencies not significantly different from normal mice. The mice were immunized the same day (d. 151) and bled on days 161, 163 and 165. Day 161 IgE and IgG_{2a} titers are shown.

3.9 CD8 T cell depletion in vivo.

OA-POL and saline treated mice (d. 0, 2, 4) were injected i.p. with anti-CD8 mAb YTS 191.1 tissue culture supernatant on days 123 and 124. On day 126, these mice were found to contain <2% of control CD8 splenic T cells as determined by flow cytometry using the unrelated mAb HO 2.2 and a second FITC labelled goat anti-mouse/rat Ig. Treatment of mice with equivalent amounts of normal rat immunoglobulin had no effect on CD8 splenic T cell frequency or the resulting OA specific IgE and IgG_{2a} response (data not shown). The mice were immunized on day 126 and bled on days 136, 138 and 140. Day 136 IgE titers are shown.

4.0 Preparation and Purification of anti-IFNγ mAb.

Rat IgG_1 mAb specific for murine IFNγ (XMG 1.2) (kindly provided by Dr. T. Mosmann, University of Alberta, Edmonton, Canada) was purified from tissue culture supernatant using the Pharmacia Biopilot system. The rat mAb obtained was 85 to 95% pure as determined by comparison of total protein present in the purified material (estimated by absorbance at 280 nm) with the concentration of rat IgG determined by ELISA. This material was used for in vivo (i.p.) injection at the times described in results.

4.1 In vivo anti-IFNγ administration

C57Bl/6 mice were treated with OA-POL (d. 0, 2, 4). Independent cohorts also received 250 ug anti-IFNγ (XMG 1.2), normal rat Ig or nothing i.p. on days 0, 1, 2, 3, 4, 5, 7 and 9 (2 mg total). Normal rat immunoglobulin was prepared from a 45% NH_4SO_4 precipitate of normal rat serum purified by gel filtration on G-200 Sephadex. On days when mice received both OA-POL and anti-IFNγ the injection of mAb was always carried out at least seven hours after the injection of OA-POL. All mice were immunized on day 70 and bled on days 80 and 84.

4.2 In vivo anti-IFNγ administration at the time of OA immunization.

C57Bl/6 mice were treated with OA-POL (d. 0, 2, 4) and immunized on day 91. Subgroups of these mice received 250 ug anti-IFNγ, normal rat Ig or nothing on days 91, 92 and 93 (750 ug total). Sera were collected 10, 12 and 14 days after immunization.

4.3 Bulk spleen cell cultures

Bulk spleen cell cultures were established at 14 x 10^6 cells per well (2 ml) in 24 well plates (Corning Science Products, Rochester, NY) for 18 to 36 hours. Culture medium consisted of RPMI 1640 supplemented with 10% fetal calf serum, 10 mM L-glutamine, penicillin (100 units/ml), streptomycin sulfate (100ug/ml), fungizone (0.25 ug/ml) (Flow Labs, Mississauga, ON), and 2 x 10^{-5} M 2-mercaptoethanol. This is designated complete culture media. Cultures (+/- OA at 1mg/ml) were incubated at 37 °C for 18 and 36 hours before harvesting supernatant to be tested for IL-4 and IFNγ. Cytokine production is expressed as units/ml calibrated against standard curves using a rmIL-4 standard provided by Dr. W. Paul (NIAID,NIH, Bethesda, MD) or WHO-NIAID IFNγ international reference reagent Gg02-901-533 (provided by Dr. C. Laughlin, NIAID,NIH, Bethesda,MD).

4.4 Bulk Culture IL-4 determination

5000 CT.4S cells (Hu-Li, 1989) in 50 ul of culture medium were added to triplicate 50 ul culture supernatant aliquots and cultured for 24 hours at 37 °C. 50 ug MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma Chemical Co.) in 10 ul RPMI 1640 was added to each well. After a further 5 hour incubation, 190 ul of stop solution (2:1 2-propanol:H_2O made 0.04N in HCL) was added to each well. Each well was then vigorously pipetted, the plate was left at 37 °C for 10 minutes and A_{570} was determined (Dynatech Instruments, Torrance CA). The specificity of the CT.4S bioassay for IL-4 was confirmed by inclusion of anti-IL-4 mAb 11B11 in some assays, which in all cases reduced the absorbance to background levels. Moreover, addition of 200 U/ml rIL-2 (Genzyme) demonstrated that this level of IL-2 yielded CT.4S absorbancies (A_{570} <0.03) indistinguishable from those observed in the absence of IL-4, confirming the insensitivity of the CT.4S line to IL-2 at these levels. The lower limits of detection are 0.2 U/ml IL-4 with quantitative measurement of cytokine above 0.5 U/ml. S.E. within any given experiment was usually < 5%.

4.5 IL-2 determination

IL-2 was measured using a procedure identical to that used to determine IL-4 levels, except that the IL-2 dependent cell line HT-2 was employed. The assay was performed in the presence of anti-IL-4 mAb 11B11, and the

specificity for IL-2 was ensured by the ability of a mixture of anti-IL-2 and anti-IL-2 receptor mAb (S4B6 provided by Dr. T. Mosmann, University of Alberta, 7D4 American Type Culture Collection, Rockville, MD) to bring the absorbance of a duplicate well to background levels. An internal standard of recombinant murine IL-2 (Genzyme, Boston, MA) ±anti-IL-2/IL-2 receptor mAb was run in each assay.

4.6 IFNγ determination.

A sandwich ELISA using purified anti-IFNγ mAbs XMG 1.2 and purified, biotinylated R4-6A2 (American Type Culture Collection, Rockville, Maryland) in combination with streptavidin-alkaline phosphatase was used. The lower limits of detection are 0.2 U/ml with quantitative measurement of cytokine above 0.5 U/ml.

4.7 Limiting Dilution Analysis

Treatment of mice/enrichment of $CD4^+$ responder population.

OA or OA-POL treatment consisted of 80 ug i.p. injections on days 0, 2 and 4. In experiments where anti-IFNγ (XMG 1.2) and NRIg were used the mice received 250 ug mAb/day i.p., starting on d -1 and continuing until day 6. mAb injections were always carried out at least seven hours after the administration of OA or OA-POL. Spleen cells were isolated from mice

sacrificed on day 9. Adherent cells and B cells were depleted by incubation for 30 minutes in 15 cm petri plates coated overnight with goat anti-mouse Ig. $CD4^+$ T cells were purified from this population by passage of the remaining cells through a CD4 T cell negative selection column (Biotex Laboratories Inc., Edmonton, Alberta). The resulting population was 92 to 96% CD4 positive with less than 0.7% contamination with B cells or $CD8^+$ T cells (as assessed by flow cytometry).

4.8 Limiting Dilution Analysis-comparison of cytokine precursor frequency in C57Bl/6 compared to BALB/c

Treatment of mice/enrichment of $CD4^+$ responder population

C57Bl/6 and BALB/c mice were treated with OA-POL on day 0, 2 and 4 and immunized along with age matched mice between 12 and 22 weeks later. Five days after immunization, spleen cells were obtained and $CD4^+$ T cells were isolated by positive selection using flow cytometry. Briefly, spleen cells were depleted of adherent and B cells by plating on goat anti-mouse Ig coated 15 cm petri plates. The resulting population was stained sequentially with anti-CD4 mAb (GK1.5) and goat anti-Rat Ig FITC (no detectable crossreactivity with mouse Ig). This population was sorted by positive selection on an EPICS fluorescent activated cell sorter. The resulting population was 95-98% CD4 positive with no detectable contamination with B cells or $CD8^+$ T cells (as

assessed by flow cytometry).

4.9 Limiting Dilution Analysis

Cultures were set up in complete culture media as described. Purified $CD4^+$ responder T cells were enumerated and graded numbers (50-102,400/well; 36 replicate wells per responder cell number) were cultured in round bottom 96 well culture plates (Corning Science Products) with 6×10^5 irradiated spleen cells (1500 rad.) and 20 U/ml rIL-2 (highly purified recombinant IL-2 obtained from Cetus Corporation Seattle, Washington). 12 wells containing the above in the absence of responder cells were included in each plate in order to provide background values in each cytokine assay employed. Duplicate plates were set up with and without the presence of OA (1 mg/ml). After incubation for 14-16 days, wells positive for growth could easily be distinguished at low level magnification (x 5). The wells were then centrifuged and washed twice. Each well was restimulated in 290 ul culture medium containing fresh irradiated (1500 rad.) APC and antigen in the absence of IL-2. Samples of supernatant harvested immediately after washing demonstrated no measurable level of residual IL-2 (< 0.1 U/ml). After an additional 48 hours, aliquots of supernatant from each well were normally assayed immediately for the presence of IFNγ, IL-4, and IL-10. In each assay wells were considered positive for cytokine production if the absorbance value obtained in the sample well was greater than three standard deviations above the mean value obtained

in wells that lacked responder CD4 T cells.

There was a very clear difference between positive cultures and those that poduced no detectible cytokine for each of the cytokines tested. Negative wells produced absorancies in each assay very similar to the APC control, while positive wells were almost always well above the three standard deviation cutoff. Although a low frequency of growth occurred without antigen at the highest responder cell numbers (2-6/36 with 51,200-102,400 responders/well), none of these wells scored positive for cytokine secretion after specific restimulation with OA.

5.0 Limiting Dilution Analysis IL-4 determination.

IL-4 levels were determined using a MTT colorimetric assay employing the IL-4 dependent cell line CT.4S (23) (provided by Dr. W. Paul, NIAID, NIH). A 70 ul aliquot of culture supernatant from each well was added to 5000 CT.4S cells in 30 ul of 15% FCS-containing culture medium in 96 well flat bottom plates (Corning Science Products) . Every second or third plate contained a two fold serial dilution of standard recombinant IL-4. After 24 hours incubation at 37 °C, 50 ug of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) in 10 ul of RPMI 1640 was added to each well. After a further 5 hours incubation, 190 ul of stop solution consisting of 2:1 2-propanol:H_20 made 0.04N in HCL was added to each well. After vigorous pipetting, the plate was left at 37 °C for 10 minutes at which time absorbance

was read at 570 nm. The specificity of this assay was assured by incubation of representative aliquots with anti-IL-4 mAb (11B11), which in all cases resulted in background absorbance indistinguishable from those observed in the absence of IL-4. As used here, this assay detects IL-4 at 0.2 U/ml and readily quantitates IL-4 levels above 0.5 U/ml, while being entirely unresponsive to IL-2 levels below 200 U/ml.

5.1 Limiting Dilution Analysis IL-10 determination.

A sandwich ELISA using purified anti-IL-10 mAbs SXC1 and purified, biotinylated SXC2 (both hybridomas kindly provided by Dr. T. Mosmann, University of Edmonton, mAb were purified by W. Stefura, University of Manitoba.) in combination with streptavidin-alkaline was used. IL-10 levels in culture supernatant were quantitated against a rIL-10 standard. The lower limit of detection is 0.2 U/ml, with quantitation of levels $\geq$0.5 U/ml.

5.2 Frequency estimations

Estimates of the precursor frequency for growth and synthesis of each cytokine tested were obtained by both the maximum likelihood and minimum X^2 methods based on the Poisson distribution relationship between the number of responding cells and the logarithm of the fraction of negative cultures. The results were consistent using either method, thus for clarity, maximum

likelihood results are presented.

5.3 Calculation of IFNγ:IL-4 and IFNγ:IL-10 frequency ratios.

The balance of CD4 T cells producing IFNγ compared to those producing IL-4 (IFNγ:IL-4), and the balance of CD4 T cells producing IFNγ compared to those producing IL-10 (IFNγ:IL-10), was approximated by determining the ratio of the frequencies of CD4 T cells producing each cytokine in limiting dilution analysis. For example, if in group a the frequency of: IFNγ producing CD4 T cells is 1/X, the frequency of IL-4 producing cells is 1/Y, and the frequency of IL-10 producing cells is 1/Z, the (IFNγ:IL-4) ratio for that group = $1/X \div 1/Y$. The (IFNγ:IL-10) ratio for this group would be $1/X \div 1/Z$. This method was used to calculate ratios for each treatment group in each experiment. The data presented in Figure 13 and Table 10 represent the arithmetic mean (±SEM) of the ratios calculated as above. i.e. for Table 10 the mean (IFNγ:IL-4) ratio = $[(1/X \div 1/Y)$ exp. 1 + $(1/X \div 1/Y)$ exp. 2 + $(1/X \div 1/Y)$ exp.3] $\div 3$.

5.4 Selection and expansion of short term CD4 T cell lines for use in cellular immunotherapy.

During analysis of limiting dilution data from OA[$Al(OH)_3$] immunized mice, a low frequency of CD4 T cells that produced substantial IFNγ and no

detectible IL-4 or IL-10 were consistently found. Dilutions of responder cells that resulted in less than 30% positive growth were isolated and restimulated with OA with irradiated normal spleen cells as APC. CD4 T cell cytokine synthesis patterns in individual wells were assessed after 48 hours. Wells which displayed significant IFNγ levels in the absence of IL-4 or IL-10, as well as, several IL-4/10 producing, but IFNγ negative wells were transferred to 24 well plates (Corning Science Products) and expanded with 1 mg/ml OA, 20 U/ml IL-2 and 3.5 x 10^6/ml irradiated (2500 rad.) normal spleen cells (APC) in complete media. After one week, supernatants were removed and replaced by fresh IL-2 containing (20 U/ml) complete medium. After an additional week, wells with significant growth were split into three wells and all cultures were restimulated as above. Once six rounds of expansion were completed the cell lines were extensively washed and grown an additional 4 days in IL-2 containing media in the absence of antigen. In a second experiment sufficient cell numbers were obtained after four additional restimulations, after which the cell lines were extensively washed and grown an additional 4 days in IL-2 containing media in the absence of antigen. In each experiment viable cells were recovered, washed, enumerated and 10 x 10^6 were transferred i.v. to naive recipients. All recipient mice were immunized the same day and boosted 28 days later. Duplicate cultures of these lines containing 50 x 10^5 cells from each cell line were restimulated with 3.5 x 10^6 irradiated APC and 1 mg/ml OA. Supernatants were assayed for IL-2, IL-4, IL-10 and IFNγ levels to ensure that

each line retained its initial cytokine synthesis pattern. Mice were bled 10 and 14 days after primary immunization and seven days after boosting.

5.5 Statistical Analysis

Antibody titers were log transformed, after which geometric means were compared using unpaired two-tailed Student's t-tests. A p value of greater than 0.05 was considered nonsignificant. Statistical significance of differences in mean IFNγ:IL-4 ratios after OA-POL treatment was determined via a paired Wilcoxon rank sum test.

Results

5.6 Reciprocal regulation of IgE and IgG_{2a} responses following treatment with glutaraldehyde-polymerized ovalbumin.

Glutaraldehyde polymerization of ovalbumin one half pH unit above its isoelectric point yields a freely soluble product of M_r. 3.5 x 10^7, termed OA-POL. Previous studies found that treatment of mice with three 80 ug OA-POL injections (i.p.) resulted in 75 to 99% inhibition of primary and secondary allergen specific IgE responses in each of 15 strains examined, when immunized with OA[$Al(OH)_3$] two weeks after treatment (HayGlass, 1991a).

Table 1 summarizes the effects of OA-POL or unmodified OA treatment on allergen specific antibody responses. C57Bl/6 mice were treated with 80 ug of OA-POL or OA on days 0, 2, and 4 and immunized with 2 ug OA adsorbed to 2 mg $Al(OH)_3$ on days 14 and 28. Results from sera collected 14 days after primary and 7 days after secondary immunization are shown. In comparison to the response of saline treated mice, OA-POL treatment led to >97% inhibition of primary and secondary IgE responses. Primary IgG_1 responses were markedly increased, but these levels became equivalent to

normal control mice in the secondary response. In contrast, IgG_{2a} production was elevated 250 fold in the primary response and remained 685 fold increased upon rechallenge.

In comparison, administration of OA had no suppressive effect on IgE production and little effect on IgG_{2a}. Instead it promoted primary IgG_1 production, which in this experiment was 360 fold increased, and remained significantly augmented after rechallenge. OA-specific IgA and IgM production was largely unaffected by either treatment.

Table 2 demonstrates the effect of OA versus OA-POL treatment on allergen specific antibody responses in BALB/c mice. In this experiment, OA-POL treatment resulted in > 87% inhibition of primary and secondary IgE. Primary IgG_1 and IgG_{2a} responses were elevated by OA-POL treatment but these differences became insignificant in the secondary response. OA treatment did not inhibit IgE or enhance IgG_{2a}, although IgG_1 was increased by 384 fold in the primary and moderately increased in the secondary response. OA-specific IgA and IgM was generally unaffected by pretreatment with either form of ovalbumin. In summary, the most significant alteration in isotype pattern caused by OA-POL treatment is the reciprocal regulation of the IgE and IgG_{2a} isotypes.

Treatment	d. 14 IgE	d. 35 IgE	d. 14 IgG1	d. 35 IgG1	d. 14 IgG2a	d. 35 IgG2a	d.14 IgA	d. 35 IgA	d. 14 IgM	d. 35 IgM
Saline	640	3200	1,200	115,400	30	55	15	25	290	870
OA-POL	20	120	74,900	168,000	7,500	37,560	10	20	430	1,360
OA	800	5000	465,200	942,500	100	435	15	20	620	2,150

OA-POL treatment induces reciprocal regulation of allergen specific IgE and IgG_{2a} responses.

Table 1. C57Bl/6 mice were treated i.p. with 80 ug OA (in saline), OA-POL (in saline) or saline 10, 12 and 14 days before immunization with 2 ug OA $Al(OH)_3$. Mice were bled 14 days after primary and 7 days after secondary (d. 28) immunization. OA specific serum Ig titers were determined as described in Materials and Methods. Data are presented as reciprocal titers. Standard errors were less than 15% in all instances.

Treatment	d. 14 IgE	d. 35 IgE	d. 14 IgG1	d. 35 IgG1	d. 14 IgG2a	d. 35 IgG2a	d.14 IgA	d. 35 IgA	d. 14 IgM	d. 35 IgM
Saline	800	6400	2,250	184,100	40	2560	15	30	760	2750
OA-POL	50	800	284,000	216,800	5150	5300	25	40	2900	2028
OA	800	8000	865,400	1.15×10^6	65	1920	40	30	1850	1920

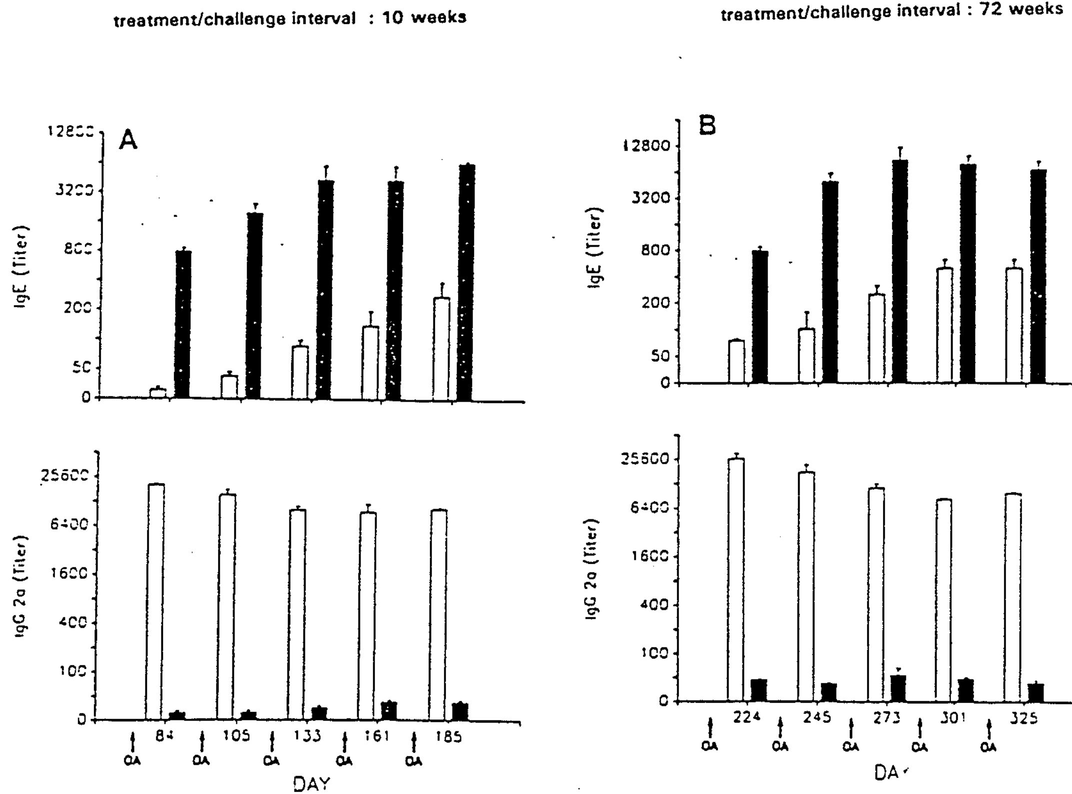
Inhibition of IgE responses in BALB/c mice immunized two weeks after OA-POL treatment.

Table 2. Mice were treated and immunized as in Table 1. OA specific serum Ig titers were determined as described in Materials and Methods. Data are presented as reciprocal titers. Standard errors were less than 15% in all instances.

5.7 Demonstration of OA-POL induced long-lived reciprocal regulation of anti-OA IgE and IgG_{2a} responses in C57Bl/6 mice.

Most in vivo studies of immunoregulation have examined time periods of 6 weeks or less. Caution is required to extrapolate from such studies to long-term regulation of IgE responses and chronic allergen exposure. To directly determine the capacity of OA-POL to induce long-term changes in murine responsiveness to native allergen, the longevity of allergen specific IgE inhibition and parallel IgG_{2a} enhancement was examined. C57Bl/6 mice were treated with 80 ug of OA-POL on days 0, 2, and 4. Separate cohorts of these mice, along with age-matched, saline treated controls, were immunized 10 (**Figure 1A**), 20, or 30 weeks later. In a second, independent experiment, OA-POL treated and age matched normal mice were immunized 15, 40, or 72 (**Figure 1B**) weeks after treatment. The mice were boosted every 28 days and bled 14 days after primary immunization and seven days after each booster.

C57Bl/6



Long-lived, reciprocal regulation of OA-specific IgE and IgG_{2a} responses in C57Bl/6 mice after OA-POL treatment.

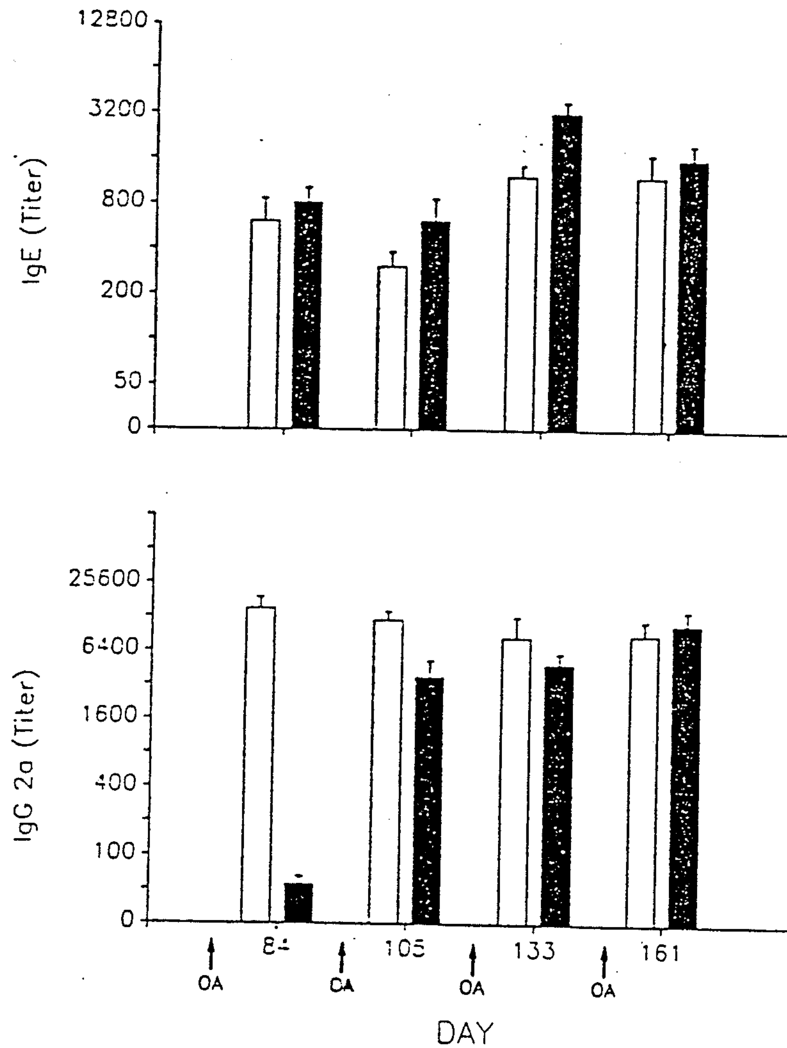
Figure 1. Mice were treated with OA-POL (□) or saline (■) on days 0, 2 and 4, then immunized (↑) with 2 ug OA $Al[(OH)_3]$ on day 70 (**1A**) or, in an independent experiment, on day 509 (**1B**). Mice were boosted at 28 day intervals thereafter and bled 14 days after primary immunization and 7 days after each boost. Geometric mean titers (±SEM) are presented from two experiments representative of six independent experiments performed.

As shown in Figure one, C57Bl/6 mice immunized 10 or 72 weeks after a single course of OA-POL treatment produce anti-OA IgE responses inhibited by greater than 90 % relative to age-matched, untreated mice. More importantly, this hypo-responsive state was maintained despite 4 additional OA[$Al(OH)_3$] booster immunizations over a period of more than 100 days.

The effects of OA-POL treatment were largely isotype selective. Total anti-OA IgG levels in the OA-POL treated groups were markedly elevated in the primary response but became moderately reduced in secondary and tertiary responses (data not shown). Analysis of the response by isotype subclass revealed that OA-POL treated mice generated IgG_{2a} levels 100 to 800 fold higher than untreated mice in both primary and secondary responses. Primary IgG1 levels were elevated, becoming variably (2 to 4 fold) decreased in secondary responses. IgA titers remained low (less than 50) in both groups, whereas primary IgM responses were moderately elevated 4 to 8 fold, but became equivalent in secondary responses (data not shown).

BALB/c

treatment/challenge interval : 10 weeks

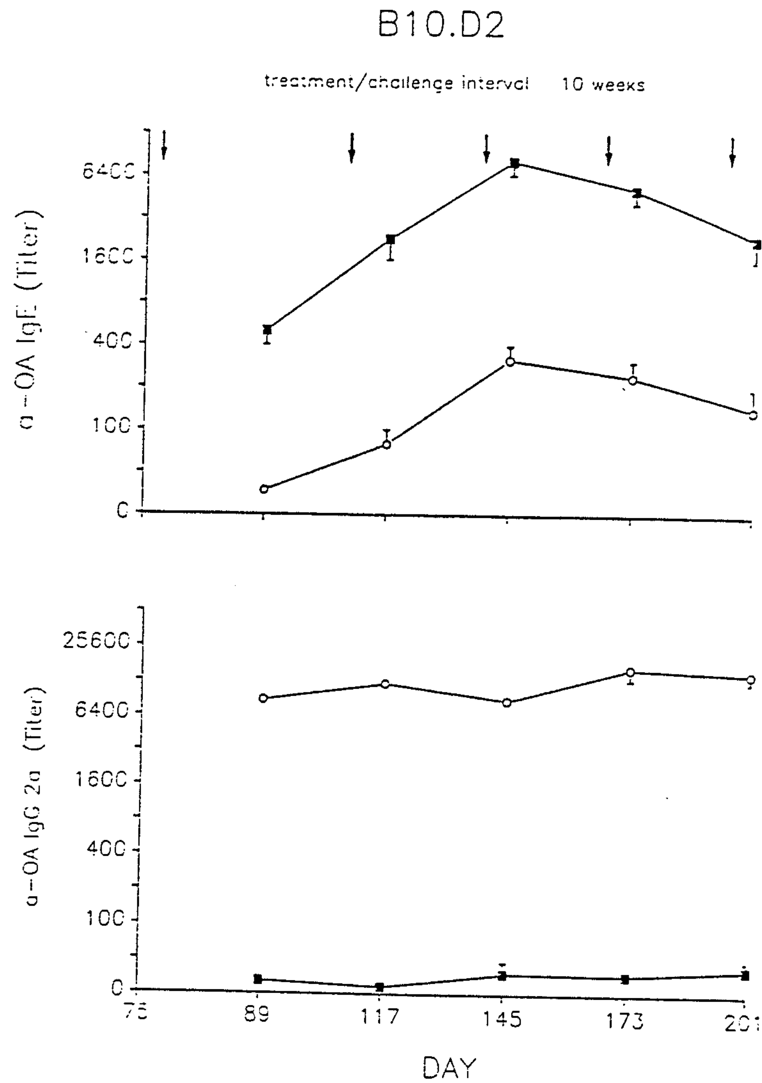


Failure of OA-POL treatment to elicit long-lived inhibition of OA-specific IgE responses in BALB/c mice.

Figure 2. Mice were treated with OA-POL as in Figure 1 [OA-POL (□), saline (■)] and immunized (↑) on day 70. Mice were boosted at 28 day intervals thereafter, and bled 14 days after primary immunization and 7 days after each boost. Geometric mean titers (±SEM) are presented from one experiment representative of six independent experiments performed.

5.8 Failure of OA-POL treated BALB/c mice to exhibit long-term inhibition of anti-OA IgE responses.

OA-POL treated BALB/c mice exhibit >90% inhibition of primary and 75 to 90% inhibition of secondary anti-OA IgE responses when immunized 2 to 4 weeks after OA-POL treatment (HayGlass, 1991a) and (Table 2). However, immunization 10 weeks or longer after treatment (**Figure 2**) consistently results in IgE responses that are indistinguishable from those elicited in untreated age-matched mice. Again, primary total anti-OA IgG levels were elevated in OA-POL compared to saline treated mice, but became similar in secondary responses (data not shown). The IgG_1 (not shown) and IgG_{2a} isotypes were enhanced in the primary response, becoming essentially equivalent to untreated age-matched controls in secondary responses. Other isotypes were only moderately affected by OA-POL treatment in long-term experiments. Therefore, although both strains exhibit strongly inhibited IgE responses when challenged 2 weeks after treatment, only the C57Bl/6 strain retains the ability to demonstrated virtually permanent reciprocal regulation of allergen specific IgE and IgG_{2a} production.



Effect of MHC haplotype on the ability of OA-POL treatment to elicit long-lived inhibition of OA-specific IgE responses.

Figure 3. B10.D2 mice ($H\text{-}2^{d}$, Igh^{b}) were treated as indicated for Figure 1 [OA-POL (o), saline (■)] and immunized (↓) on day 75, and at monthly intervals thereafter. Geometric mean titers (±SEM) are presented.

5.9 The inability to elicit long-term inhibition of anti-OA IgE responses is not MHC haplotype determined.

Given the finding that OA-POL treated C57Bl/6 ($H-2^b$) mice exhibit long-lasting inhibition of allergen specific IgE responses, while BALB/c ($H-2^d$) mice do not, we investigated the genetic basis for this difference by utilizing MHC congenic mice. We probed the role of the MHC haplotype in determining the ability to express virtually permanent IgE inhibition following administration of this chemically modified allergen.

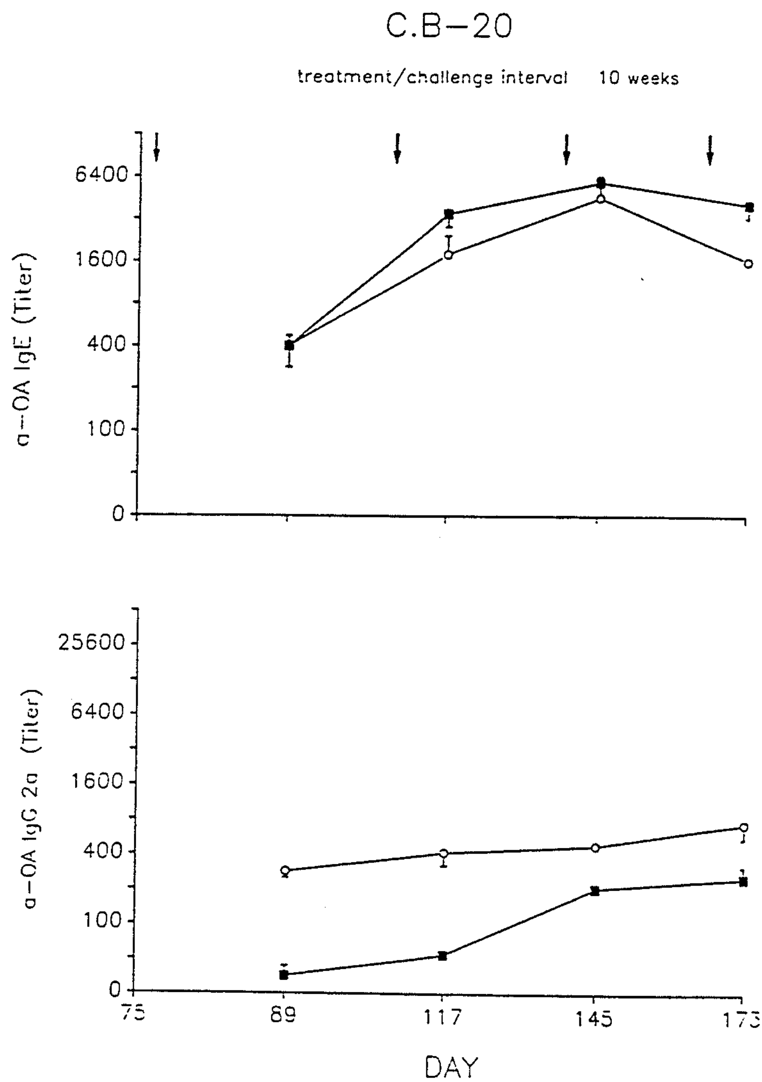
OA(alum) immunization of B10.D2 ($H-2^d$) mice between 10 weeks (**Figure 3**) and 54 weeks after administration of a single course of OA-POL treatment resulted in $\geq$ 90% inhibition of primary and secondary IgE responses relative to those generated in age-matched saline treated control mice. As in C57Bl/6 mice, administration of multiple OA [$Al(OH)_3$] booster immunizations over the subsequent three months did not diminish IgE suppression. These changes in IgE responsiveness induced by OA-POL were paralleled by >350 fold increases in antigen-specific IgG_{2a} levels in both primary and tertiary responses.

In the reciprocal experiment, OA-POL treated BALB/b ($H-2^b$) mice exhibited normal (ie. not inhibited) anti-OA IgE responses and weak secondary IgG_{2a} responses (ELISA titer <1300) when immunized 10 weeks after OA-POL treatment (data not shown). A second $H-2^d$ strain, DBA/2 failed to

demonstrate OA-POL induced long-term IgE inhibition. Taken together, these results support the hypothesis that the H-2^d haplotype is not responsible for the failure of BALB/c mice to exhibit long-lived reciprocal regulation of IgE and IgG_{2a} responses following administration of this modified allergen.

6.0 The failure of OA-POL treated BALB/c mice to demonstrate long-term inhibition of anti-OA IgE responses is not Igh haplotype determined.

In a second series of experiments, strain combinations congenic at Igh loci (immunoglobulin heavy chain) were tested to determine if the capacity to generate long-lived inhibition of IgE responses was Igh-linked. Although C.B-20 (Igh^b on BALB/c background) mice exhibit >85% decreases in their allergen specific IgE responses if immunized 10 days after OA-POL treatment, they exhibit anti-OA IgE responses equivalent to age matched controls if immunized ≥10 weeks after treatment (**Figure 4a**). These results indicate that the presence of the Igh^b haplotype is not in itself sufficient for the ability of OA-POL to induce long-term split tolerance.



Effect of Igh loci on the ability of OA-POL treatment to elicit long-lived inhibition of OA-specific IgE responses.

Figure 4 a) C.B-20 mice (H-$2^{d,}$ Igh^{b}) were treated as indicated for Figure 1 [OA-POL (o), saline (■)] and immunized (↓) on day 75, and at monthly intervals thereafter. Geometric mean titers (±SEM) are presented.

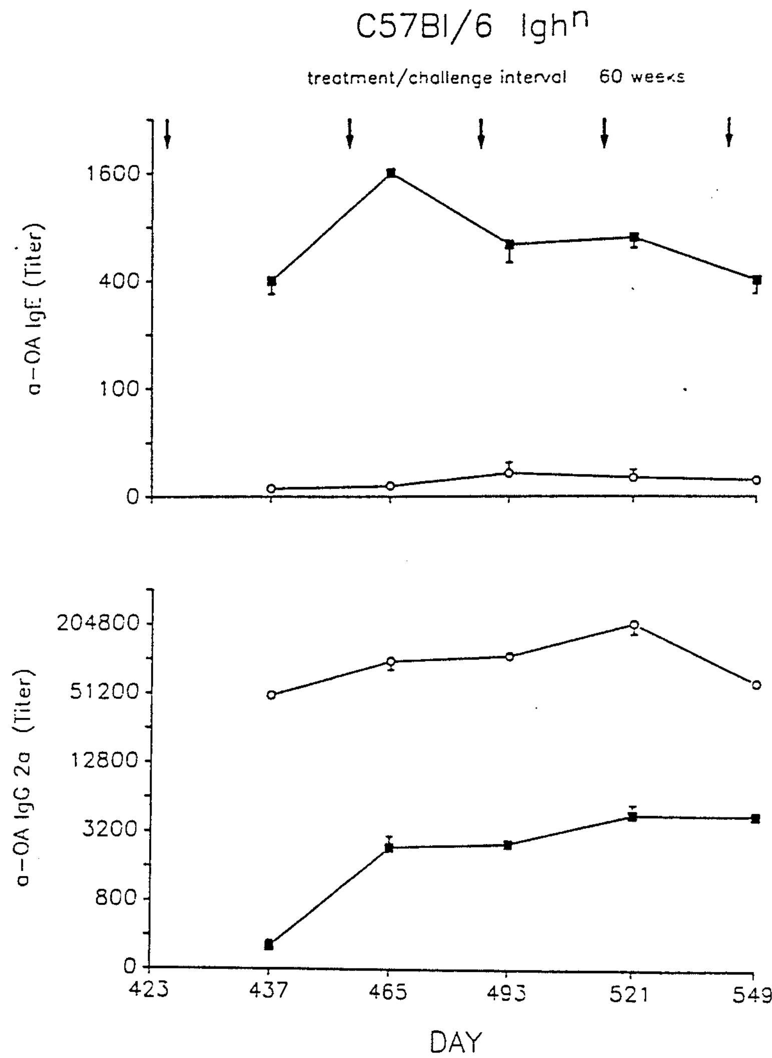


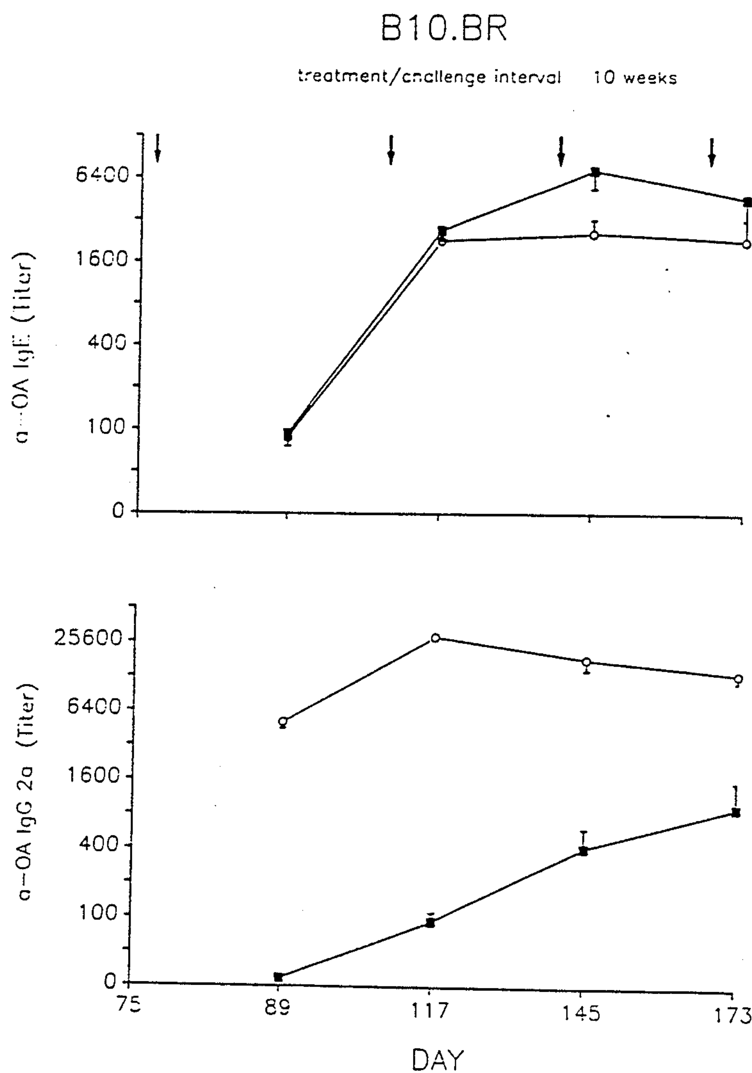
Figure 4 b) C57Bl/6 mice (H-2^{b,} Igh^n) were treated as indicated for Figure 1 [OA-POL (o), saline (■)] and immunized (↓) on day 423, and at monthly intervals thereafter. Geometric mean titers (±SEM) are presented.

To confirm that the Igh locus was not responsible for the expression of long-term anti-OA IgE decreases, a strain Igh congenic to C57Bl/6 (Igh^b) was examined. Here, a single course of OA-POL treatment resulted in > 90% inhibition of IgE responses whether C57Bl/6 Igh^n mice were immunized 12 or 60 (**Figure 4b**) weeks later. As in C57Bl/6 (Igh^b) mice, inhibition of IgE responses was not affected by multiple monthly booster immunizations. As previously, IgE inhibition was paralleled by strong allergen specific IgG_{2a} responses, typically giving ELISA titers in excess of 100,000. The results of these two experiments establish that the Igh^b and Igh^n alleles are not in themselves solely responsible for expression of reciprocal regulation of IgE and IgG_{2a} OA-specific responses in C.B-20 or C57Bl/6 mice, respectively.

An IgH restriction was also not apparent at the effector level. Spleen cells from C57Bl/6 IgH^b mice treated with OA-POL 15 weeks later inhibited the IgE response of naive IgH^n recipients by 10 fold while enhancing recipient IgG_{2a} by 235 fold. The reciprocal experiment was also performed with similar results.

6.1 Effects of background genes

Although the $H\text{-}2^d$ and $H\text{-}2^b$ haplotypes did not influence the ability of OA-POL to elicit long-term inhibition of IgE responses in the BALB or B10. background, other MHC haplotypes did have a regulatory effect. CBA/J ($H\text{-}2^k$) mice have formerly been shown to demonstrate long-lived IgE inhibition after



Effect of background genes on the ability of OA-POL treatment to elicit long-lived inhibition of OA-specific IgE responses.

Figure 5. B10.BR mice (H-2^k, Igh^b) were treated as indicated for Figure 1 [OA-POL (o), saline (■)] and immunized (↓) on day 75, and at monthly intervals thereafter. Geometric mean titers (±SEM) are presented.

treatment with OA-POL (HayGlass, 1984). Thus it was surprising that OA-POL treated B10.BR mice (H-2^k) consistently demonstrated anti-OA IgE levels equivalent to those seen in untreated controls, when immunized 10 weeks or more after treatment (**Figure 5**). The lack of a connection between B10. genes and long-lived IgE inhibition was confirmed in B10.A mice (H-2^a) wherein normal anti-OA IgE responses were obtained if mice were challenged 10 weeks or longer after OA-POL treatment. These results indicate that the expression of OA-POL induced effects on IgE responses are not solely dependent on B10. background genes. **Table 3** summarizes the results of the intact animal studies.

Demonstrate long-lived IgE inhibition	Do not demonstrate long-lived IgE inhibition
C57Bl/6 (H-2^b, Igh^b) *L. major* resistant	BALB/c (H-2^d, Igh^a) *L. major* susceptible
B10.D2 (H-2^d, Igh^b) *L. major* resistant	BALB/b (H-2^b, Igh^a) *L. major* susceptible
CBA (H-2^k) *L. major* resistant	DBA/2 (H-2^d) *L. major* susceptible
C57Bl/6 (H-2^b, Igh^n)	CB.20 (H-2^d, Igh^b)
	B10.BR (H-2^k, Igh^b)
	B10.A (H-2^a, Igh^b)

Strain Summary.

Table 3. The table summarizes the capacity of OA-POL to induce long-term IgE inhibition in the mouse strains tested. The apparent parallel between the ability of OA-POL to induce long-lived IgE inhibition in a strain and its resistance to infection with *Leishmania major* is shown.

6.2 Effects of administration of OA-POL on other isotypes

Primary OA-specific total IgG (IgG_{1-3}) responses were markedly enhanced in all OA-POL treated groups, but became equivalent or modestly reduced in secondary and tertiary responses. This observation was common to each of the strains tested, independent of whether IgE responses were inhibited. IgA levels in OA-POL treated mice were variably enhanced 0 to 8 fold. Similarly, IgM levels were variably elevated 2 to 16 fold in primary and 0 to 6 fold in secondary responses (data not shown).

6.3 A single 20 ug dose of OA-POL is sufficient to induce long-lived IgE inhibition in C57Bl/6 mice.

A single course of treatment with three 80 ug injections of OA-POL consistently induced long-lived reciprocal regulation of IgE and IgG_{2a} responses in C57Bl/6 mice. To determine the minimum dose and number of injections needed to elicit these effects, mice were treated with three 80, 20, or 5 ug doses of OA-POL on alternate days or were given a single injection of 240, 80, 20, or 5 ug OA-POL. All mice were OA(alum) immunized ten weeks after treatment and boosted 28 days later. As illustrated in **table 4**, three injections of either 80 or 20 ug OA-POL effectively induced long-lived IgE inhibition and

OA-POL Treatment	d.14 IgE	d.35 IgE	d. 14 IgG 2a	d.35 IgG 2a
None	1600	4000	25	40
3 x 80 ug	40	160	15,200	13,075
3 x 20 ug	80	80	17,480	19,620
3 x 5 ug	1600	3200	1,450	1,790
1 x 240 ug	80	80	24,625	17,600
1 x 80 ug	40	320	19,700	14,800
1 x 20 ug	160	320	4,650	6,200

How much OA-POL is required to induce long-lived IgE inhibition in C57Bl/6 mice?

Table 4. To determine the dose and number of injections needed to induce long-term IgE inhibition, C57Bl/6 mice were given 80, 20 or 5 ug OA-POL on days 0, 2 and 4. Other groups of mice received a single injection of 240, 80, 20 or 5 ug OA-POL on day 0. All mice were immunized on day 70 and boosted on day 98. OA-specific reciprocal IgE and IgG_{2a} titers are shown from sera collected 14 days after primary and 7 days after secondary immunization. Standard errors were less than 15% in all instances. Results are presented from one of two experiments performed.

IgG_{2a} enhancement in both primary and secondary responses. In contrast, three 5 ug doses failed to significantly inhibit IgE responses but still resulted in 50 fold enhancement of primary, and 20 fold increases in secondary, IgG_{2a} levels. Mice given a single dose of 240, 80, or 20 ug OA-POL induced effects similar to the standard treatment protocol. However, a single injection of 5 ug led to no significant inhibition of IgE nor to any strong enhancement of IgG_{2a}. These results indicate that a single injection of 20 ug OA-POL leads to long-lived reciprocal regulation of IgE and IgG_{2a} in primary and secondary C57Bl/6 responses.

6.4 Is the lack of long-lived IgE inhibition in some murine strains dose related? Use of 3 x 800 ug versus 3 x 80 ug OA-POL

To determine if our inability to elicit long-lived IgE hyporesponsiveness in some strains was dose related, C57Bl/6, BALB/c, DBA/2, and (C57Bl/6 x BALB/c) F1 hybrid mice were treated with three 80 or 800 ug doses of OA-POL. Twelve weeks after treatment, cohorts of mice were OA(alum) immunized and boosted 28 days later. **Table 5** shows that all three strains that fail to demonstrate long-lived IgE inhibition when treated with 3 x 80 ug OA-POL also fail to demonstrate inhibition when treated with 3 x 800 ug. In should also be noted that this increase in treatment dosage did not enhance the IgE inhibition found in C57Bl/6 mice beyond the 90-95% decrease normally

Strain	OA-POL Treatment	d.14 IgE	d.35 IgE	d.14 IgG_{2a}	d.35 IgG_{2a}
C57Bl/6	None	2000	6400	40	55
	3 x 80 ug	40	160	36,400	34,950
	3 x 800 ug	50	200	152,800	102,300
BALB/c	None	1600	6400	70	2,500
	3 x 80 ug	800	3200	7,450	6,200
	3 x 800 ug	2000	6400	9,450	7,800
DBA/2	None	640	1600	15	420
	3 x 80 ug	640	800	1,480	975
	3 x 800 ug	400	1600	6,955	7,200

Is the lack of OA-POL induced long-lived IgE inhibition in some strains dose dependent?

Table 5. To determine whether the inability to demonstrate OA-POL induced long-lived IgE inhibition is dose related, C57Bl/6, BALB/c, and DBA/2 mice were treated with either 80 or 800 ug OA-POL, or saline on days 0, 2 and 4. All mice were immunized on day 70 and boosted on day 98. OA-specific reciprocal IgE and IgG_{2a} titers are shown from sera collected 14 days after primary and 7 days after secondary immunization. Standard errors were less than 15% in all instances.

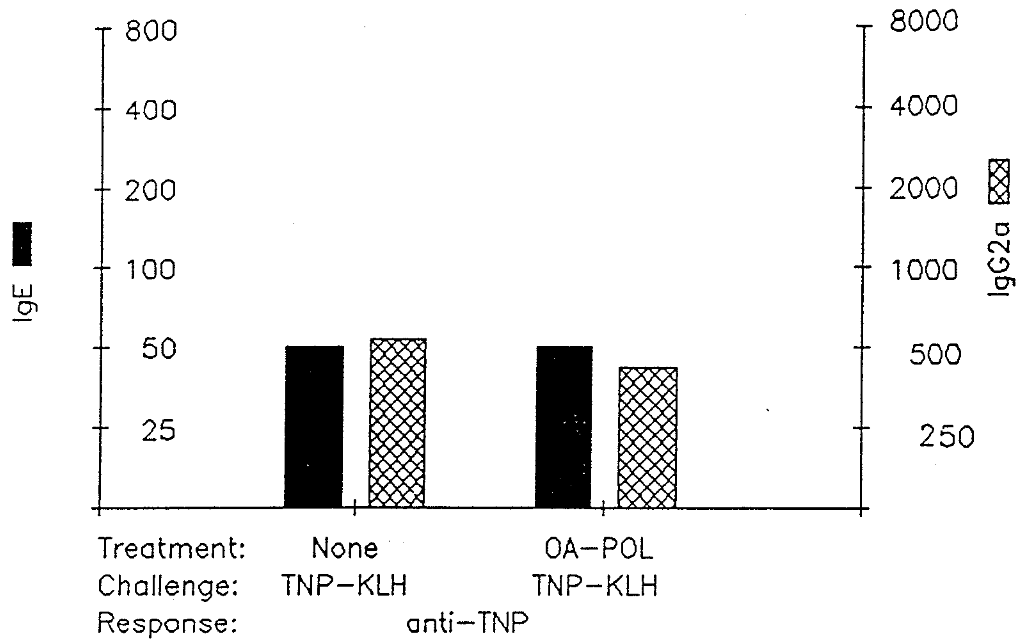
induced by 3 x 80 ug, although IgG_{2a} responses were on average 3-4 fold higher in the 3 x 800 ug treated group.

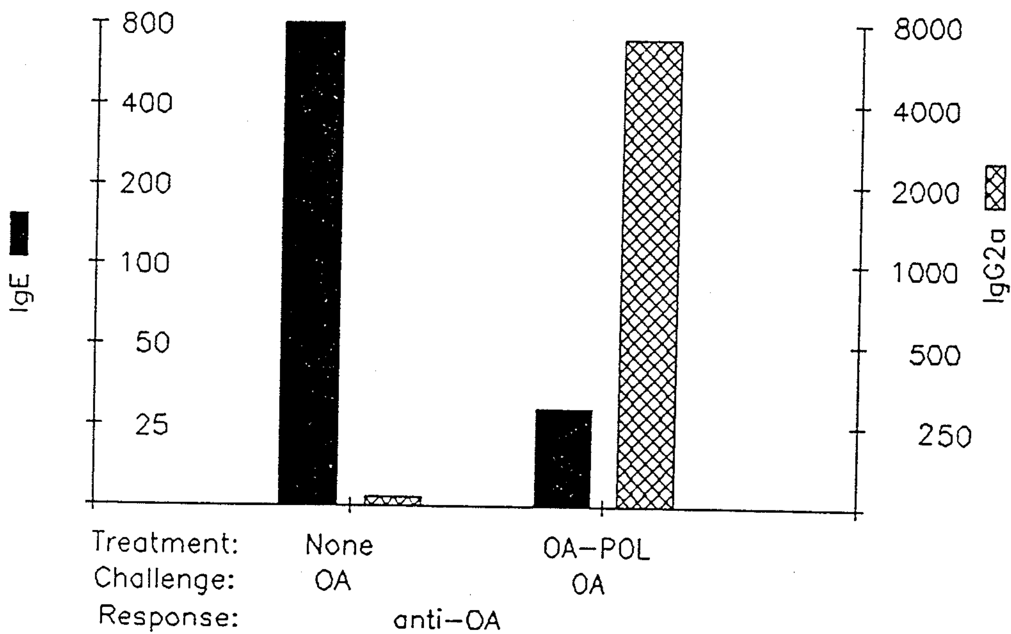
6.5 Antigenic specificity

The specificity of this long-lived inhibition of IgE responses was demonstrated upon challenge of saline or OA-POL treated C57Bl/6 mice six months after treatment. In the experiment shown (**Figure 6**), OA-POL pretreatment resulted in 94% inhibition of OA specific IgE responses and 350 fold increases in anti-OA IgG_{2a} production. In contrast, IgE and IgG_{2a} responses to TNP were unaffected by OA-POL treatment prior to TNP_{22}-KLH immunization. Similar results were obtained in C57Bl/6 Igh^n mice (data not shown).

6.6 Longevity and T cell dependency of IgE inhibition in C57Bl/6 mice.

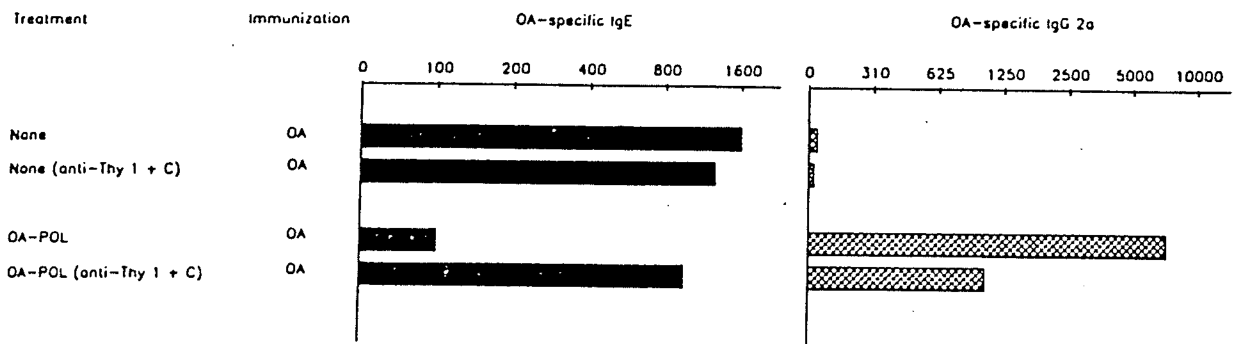
OA-POL induced inhibition of IgE responses in C57Bl/6 mice is actively mediated as demonstrated by the ability of 80 x 10^6 spleen cells from mice treated with OA-POL greater than 10 weeks earlier to consistently inhibit the development of IgE responses when transferred i.v. to naive recipients. Indeed, the longevity of the changes induced as a consequence of OA-POL treatment was such that transfer of 40 x 10^6 spleen cells obtained 60 weeks





Antigenic specificity of OA-POL's effects on IgE and IgG_{2a} responses.

Figure 6. C57Bl/6 mice were treated with OA-POL or saline and 185 days later challenged with TNP_{22}-KLH (top panel) or OA (bottom panel). Peak IgE (■) and IgG_{2a} (⊠) responses 10 days later are indicated.



OA-POL induced IgE inhibition is T cell dependent.

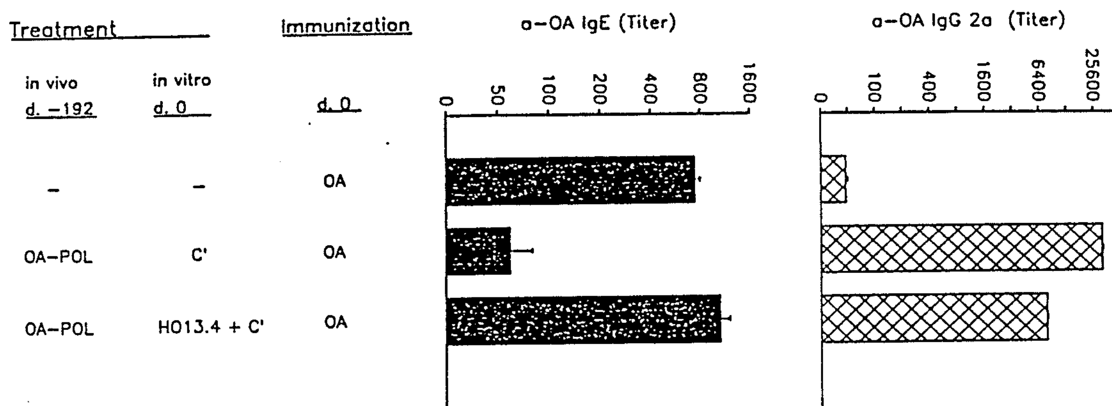
Figure 7. Six months after OA-POL treatment 80 x 10^6 spleen cells, or T depleted spleen cells, were transferred i.v. to normal recipients. All recipients were immunized immediately after cell transfer and bled 10 days later. Treatment with anti-Thy 1.2 (HO13.4 tissue culture supernatant) and complement resulted in 41% cell lysis while complement alone killed 8% in this experiment.

after a single course of OA-POL treatment led to a 10 fold reduction in OA-specific IgE responses in the recipient mice (PCA titer 80 vs 800 in recipients of spleen cells from OA-POL treated and untreated mice respectively.)

To examine the mechanism by which IgE responses were inhibited, whole or T cell depleted spleen cell populations obtained from mice treated with OA-POL six months earlier were transferred to normal recipients which were immunized the same day (**Figure 7**). Recipients of T cell populations from OA-POL treated mice exhibited 90% inhibition of IgE responses concomitant with 400-600 fold increases in anti-OA IgG_{2a}, in comparison to mice receiving normal or T cell depleted spleen cells. The increases in IgG_{2a} production were reduced by 60-80% if donor T cells were depleted prior to transfer.

6.7 T cell dependence in C57Bl/6 Igh^n mice.

To determine if OA-POL induced IgE suppression was T cell dependent in a second strain, a similar experiment was carried out in C57Bl/6 Igh^n mice (**Figure 8**). Transfer of 80 x 10^6 spleen cells to normal recipients from mice treated 192 days earlier resulted in 87% inhibition of allergen specific IgE production and 320 fold increases in IgG_{2a} relative to normal controls. *in vitro* T cell depletion before transfer abrogated the ability of the transferred



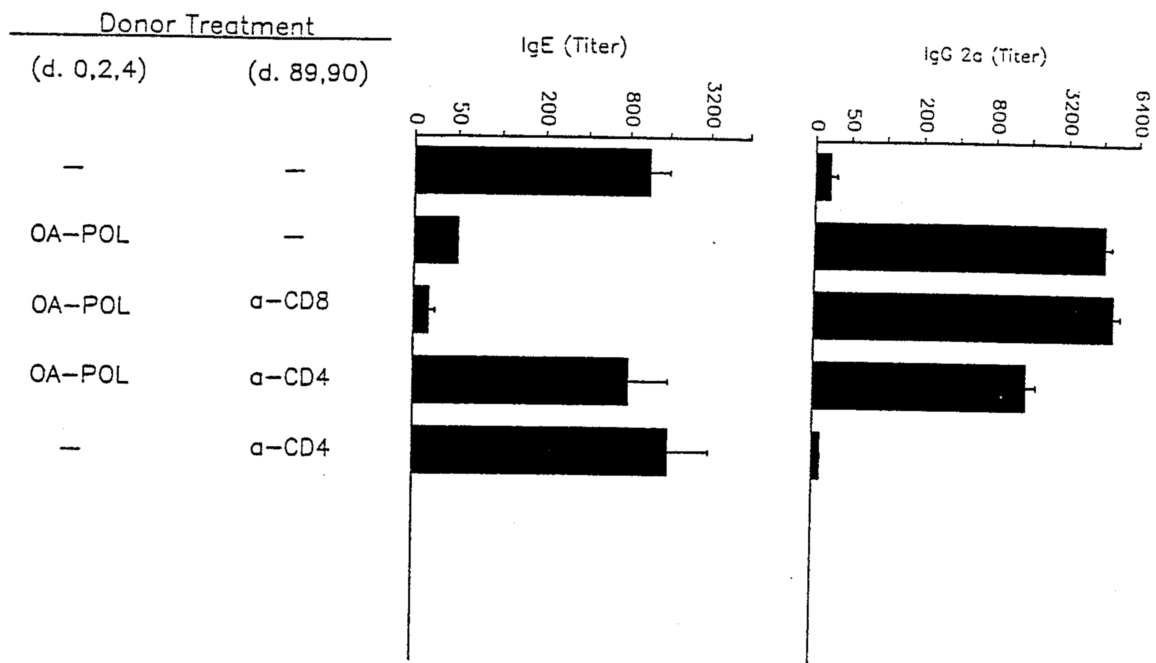
T cell dependence of OA-POL induced IgE inhibition in C57Bl/6 Igh^n mice.

Figure 8. Intact or T cell depleted spleen cell populations were transferred into normal recipients from C57Bl/6 Igh^n mice treated with OA-POL 192 days earlier. Recipients were immunized the same day and bled 10 days later. Geometric mean titers (±SEM) are shown from one of two experiments performed.

cells to inhibit IgE responses. Moreover, recipients of T cell depleted spleen cell populations from OA-POL treated donors exhibited a 70% decrease in allergen specific IgG_{2a} levels relative to those observed following transfer of complement treated spleen cells from OA-POL treated donors.

6.8 *In vivo* depletion of CD4 but not CD8 T cells ablates the ability to transfer IgE inhibition to naive recipients.

In vivo depletion of CD4 or CD8 T cells with anti-CD4 or anti-CD8 mAb was used to characterize the role of these subsets in the effects elicited by this modified allergen. Following OA-POL treatment (d. 0, 2, 4), mice were depleted of $CD4^+$ or $CD8^+$ cells via i.p. injection of the appropriate mAb on days 89 and 90. On day 91 anti-CD4 and anti-CD8 treated mice were found to contain less than 3% of control splenic CD4 or CD8 T cells respectively as assessed by flow cytometry. The same day 90×10^6 spleen cells were transferred from each group to normal recipients. As a control for effects of potentially carried over anti-CD4 mAb, the same number of cells were transferred from CD4 depleted donors that had not been treated with OA-POL. All recipients were OA(alum) immunized the same day and bled 10 (**Figure 9**), 12 and 14 days later.



CD4 T cell dependence of OA-POL induced IgE inhibition.

Figure 9. C57Bl/6 mice were treated with OA-POL or nothing (d. 0, 2, 4), and then CD4-depleted or CD8-depleted by in vivo treatment with YTS 191.1 or YTS 169.4 on days 89 and 90. On day 91, 90 x 10^6 spleen cells from these donors were transferred i.v. to normal recipients, immediately prior to immunization. Cells were also transferred from nontreated, CD4 depleted mice as a control for possible in vivo effects of passively transferred anti-CD4 mAb. CD4-depleted and CD8 depleted donor cell populations exhibited <3% of normal $CD4^+$ or $CD8^+$ spleen cells at the time of transfer as assessed by flow cytometry. OA-specific IgE and IgG_{2a} geometric mean titers (±SEM) 10 days after immunization are shown. Results are presented from one experiment representative of two performed.

Recipients of spleen cells from OA-POL treated donors produced IgE responses that were, at most, 7% of those seen in normal controls, while IgG_{2a} responses were 380 fold greater. Depletion of $CD8^{+}$ cells from the transferred population was without effect on these reciprocal changes in IgE and IgG_{2a} synthesis. In contrast, recipients of CD4 depleted spleen cells from OA-POL treated mice, produced anti-OA IgE responses not different than those of untreated, $OA[Al(OH)_3]$ immunized mice. Consistent with earlier transfer studies, the elevated IgG_{2a} response seen in recipients of cells from OA-POL treated donors was 4.4 fold reduced when CD4 depleted, OA-POL treated donors were used. The same number of cells transferred from a CD4 depleted but not OA-POL treated group as a control for the *in vivo* effects of possibly transferred anti-CD4 mAb failed to detectibly influence OA specific IgE or IgG_{2a} production.

6.9 CD4 T cell dependence of long-lived IgE inhibition

As a complementary approach to verify the CD4 T cell dependence of OA-POL induced IgE inhibition (**Table 6**), naive or OA-POL treated (d 0, 2, 4) C57Bl/6 mice were administered anti-CD4 mAb (YTS 191.1) i.p. on days 123 and 124. On day 126, parallel groups of anti-CD4 treated mice contained <2% of control CD4+ T cells as determined by flow cytometry. One month

Treatment		anti-OA IgE		anti-OA IgG2a	
d. 0,2,4	d.123,124	d. 161	d. 163	d. 161	d. 163
None	None	800	1600	62	52
None	anti-CD4	640	1280	20	40
OA-POL	None	100	160	12,960	11,320
OA-POL	anti-CD4	800	1600	10,880	9,775

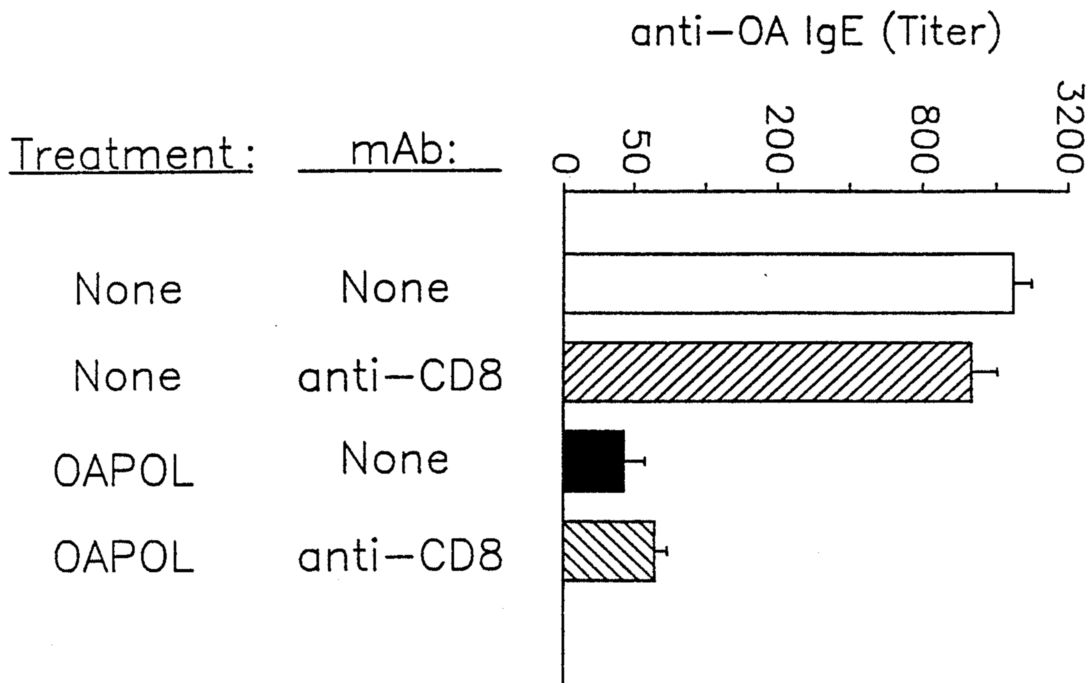
Inhibition of IgE responses following administration of OA-POL is abolished by *in vivo* treatment with anti-CD4 mAb.

Table 6. OA-POL treated and age matched normal C57Bl/6 mice were depleted of $CD4^+$ cells *in vivo* 123 days after OA-POL treatment via i.p. injection of anti-CD4 mAb (YTS 191.1) as described in materials and methods. 28 days later, when $CD4^+$ T cell levels had returned to control levels, all groups were immunized and bled 10 days later. Reciprocal IgE and IgG_{2a} titers are presented. Standard errors were less than 15% in all instances.

later (day 151), a time when anti-CD4 treated mice had regenerated their CD4 T cell repertoire and exhibited CD4 T cell frequencies equivalent to normal nondepleted mice, all groups were OA(alum) immunized. As previously, OA-POL treated mice generated 8 to 10 fold decreased allergen specific IgE paralleled by 210 fold enhanced IgG_{2a} levels relative to untreated, OA(alum) immunized mice. *in vivo* depletion of CD4 T cells from OA-POL treated mice, abolished their capacity to inhibit IgE responses but was largely without effect on IgG_{2a} responsiveness.

7.0 *In vivo* depletion of CD8 T cells does not abrogate long-term IgE inhibition.

In order to assess the contribution of CD8 T cells in this system, OA-POL treated (d. 0, 2, 4) and normal mice were depleted of $CD8^+$ cells by injection of anti-CD8 mAb (YTS 169.4) on days 123 and 124. On day 126, anti-CD8 treated mice contained less than 2% $CD8^+$ spleen cells. All mice were immunized the same day and bled 10 (**Figure 10**) and 14 days later. CD8 depletion of normal mice did not affect their ability to produce an IgE response. OA-POL treated mice demonstrated 97% suppression of IgE. This level of inhibition was not significantly affected by CD8 T cell depletion. This treatment also had no detectable effect on the quantity of IgG_{2a} produced (data not shown).



OA-POL induced inhibition of IgE responses is not altered by in vivo depletion of CD8 T cells.

Figure 10. To determine the contribution of CD8 T cells to OA-POL induced long-term IgE inhibition, normal and OA-POL treated mice were depleted of CD8 T cells by injection of anti-CD8 mAb (YTS 169.4) 123 and 124 days after treatment. On day 126 these mice were found to have $<2\%$ of control CD8 levels. All mice were immunized the same day and bled 10 (shown), 12 and 14 days later. Geometric mean IgE titers ($\pm$SEM) are presented.

Collectively, the *in vivo* depletion studies indicated that OA-POL induced long-term changes in IgE synthesis are CD4 T cell dependent. The relative lack of impact of CD4 T cell depletion on increases in IgG_{2a} synthesis is consistent with the hypothesis that much of the effect on this isotype is dependent on induction of long-lived $B_{\gamma 2a}$ memory cells by OA-POL treatment.

The striking reciprocal regulation of IgE and IgG_{2a} responses *in vivo*, taken with the observation that depletion of CD4 but not CD8 T cells in OA-POL treated donors ablates the ability to transfer IgE inhibition, suggested that pre-administration of this chemically modified allergen biases the pattern of CD4 T cell cytokine synthesis elicited by subsequent exposure to native allergen toward an IFNγ dominated response. This hypothesis was tested using three independent approaches.

7.1 Administration of anti-IFNγ mAb coincident with OA-POL treatment abrogates IgE inhibition and decreases the enhancement of IgG_{2a} synthesis.

To explore the role of IFNγ in the *induction* of long-term reciprocal regulation of IgE and IgG_{2a}, highly purified anti-IFNγ mAb (XMG1.2) was administered concomitant with OA-POL treatment. C57Bl/6 mice were treated with 80 ug of OA-POL on days 0, 2 and 4, with subgroups of these mice also receiving 250 ug anti-IFNγ, normal rat immunoglobulin (NRIg), or nothing i.p.

Treatment		anti-OA IgE		anti-OA IgG2a	
d. 0,2,4	d.0-5,7,9	d. 80	d. 84	d. 80	d. 84
None	**None**	**1,905**	**1,131**	**55**	**83**
None	**anti-IFNγ**	**1,270**	**1,131**	**51**	**54**
OA-POL	**None**	**119**	**70**	**7,482**	**9,744**
OA-POL	**anti-IFNγ**	**800**	**566**	**1,116**	**780**

Sensitivity of long-lived IgE inhibition to anti-IFNγ mAb administration at the time of OA-POL treatment.

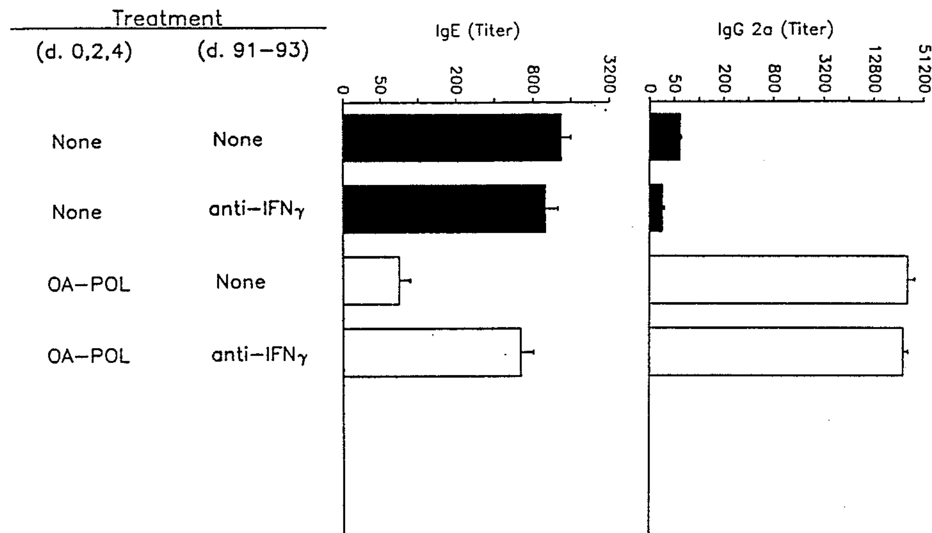
Table 7. C57Bl/6 mice were treated with OA-POL on days 0, 2 and 4. Separate cohorts of these mice also received anti-IFNγ mAb (250 ug/day), normal rat immunoglobulin (250 ug/day, data not shown) or nothing on days 0, 1, 2, 3, 4, 5, 7 and 9. Groups of untreated mice were treated similarly. Ten weeks later, all groups were immunized and antibody responses determined 10 and 14 days later. Data are geometric mean titers. Standard errors were less than 15% in all instances and are omitted for clarity.

on days 0, 1, 2, 3, 4, 5, 7 and 9 (2 mg total). Normal control mice were also treated with anti-IFNγ or NRIg in the absence of OA-POL. Ten weeks later (d. 70), all mice were immunized and bled 10 (**Table 7**) and 14 days thereafter.

Treatment of normal mice with anti-IFNγ and normal or OA-POL treated mice with NRIg did not detectably affect the immune response of these mice. As would be expected, OA-POL treated mice produced IgE titers reduced by greater than 90% compared to normal mice, concomitant with 115 to 140 fold increases in IgG_{2a}. However, administration of anti-IFNγ during OA-POL treatment blocked IgE inhibition, resulting in virtually normal IgE responses and 7-12 fold decreases in IgG_{2a} (80 - 90% reductions) compared to OA-POL treated mice that did not receive anti-IFNγ. Therefore, early production of IFNγ during OA-POL treatment is required for induction of the capacity to inhibit IgE responses elicited by native OA immunization 10 weeks later.

7.2 Administration of anti-IFNγ at the time of OA[Al(OH)$_3$] immunization abrogates OA-POL induced anti-OA IgE inhibition but does not significantly alter the enhancement of anti-OA IgG_{2a} responses.

To examine the role of IFNγ in the *expression* of long-term IgE inhibition, anti-IFNγ mAb was administered coincident with OA(alum) immunization of C57Bl/6 mice. OA-POL treated and age-matched normal mice were



In vivo administration of anti-IFNγ mAb at the time of OA immunization blocks IgE inhibition, but not IgG_{2a} enhancement, elicited by OA-POL treatment given 91 days earlier.

Figure 11. OA-POL treated C57Bl/6 mice, and age matched untreated controls, were immunized on day 91. The same day, and for two consecutive days thereafter, independent subgroups of treated and normal immunized mice were injected i.p. with purified anti-IFNγ mAb XMG 1.2 (250 ug/day, i.p.), normal rat immunoglobulin (250 ug/day, not shown), or nothing. IgE and IgG_{2a} responses 10 (shown), 12 and 14 days later were assessed. Geometric mean titers (±SEM) are from one of four experiments performed.

immunized 13 weeks after treatment on day 91. On days 91-93, groups of these mice received 250 ug of anti-IFNγ mAb (XMG 1.2), normal rat immunoglobulin (NRIg) or nothing i.p.. The mice were bled 10 (shown), 12 and 14 days later. Administration of XMG 1.2 or NRIg (data not shown) to control OA(alum) immunized mice did not alter IgE or IgG_{2a} responses. As previously, OA-POL treated mice displayed a 90% reduction in anti-OA IgE and a 560 fold increase in IgG_{2a} **(Figure 11)**. However, administration of anti-IFNγ to OA-POL treated mice resulted in elevated IgE production, equivalent to that seen in normal controls. Of interest was the finding that increases in anti-OA IgG_{2a} levels induced by OA-POL treatment were not significantly altered by anti-IFNγ administration at the time of immunization. This observation, along with the ability of anti-IFNγ administration during OA-POL treatment to block the majority of IgG_{2a} enhancement, supports the hypothesis that OA-POL treatment results in early induction of an isotype switch to IgG_{2a} and production of $B_{\gamma 2a}$ memory cells. Therefore long-lived inhibition of anti-OA IgE demonstrated in C57Bl/6 mice requires the production of IFNγ during the immune response toward native allergen.

Collectively the *in vivo* anti-IFNγ studies suggest that administration of this chemically modified allergen leads to long-lived, IFNγ dependent inhibition of IgE responsiveness *in vivo*. The necessity for production of this cytokine both during OA-POL treatment and after OA immunization several months later

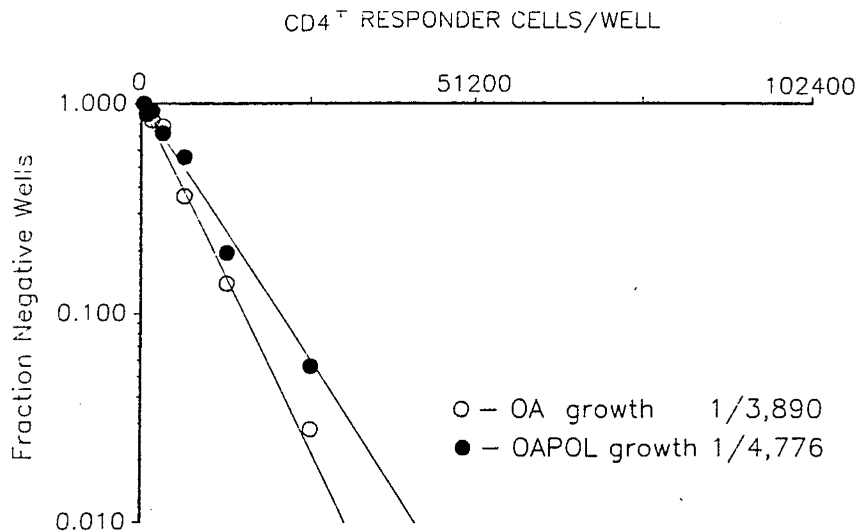
is consistent with a virtually permanent shift in the OA-specific T cell repertoire *in vivo*, from a response dominated by Th2-like patterns of cytokine synthesis (IL-4/IgE) to one dominated by Th1-like responses (IFNγ/IgG_{2a}). The clear implication is that cytokine synthesis patterns elicited upon antigen exposure *in vivo* are affected by the physical nature of the sensitizing/immunizing antigen and can be manipulated via administration of appropriately modified forms of antigen.

7.3.1 Analysis of the precursor frequency of CD4 T cells capable of IFNγ, IL-4 or IL-10 synthesis.

Accumulated evidence suggested that OA-POL was inducing cytokine synthesis patterns distinct from those induced following exposure to native OA. We utilized limiting dilution analysis to determine the frequencies of IFNγ, IL-4 and IL-10 synthesis within the CD4 T cell repertoire after OA-POL compared to OA treatment. We initially investigated the frequency of allergen specific CD4 T cells secreting IFNγ, IL-4, and IL-10 in native versus chemically modified allergen treated mice. Cytokine synthesis patterns were determined in these experiments in the absence of additional OA(alum) immunization in

order to directly compare the cytokine patterns induced by native compared to chemically modified allergen.

C57Bl/6 mice were treated i.p. with 80 ug OA or OA-POL on days 0, 2 and 4. On day 9, the mice were sacrificed and highly enriched $CD4^+$ T cell populations were isolated. Titrations of limiting numbers of these responder cells along with 6×10^5 irradiated normal spleen cells, and 20 U/ml rIL-2 were cultured in the presence or absence of OA for 16 days. At this time, growth was scored microscopically. All cultures were then washed and restimulated with fresh antigen and irradiated APC. After 48 hours, individual aliquots of supernatant from each well were harvested and tested for the presence of IL-4, IFNγ and IL-10. In the absence of antigen, no growth was noted at concentrations of CD4 T cells used for analysis. At very high CD4 T cell concentrations from 0-6/36 cultures scored positive for growth. However, these cultures failed to secrete any of the tested cytokines when restimulated with antigen/APC.



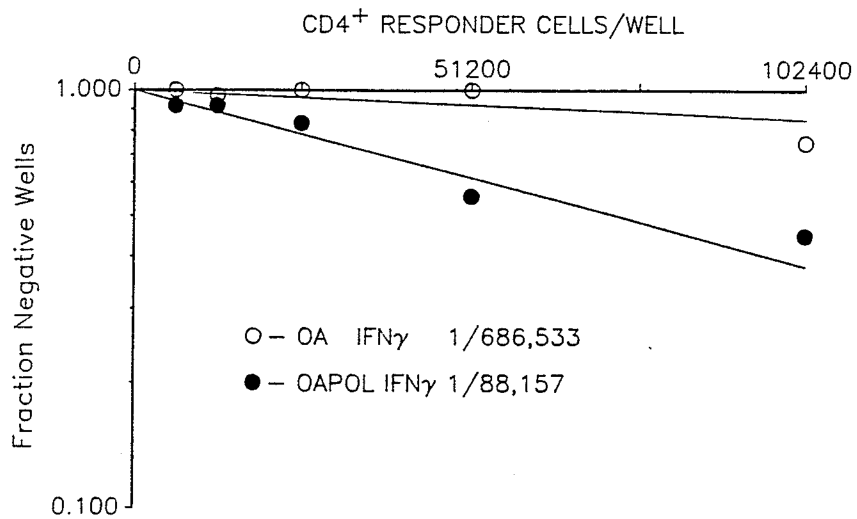
OA specific Precursor Frequency

	Treatment		Comparison
Exp. #	OA	OA-POL	OA-POL/OA
1	1/2,446 (1/2009 - 1/3127)	1/1,670 (1/1361 - 1/2158)	1.4
2	1/3,890 (1/3,183 - 1/5,002)	1/4,766 (1/3,893 - 1/6,141)	0.82
3	1/21,682 (1/17,809 - 1/27,723)	1/31,540 (1/25,782 - 40,609)	0.68

Comparison of the frequency of IFNγ, IL-4, and IL-10 producing allergen specific CD4+ cells from mice treated with native versus chemically modified allergen.

Frequency of antigen specific growth in OA and OA-POL treated mice.

Figure 12 a) C57Bl/6 mice were treated with OA or OA-POL (d. 0, 2, 4) and sacrificed on day 9. Graded numbers of highly enriched CD4+ splenic T cells (92 to 96% CD4 positive as assessed by flow cytometry) were cultured as described in materials and methods. Each well was scored for growth and aliquots of supernatant were assayed for the presence of IL-4 by CT.4S bioassay, and IFNγ and IL-10 by specific sandwich ELISAs. Estimates of the frequency of responding cells were obtained by the maximum likelihood method based on Poisson statistics. Frequency estimates for antigen specific growth including the 95% confidence intervals, from three experiments performed, are summarized in the attached table .

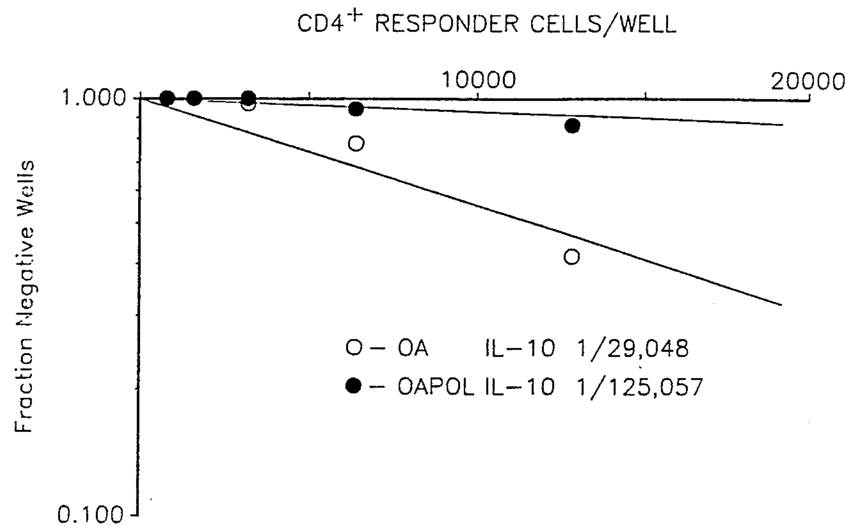


IFNγ Precursor Frequency

	Treatment		Comparison
Exp. #		OA-POL	OA-POL/OA
1	1/161,791 (101,684 - 395,685)	1/31,981 (24,816 - 44,961)	5.1
2	1/686,533 (423,701 - 1,808,211)	1/88,157 (70,402 - 117,887)	7.8
3	1/3,519,754 (1,475,18 1 - 9,118,968)	1/200,110 (146,383 - 316,149)	17.6

Increased IFNγ precursor frequency after OA-POL compared to OA treatment.

Figure 12 b) Estimates of the frequency of IFNγ producing CD4 T cells were obtained by the maximum likelihood method based on Poisson statistics. Frequency estimates including the 95% confidence intervals, from three experiments performed, are summarized in the attached table.

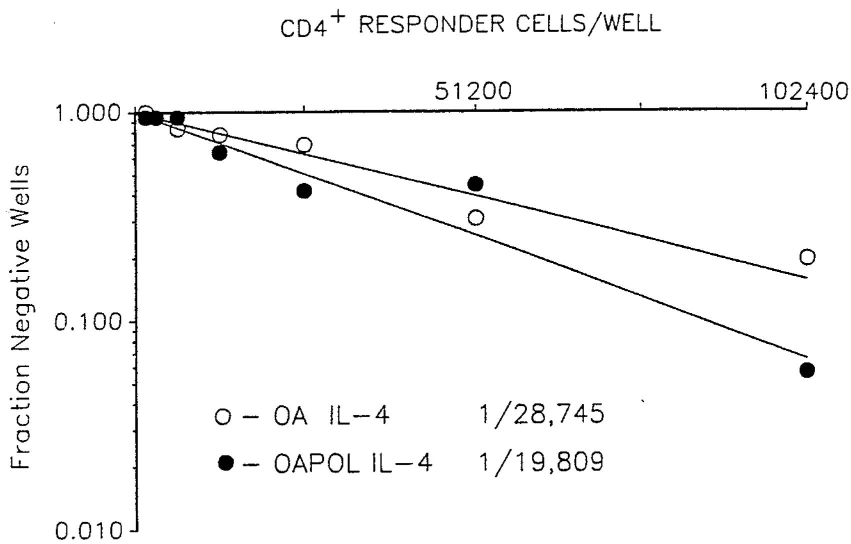


IL-10 Precursor Frequency

	Treatment		Comparison
Exp. #	**OA**	**OA-POL**	**OA-POL/OA**
1	**1/29,048** (20,943 - 47,390)	**1/125,057** (71,830 - 482,967)	**0.23**
2	**1/348,109** (214,873 - 916,236)	**1/1,164,613** (546,331 - 8,843,147)	**0.30**
3	**1/440,255** (257,927 - 1,502,033)	**< 1/5,092,956** (1,913,308 - 7,614,925)	**0.09**

Decreased IL-10 precursor frequency induced by OA-POL treatment.

Figure 12 c) Estimates of the frequency of IL-10 producing CD4 T cells were obtained by the maximum likelihood method based on Poisson statistics. Frequency estimates including the 95% confidence intervals, from three experiments performed, are summarized in the attached table.



IL-4 Precursor Frequency

	Treatment		Comparison
Exp. #	OA	OA-POL	OA-POL/OA
1	1/24,187 (19,246 - 32,543)	1/12,122 (9,988 - 15,415)	2.0
2	1/28,745 (23,719 - 36,473)	1/19,809 (16,278 - 25,297)	1.5
3	1/164,427 (122,692 - 249,169)	1/135,930 (103,014 - 199,757)	1.2

Similar IL-4 precursor frequencies in mice treated with OA or OA-POL.

Figure 12 d) Estimates of the frequency of IL-4 producing CD4 T cells were obtained by the maximum likelihood method based on Poisson statistics. Frequency estimates including the 95% confidence intervals, from three experiments performed, are summarized in the attached table.

Figure 12 a) shows results from one of three experiments. The frequency of antigen specific growth was very similar in mice treated with native OA or OA-POL (OA 1/3890 versus OA-POL 1/4766 $p > 0.05$). In each of three experiments, no significant difference in the frequency of antigen specific proliferation was noted.

In contrast to the similar frequencies of antigen specific proliferation, the frequency of CD4 T cells secreting IFNγ was strongly altered by treatment with chemically modified allergen (**Figure 12 b**). In the experiment shown, the frequency of CD4 T cells capable of IFNγ secretion was increased 7.8 fold in OA-POL compared with that found in OA treated mice. Over three experiments, the frequency of CD4 T cells capable of IFNγ secretion was increased on average 10 fold in chemically modified versus native allergen treated mice.

Analysis of the frequency of CD4 T cells capable of IL-10 secretion demonstrated that although CD4 T cells producing IL-10 were detectable in mice treated with native OA, positive wells were very rare (**Figure 12 c**) or not detectable (see experiment 3) in mice treated with chemically modified allergen. In three experiments, the frequency of IL-10 secreting CD4 T cells was reduced 3 to 12 fold (average 6.4) in chemically modified treated compared with native allergen treated mice.

In contrast, the frequency of CD4 T cells capable of IL-4 synthesis was similar in OA and OA-POL treated groups (OA 1/28,745 versus OA-POL 1/19,809 $p > 0.05$) (**Figure 12 d**). In two of three experiments there was no significant difference in IL-4 precursor frequency between these two treatment protocols.

7.4 Effect of anti-IFNγ treatment on precursor frequencies of IFNγ, IL-4 and IL-10 secreting CD4 T cells in OA and OA-POL treated mice.

Previous experiments demonstrated that *in vivo* administration of anti-IFNγ mAb during OA-POL treatment, or at the time of OA immunization ten weeks later, restores normal IgE production (**Table 7, Figure 11**). Here, we directly determined the effects of *in vivo* IFNγ neutralization on the frequency of CD4 T cells able to secrete IFNγ, IL-4 and IL-10 in OA and OA-POL treated mice. Mice were treated with 80 ug OA or OA-POL (d. 0, 2, 4) and 250 ug of either anti-IFNγ or normal rat immunoglobulin (NRIg) (d. -1, 0, 1, 2, 3, 4, 5, 7).

NRIg had no significant effect on the frequencies of IFNγ, IL-4, or IL-10 secreting CD4 T cells (**Table 8**). Comparison of the frequency of CD4 T cells capable of IFNγ or IL-10 production between OA-POL/NRIg and OA/NRIg treated mice demonstrated that OA-POL/NRIg treatment induced a 6.3 fold increase in the frequency of IFNγ secretion paralleled by a significant ($p < .05$)

Precursor Frequency

Treatment	IFNγ	IL-4	IL-10
OA/NRIg	1/ 217,549 (109,867 - 10,940,200)	1/ 20,025 (15,115 - 29,658)	1/37,930 (31,325 - 48,066)
OAPOL/NRIg	1/34,509 (24,475 - 58,491)	1/12,682 (9,926 - 17,558)	1/87,325 (69,867 - 116,411)
OA/anti-IFNγ	1/175,004 (93,254 - 1,418,517)	1/6,658 (5,167 - 9,359)	1/8,319 (6,307 - 12,218)
OAPOL/anti-IFNγ	1/41,260 (28,675 - 73,535)	1/ 3,205 (2,467 - 4,574)	1/8,233 (6,248 - 11,860)

Effect of anti-IFNγ treatment on the precursor frequencies of IFNγ, IL-4 and IL-10 secretion in OA and OA-POL treated mice.

Table 8. $CD4^+$ T cells obtained from mice treated with anti-IFNγ or normal rat Ig simultaneous with OA or OA-POL immunization, as described in materials and methods, were cultured and analyzed as in figure 12. The precursor frequency of IFNγ, IL-4 and IL-10 synthesis in one experiment representative of three performed are presented. 95% confidence intervals are included in brackets.

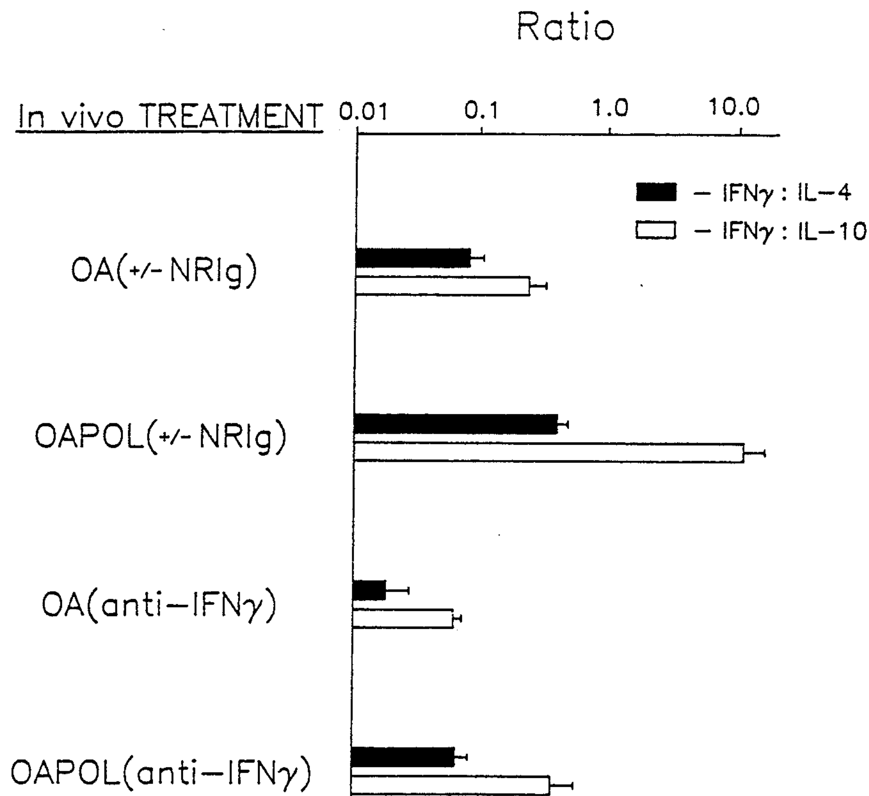
2.3 fold decrease in the frequency of IL-10 secretion. No significant difference in the frequency of IL-4 secretion was observed between OA-POL/NRIg and OA/NRIg treated mice, consistent with previous experiments **(Figure 12 d)**.

Anti-IFNγ treatment did not significantly affect the frequency of IFNγ secretion in OA or OA-POL treated mice relative to mice treated with OA or OA-POL and NRIg. Thus, we observed a 4.3 fold increase in IFNγ secreting CD4 T cells in OA-POL/anti-IFNγ compared to OA/anti-IFNγ treated mice. However, this treatment had a pronounced effect on IL-4 and IL-10 precursor frequency. In OA treated mice, administration of anti-IFNγ mAb caused a 3 fold increase in IL-4 precursor frequency (average of three experiments 6.5 fold) concurrent with a 4.6 fold increase in IL-10 frequency (average of three experiments 4.2 fold) compared to mice receiving OA and NRIg. In OA-POL/anti-IFNγ treated mice there was a 4 fold increase in IL-4 precursor frequency (average of three experiments 4.3 fold) paralleled by a 10.6 fold increase in the IL-10 precursor frequency (average of three experiments 23.8 fold) relative to mice receiving OA-POL and NRIg. With the majority of *in vivo* IFNγ neutralized, no significant difference in the frequency of IL-10 secretion was observed between OA and OA-POL treated mice (Exp.1 OA-POL/anti-IFNγ 1/8,319, OA/anti-IFNγ 1/8,233/ Exp.2 OA-POL/anti-IFNγ 1/26,843, OA/anti-IFNγ 1/57,127/ Exp.3 OA-POL/anti-IFNγ 1/29,441, OA/anti-IFNγ 1/21,791.). Collectively these data demonstrate that at least a portion of the enhanced IFNγ precursor frequency

induced by OA-POL treatment is independent of IFNγ itself. The data also argues that IFNγ may have the ability to regulate the expansion of Th2-like clones *in vivo* as demonstrated by the increases in IL-4 and IL-10 precursor frequency after anti-IFNγ treatment of both OA and OA-POL treated mice.

7.5 Increases in the ratio of CD4 T cells capable of IFNγ versus IL-4 or IFNγ versus IL-10 synthesis induced by OA-POL treatment are blocked by *in vivo* administration of anti-IFNγ mAb.

A number of experimental systems have suggested that the balance in which Th1 and Th2 cytokines are elicited *in vivo* can dictate the direction of the immune response and thus, the pattern of isotypes produced. We therefore examined the ratio between the frequencies of CD4 T cells capable of IFNγ secretion and those capable of IL-4 or IL-10 secretion in mice treated with OA(±NRIg), OA-POL(±NRIg), OA(anti-IFNγ) and OA-POL(anti-IFNγ). The mean ratios of CD4 T cells producing IFNγ versus IL-4 (IFNγ:IL-4) and IFNγ versus IL-10 (IFNγ:IL10) were calculated from all experiments presented in Figure 12 and Table 8 and are presented in **Figure 13**. The increase in the frequency of CD4 T cells producing IFNγ after OA-POL treatment produced a ratio between IFNγ and IL-4 precursor frequencies (IFNγ:IL-4) that was **5 fold** higher in OA-POL(±NRIg) compared with OA(±NRIg) treated mice.



Administration of anti-IFNγ mAb coincident with OA-POL treatment reverses the increase in the ratio of CD4 T cells producing IFNγ:IL-4 and IFNγ:IL-10 induced by chemically modified allergen.

Figure 13. Mean ratios illustrating the balance between CD4 T cells producing IFNγ versus IL-4 (IFNγ:IL-4) or IFNγ versus IL-10 (IFNγ:IL-10) in mice treated with OA(±NRIg), OA-POL(±NRIg), OA(anti-IFNγ), or OA-POL(anti-IFNγ). The data presented on a log scale represent mean ratios (±SEM) from the experiments described in Figure 12 and Table 8. The ratios were calculated as described in materials and methods.

In OA(±NRIg) treated mice, the frequency of CD4 T cells capable of IL-10 secretion was consistently higher than the frequency of CD4 T cells capable of IFNγ secretion (mean IFNγ:IL10 ratio = **0.25**). In comparison, following OA-POL(±NRIg) treatment the frequency of CD4 T cells capable of IFNγ secretion was **higher** than the frequency of CD4 T cells capable of IL-10 secretion (mean IFNγ:IL-10 ratio = **11.3**) Thus the balance between CD4 T cells capable of IFNγ versus IL-10 production is **46** fold higher in OA-POL(±NRIg) compared to OA(±NRIg) treated mice.

These data demonstrate that OA-POL treatment leads to a shift toward IFNγ production in the balance of IFNγ to IL-4 and particularly IFNγ to IL-10 producing CD4 T cells. This consistent reciprocal regulation of IFNγ and IL-10 precursor frequencies induced by OA-POL closely parallels the characterized reciprocal changes in isotype pattern. The data are consistent with preferential commitment of the CD4 T cell response to ovalbumin toward a Th1-like pattern of cytokine synthesis following treatment with chemically modified allergen.

The selective increases in IL-4 and IL-10 precursor frequencies caused by the administration of anti-IFNγ generates substantial *decreases* in the balance of IFNγ:IL-4 and IFNγ:IL-10 producing CD4 T cell ratios in OA-POL/anti-IFNγ compared to OA-POL(±NRIg) treated mice. Specifically, anti-IFNγ treatment changed the ratio of IFNγ to IL-4 (IFNγ:IL-4) precursor frequency from that

observed in OA-POL(±NRIg) immunized mice (**0.413**) to **0.067**, a ratio very similar to that found in OA(±NRIg) immunized mice (**0.** 082)

Anti-IFNγ caused the ratio of CD4 T cells capable of IFNγ versus IL-10 production (IFNγ:IL-10) to change from a balance where the IFNγ precursor frequency dominated (OA-POL(±NRIg) mean [IFNγ:IL-10] ratio = **11.3**) to a ratio dominated by IL-10 (OA-POL/anti-IFNγ mean [IFNγ:IL-10] ratio = **0.385**), a 29 fold decrease. This ratio is similar to that found in OA(±NRIg) treated mice (OA(±NRIg) mean [IFNγ:IL-10] ratio = **0.25**).

In summary, anti-IFNγ administration converts the IFNγ:IL-4 and IFNγ:IL-10 ratios produced by OA-POL treatment to values consistent with those produced by mice treated with unmodified allergen. This change in the relative precursor frequency ratios helps explain our previous finding that anti-IFNγ administration during OA-POL treatment allows production of normal IgE levels (Table 7). Therefore anti-IFNγ administration converts the cytokine balance induced by OA-POL from that associated with suppressed IgE and elevated IgG_{2a} levels to one associated with a strong IgE response and 100-1000 fold lower IgG_{2a} synthesis.

In Vivo	In Vitro	IL-4 (U/ml)	IFN-γ (U/ml)
C57BL/6			
OVA		3.1	7.6
OVA	OVA	14.7	22.6
OVA-POL/OVA		2.4	14.1
OVA-POL/OVA	OVA	2.8	61.3
BALB/c			
OVA		2.6	8.4
OVA	OVA	15.9	33.0
OVA-POL/OVA		3.1	9.1
OVA-POL/OVA	OVA	11.4	24.0

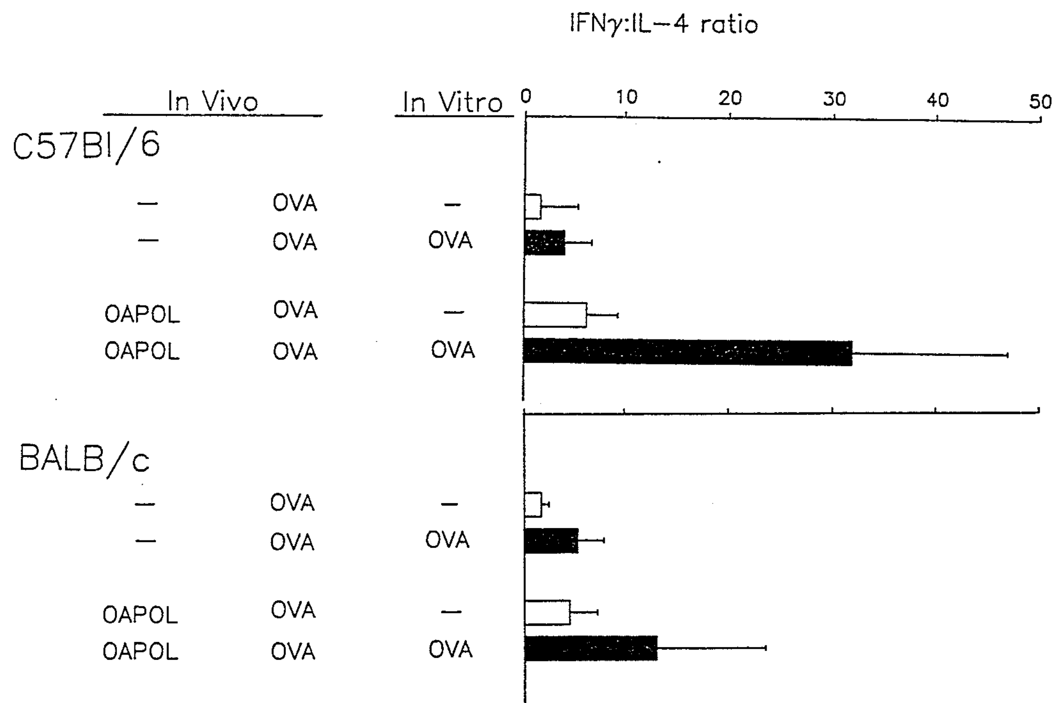
Administration of OA-POL leads to increased IFNγ and decreased IL-4 production elicited upon in vitro restimulation with unmodified allergen in C57Bl/6, but not BALB/c mice.

Table 9. C57Bl/6 and BALB/c mice were OA-POL treated (d. 0, 2, 4) or left untreated and immunized 126 days later. Spleen cell bulk cultures were established from mice sacrificed 5 days after immunization. IL-4 and IFNγ production (expressed as U/ml) was determined in replicate cultures. Standard error within an experiment was <5% for IL-4, <10% for IFNγ and is omitted for clarity. One experiment representative of 3 carried out with BALB/c and 1 representative of 10 with C57BL/6 mice are shown.

7.6 Long-lived changes in the ratio of IFNγ:IL-4 synthesis are associated with successful long-term inhibition of IgE responses.

In this model, OA-POL treatment is successful in the induction of long-lived IgE inhibition in C57Bl/6 but not BALB/c mice. We utilized these prototype strains to characterize the cytokine synthesis patterns which are associated with successful allergen immunotherapy in this experimental animal model.

C57Bl/6 and BALB/c mice were treated with OA-POL and immunized 18 weeks later. Five days after immunization, overnight spleen cell cultures were initiated. IFNγ and IL-4 content in 18 hour replicate culture supernatants were determined by sandwich ELISA and CT.4S MTT colorimetric bioassay respectively (**Table 9**). Markedly decreased IL-4 production ($p < 0.0001$) and increased IFNγ production ($p = 0.0006$) was observed in OA-POL treated, OA immunized C57Bl/6 mice as compared to immunized, but untreated, controls. In contrast, IL-4 and IFNγ synthesis by BALB/c mice was not significantly affected by identical OA-POL treatment.



Administration of OA-POL biases the balance of IFNγ:IL-4 synthesis toward IFNγ in cases of successful induction of long-term IgE inhibition.

Figure 14. Summary of the mean IFNγ:IL-4 ratio in OA-POL treated, OA(alum) immunized versus nontreated, OA(alum) immunized C57Bl/6 and BALB/c mice. Mean ratios from 10 experiments in C57Bl/6 and three in BALB/c are presented.

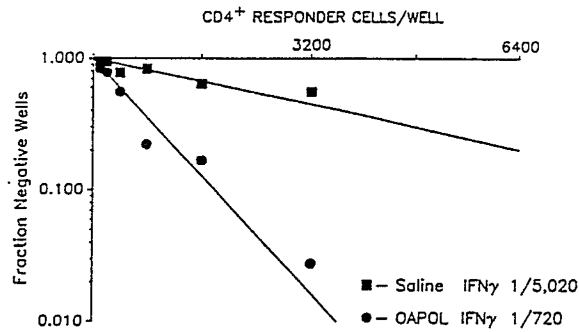
The balance in which IFNγ and IL-4 are produced in response to antigen specific stimulation is thought to be important in the regulation of the IgE isotype. One approach to evaluating cytokine production is to examine the ratio in which these antagonistic cytokines are invoked upon allergen specific *in vitro* restimulation of spleen cells directly ex vivo (**Figure 14**). Over 10 experiments in C57Bl/6 mice, the IFNγ:IL-4 ratio was increased an average of 7 fold after OA-POL treatment ($p < 0.0076$). In three experiments in BALB/c mice, there was only minor increases in the IFNγ:IL-4 ratio under the same long-term conditions used ($0.1 > p > 0.05$). Thus, OA-POL treatment induces long-lived changes in the ratio of IFNγ:IL-4 synthesis elicited in C57BL/6, but not BALB/c mice.

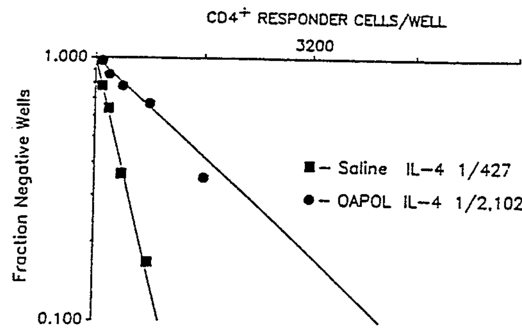
This evidence of increased IFNγ:IL-4 synthesis in the OA-specific repertoire of spleen cells from OA-POL treated C57BL/6, but not untreated C57Bl/6 or treated/untreated BALB/c mice, taken with the changes in OA-specific IgE and IgG_{2a} responses observed *in vivo* following administration of anti-IFNγ mAb is consistent with the induction of a long-lived bias toward IFNγ production within the allergen specific T cell repertoire in cases of successful long-term reciprocal regulation of IgE and IgG_{2a} responses *in vivo*.

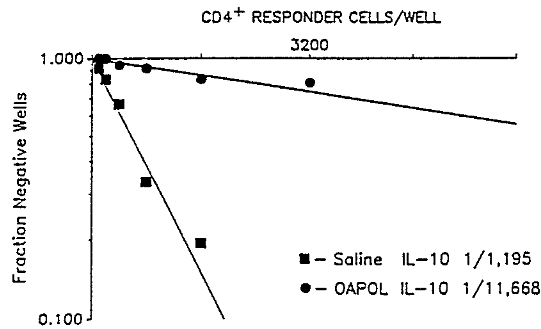
7.7 Long-lived reciprocal regulation of IFNγ and IL-4/IL-10 precursor frequencies is associated with successful long-term inhibition of IgE responses.

One difficulty with bulk culture is the inability to distinguish whether changes in the quantity of cytokine produced are the result of direct changes in the precursor frequency of cells producing the cytokine or the result of cross-regulation between cytokines produced in culture. We utilized limiting dilution analysis to directly characterize the effects of OA-POL treatment on cytokine synthesis patterns within the CD4 T cell repertoire in two strains representative of successful (C57Bl/6) and failed (BALB/c) induction of long-lived IgE inhibition. C57Bl/6 and BALB/c mice were treated with OA-POL and OA(alum) immunized 12 to 22 weeks later. Five days after immunization, CD4 T cells were enriched and limiting dilution analysis cultures were established as previously described. The results from one of three independent experiments are shown in **Figure 15** (C57Bl/6) and **16** (BALB/c).

In C57Bl/6 mice, the frequency of IFNγ secreting CD4 T cells was 6-8 fold higher in OA-POL treated, OA(alum) immunized groups compared to untreated, OA(alum) immunized groups. The frequency of IL-4 secreting CD4 T cells was reduced 2-5 fold, while the frequency of IL-10 production was reduced by 4-10 fold in OA-POL treated, OA immunized mice.

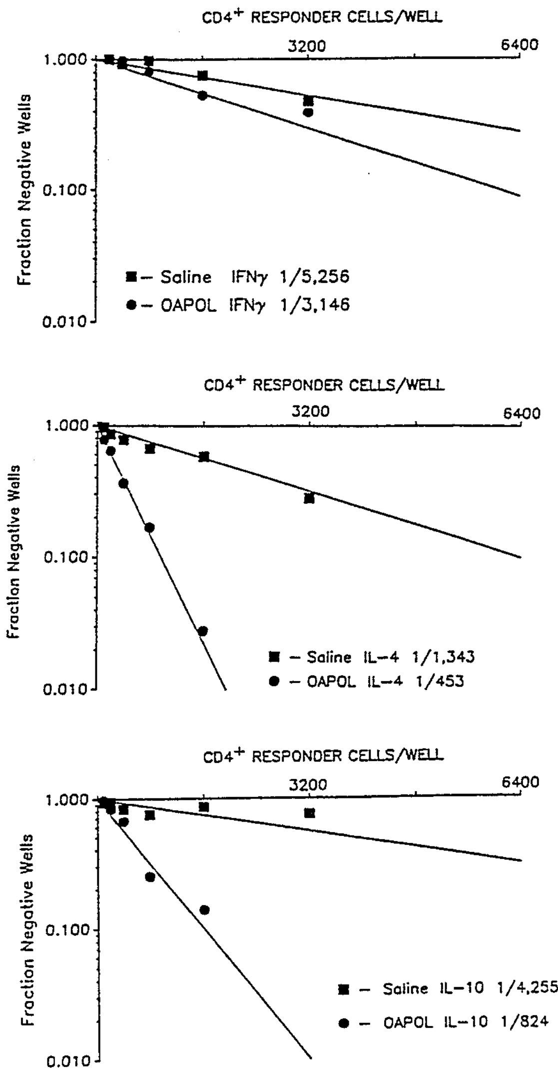






Successful inhibition of IgE responses is associated with long-lived reciprocal regulation of IFNγ and IL-4/IL-10 $CD4^+$ precursor frequencies.

Figure 15. Increased IFNγ and decreased IL-4 and IL-10 precursor frequencies are associated with successful induction of long-lived IgE inhibition. In three independent experiments C57Bl/6 and BALB/c mice were treated with OA-POL (d. 0, 2, 4) or saline and immunized 12, 18 (shown), or 25 weeks later. Five days after immunization limiting dilution cultures were established as described in materials and methods. The precursor frequency of IFNγ, IL-4 and IL-10 synthesis by $CD4^+$ cells in OA-POL treated, OA immunized compared to saline treated, OA immunized C57Bl/6 mice are presented. The data is from one experiment representative of three performed.



OA-POL treated BALB/c mice fail to demonstrate long-lived increases in IFNγ or decreases in IL-4 or IL-10 CD4$^+$ precursor frequencies.

Figure 16. BALB/c mice were treated and analyzed as described in figure 14. The CD4 T cell precursor frequency of IFNγ, IL-4 and IL-10 synthesis in OA-POL treated, OA immunized compared to saline treated OA immunized BALB/c mice are presented. The data is from the same experiment shown in figure 15.

In contrast, OA-POL treated, OA immunized BALB/c mice consistently failed to demonstrate an increase in IFNγ precursor frequency (<1.8 fold increased in each of 3 experiments, $p > 0.05$). Surprisingly, OA-POL treated, OA immunized BALB/c mice displayed 3-5 fold increases in IL-4 precursor frequency, concomitant with 4-6 fold increases in the frequency of IL-10 secretion compared to untreated, OA immunized mice. These results provide direct evidence for a genetically controlled shift in the balance of CD4 T cells toward Th1 like responses in cases of successful induction of long-lived IgE inhibition.

7.8 OA-POL treatment redirects the balance in the frequency of OA specific CD4+ T cells capable of IFNγ versus IL-4/IL-10 synthesis in successful immunotherapy.

As demonstrated above, the balance between CD4 T cells capable of Th1-like (IFNγ dominant) versus Th2-like (IL-4 or IL-10) cytokine synthesis demonstrated that OA-POL induced inhibition of IgE responses in C57Bl/6 mice were associated with increases in the frequency of IFNγ to IL-4 (IFNγ:IL-4) and IFNγ to IL-10 (IFNγ:IL-10) producing CD4 T cells, which persists a minimum of 24 weeks.

	Treatment	mean IFNγ:IL-4	mean IFNγ:IL-10
C57Bl/6	Saline/OA(alum)	0.091 ± 0.006	0.170 ± 0.068
C57Bl/6	OA-POL/OA(alum)	1.601 ± 1.317	9.165 ± 7.041
BALB/c	Saline/OA(alum)	0.271 ± 0.015	0.712 ± 0.098
BALB/c	OA-POL/OA(alum)	0.123 ± 0.021	0.275 ± 0.013

OA-POL treatment redirects the balance between the frequency of OA specific CD4 T cells capable of IFNγ versus IL-4/IL-10 synthesis in successful immunotherapy.

Table 10. The ratio between the precursor frequency of CD4 T cells capable of IFNγ versus IL-4 or IFNγ versus IL-10 synthesis (IFNγ:IL-4 and IFNγ:IL-10) in OA-POL/OA[$Al(OH)_3$] compared to saline/OA[$Al(OH)_3$] immunized C57Bl/6 and BALB/c mice was calculated in each of three experiments as described in materials and methods. Arithmetic means ± S.E. of frequency ratios from three experiments are presented.

Analysis of the balance between CD4 T cells capable of IFNγ versus IL-4 or IFNγ versus IL-10 synthesis further demonstrated that OA-POL induced long-lived inhibition of IgE responses in C57Bl/6 mice was associated with the maintenance of increases in the frequency ratio of IFNγ to IL-4 (IFNγ:IL-4) and IFNγ to IL-10 (IFNγ:IL-10) synthesis.

Table 10 shows the mean IFNγ:IL-4 and IFNγ:IL-10 ratio in C57Bl/6 and BALB/c mice from three independent experiments. In C57Bl/6 mice, OA-POL treatment induced a 17.6 fold increase in the IFNγ:IL-4 frequency ratio concomitant with a striking 54 fold increase in the IFNγ:IL-10 frequency ratio in OA-POL/OA versus untreated/OA immunized mice. These changes parallel the 10 fold decreases in allergen specific IgE and 100-1000 fold increases in IgG_{2a} elicited by this chemically modified allergen.

In BALB/c mice the failure to demonstrate long-lived OA-POL induced IgE inhibition or IgG_{2a} enhancement is associated with a 2.2 fold <u>decrease</u> in the balance of CD4 T cells that produce IFNγ versus IL-4 and a 2.6 fold <u>decrease</u> in the ratio of IFNγ compared to IL-10 secreting CD4 T cells in OA-POL/OA compared to untreated/OA immunized mice i.e. a balance shifted toward IL-4/IL-10 (Th2 like) cells. Therefore the ratio between the frequency of IFNγ and IL-4 (IFNγ:IL-4) or IFNγ and IL-10 (IFNγ:IL-10) secreting CD4 T cells is increased in cases of successful immunotherapy supporting the contention that

administration of this chemically modified allergen leads to a long-lived bias toward IFNγ synthesis in the CD4 T cell repertoire of mice exhibiting long-lived IgE inhibition. These results also demonstrate that in addition to the development of a population of IL-4 producing CD4 T cells (Finkelman, 1988b) prominent IgE synthesis requires the relative lack of CD4 T cells producing IFNγ. Thus the ratio between the frequency of IFNγ versus IL-4 producing CD4 T cells is a central factor dictating the induction of IgE responses.

7.9 Cellular Immunotherapy with IFNγ/IL-2 producing CD4 T cell lines inhibits primary and secondary IgE responses.

During analysis of limiting dilution data from OA[Al(OH)$_3$] immunized mice, a low frequency of CD4 T cells that produced substantial IFNγ and no detectible IL-4 or IL-10 were consistently found. Because we had previously demonstrated that inhibition of IgE responses was dependent on IFNγ production we investigated the ability to directly manipulate cytokine synthesis and thus the resulting isotype pattern by directly manipulating the balance between IFNγ and IL-4/10 secreting CD4 T cells *in vivo*. CD4 T cell cytokine synthesis patterns were assessed in individual wells from limiting dilution responder cell numbers that resulted in less that 30% of wells showing positive growth. Wells which displayed significant IFNγ levels in the absence

of IL-4 or IL-10 along with several IL-4/10 but IFNγ negative wells were expanded *in vitro* with antigen, IL-2 and irradiated APC. After six rounds of expansion (four rounds in the second experiment) the cells were extensively washed and grown an additional 4 days in IL-2 containing media in the absence of antigen. After additional washing, viable cells were recovered, enumerated and 10×10^6 cells were transferred i.v. to naive recipients.

5×10^4 cells from each line were restimulated with fresh irradiated normal spleen cells ± 1 mg/ml OA the same day. The production of IFNγ, IL-2, IL-4, and IL-10 were determined as described in materials and methods. In the absence of OA, no detectible cytokine was produced. All recipients were immunized the same day and boosted 28 days later. Mice were bled 10 and 14 days after primary immunization and seven days after boosting. **Tables 11a** and **11 b** show results from two experiments.

The IgE response of recipient mice was not affected by the transfer of clones that produced IL-4 or IL-4 and IL-10. In contrast, all three CD4 T cell lines that produced relatively high levels of IFNγ and IL-2 inhibited the recipient primary IgE response by 87-97% and secondary responses by at least 75%. IgG_{2a} levels were increased between 10 and 40 fold in these mice (data not shown). A CD4 T cell line that produced high levels of IFNγ, but low or no IL-2 (29A) failed to mediate significant IgE inhibition. A fourth line (45F)

Exp. 1	CYTOKINE Synthesis				IgE Titer	
Donor Cell Line	IFNγ (U/ml)	IL-2 (U/ml)	IL-4 (U/ml)	IL-10 (U/ml)	day 10	day 35
None	---	---	---	---	4031	5079
811E	< 0.5	< 0.5	838	< 0.5	3200	6400
75E	3.8	2.7	962	< 0.5	4031	4031
74D	< 0.5	< 0.5	368	94	2016	3200
89F	576	42	< 0.5	< 0.5	79	200
72F	1748	22	4.3	< 0.5	63	160
43G	720	28	< 0.5	< 0.5	126	800
45F	981	12	< 0.5	< 0.5	252	1008
29A	1109	3.2	< 0.5	< 0.5	2016	3200

Cellular Immunotherapy with IFNγ and IL-2 producing CD4 T cell lines inhibit primary and secondary IgE responses.

Table 11 a). CD4 T cell lines were established from limiting dilution analysis cultures as described in materials and methods. These lines were expanded via bi-weekly restimulation with OA, IL-2 and fresh irradiated APC. After sufficient expansion the lines were washed and cultured an additional four days in IL-2 containing media in the absence of OA. After additional washing 10×10^6 cells from lines that produced IFNγ ± IL-2, or IL-4 ± IL-10 were transferred i.v. to normal recipients immediately before immunization with OA(alum). The recipients were boosted 28 days later and bled 10 and 14 days after primary and 7 days after secondary immunization. The cytokine profile obtained by restimulation of each line in vitro on the day of transfer are included to ensure that each line maintained its characterized cytokine synthesis pattern. The cytokine data represents the arithmetic mean of duplicate cytokine determinations, as described in materials and methods. The IgE data represents the geometric mean of triplicate PCA titer determinations. Standard errors were less than 15% in all instances.

Exp. 2	CYTOKINE Synthesis				IgE Titer	
Donor Cell Line	IFNγ (U/ml)	IL-2 (U/ml)	IL-4 (U/ml)	IL-10 (U/ml)	day 10	day 35
None	—	—	—	—	1600	4031
811E	< 0.5	< 0.5	692	< 0.5	1600	3200
75E	< 0.5	2.6	487	< 0.5	2015	5079
74D	< 0.5	< 0.5	1073	112	1270	3200
89F	420	32	< 0.5	< 0.5	50	504
72F	838	15	1.7	< 0.5	160	504
43G	543	18	< 0.5	< 0.5	126	635
45F	960	2.0	< 0.5	< 0.5	1270	1600
29A	1398	< 0.5	< 0.5	< 0.5	1600	5079

Cellular Immunotherapy with IFNγ and IL-2 producing
CD4 T cell lines inhibit primary and secondary IgE responses.

Table 11 b). The experiment was carried out as described for Table 11 a).

inhibited recipient IgE responses in experiment 1 (Table 11 a), but failed to mediate suppression in a second experiment, at a time when it had lost the ability to produce IL-2 (Table 11 b). Therefore short term *in vitro* expanded Th1-like CD4 T cells that produce IFNγ and IL-2 can mediate significant inhibition of both primary and secondary IgE responses in a cellular allergy immunotherapy model.

Discussion

One of the major challenges in allergy research is the development of therapies that safely induce permanent inhibition of allergen-specific IgE synthesis. Although allergen immunotherapy is widely utilized in some countries, current approaches do not meet this objective. Strategies presently in use involve multiple injections of allergen extracts with the objective of lowering reactivity to the allergen. However, the benefits of this approach vary widely and serious side effects, though uncommon, do occur (Committee, 1986; Lockey, 1987). As such the use of this form of therapy continues to be examined in terms of safety and efficacy (Thompson, 1989). In response chemically modified allergens have been devised that display strongly reduced antigenicity. Although this reduced ability to cross-link mast cell bound antibodies allows for abbreviated treatment regimes and is thought to increase safety, the efficacy of such preparations in clinical trials and in ongoing clinical use has not resulted in a marked improvement in terms of reducing patient symptoms (Ewan, 1989).

Most studies of IgE regulation have focused on short term studies of antibody production (reviewed, Sehon, 1982; Ishizaka, 1989). Although valuable as an experimental tool, extrapolation from such murine models to clinical trials has often proven to be disappointing (Norman, 1982). We speculated that this may be due, in part, to the brief periods (4-6 weeks or less) over which most animal experiments had been carried out. To assess the potential utility of this model for further studies of IgE regulation, we examined the longevity and resilience of the effects of allergen immunotherapy on IgE and IgG_{2a} synthesis over extended periods of time (up to 70 weeks) and following multiple re-exposures to allergen.

The major finding of this study was the ability of OA-POL treatment to induce an essentially permanent shift in the pattern of responsiveness of C57BL/6 mice to native OA. In spite of multiple re-exposures to the sensitizing allergen, IgE responses of OA-POL treated C57Bl/6 were never greater than 10% of those observed in age-matched control mice even when treated groups were immunized 70 weeks after a single course of treatment. This decreased capacity to produce IgE was paralleled by 100-1000 fold increases in anti-OA IgG_{2a} responses, which persisted throughout the study. The dominant reciprocal changes in IgE and IgG_{2a} induced by OA-POL led us to hypothesize that this modified allergen may inhibit IgE responses through the induction of allergen specific IFNγ production.

In marked contrast, although OA-POL treated BALB/c mice display strongly inhibited anti-OA IgE levels when immunized two weeks after treatment, these mice mount IgE responses not significantly different from age-matched controls if first immunized 10 weeks or longer after treatment (Figure 2). This finding demonstrates that the induction of permanent IgE hypo-responsiveness by this chemically modified allergen is not universal, and also illustrates the importance of determining the prolonged effect that allergen immunotherapy has on the subsequent immune response. The identification of a strain in which OA-POL treatment is effective in inducing long-lived IgE inhibition and another where identical treatment fails to induce a permanent shift in IgE responsiveness, suggests that this system is well suited to analysis of factors which govern induction of allergic responses and the mechanisms involved in the prolonged inhibition of the IgE isotype.

Evidence that this isotype selective, CD4 T cell dependent inhibition of IgE synthesis is attributable to alterations in the pattern of cytokine synthesis elicited *in vivo* is provided by our findings that: (1) the effects on IgE synthesis were susceptible to *in vivo* administration of anti-IFNγ mAb either at the time of chemically modified allergen treatment, or during OA[$Al(OH)_3$] immunization $\geq$10 weeks later, (2) in a limiting dilution analysis system the balance in CD4 T cells capable of IFNγ versus IL-4 or IFNγ versus IL-10 production was elevated after OA-POL compared to OA treatment, (3) an increased ratio of

IFNγ:IL-4 synthesis was observed following bulk culture of allergen stimulated spleen cells from OA-POL treated, OA[Al$(OH)_3$] immunized in comparison to untreated, OA[Al$(OH)_3$] immunized C57Bl/6, but not BALB/c mice, (4) in C57Bl/6 mice the elevated level of IFNγ competent CD4 T cells is maintained for at least 22 weeks after treatment, and is paralleled by decreases in the frequency of cells capable of producing the Th2 associated cytokines IL-4 and IL-10; while OA-POL treated BALB/c mice demonstrate a long-term bias toward IL-4 and IL-10 production.

Collectively, these findings support the hypothesis that in cases of successful induction of long-term IgE inhibition, administration of this modified allergen elicits a genetically controlled, durable shift in the balance of cytokine synthesis within the allergen-specific CD4 T cell repertoire from the Th2-like (IL-4/IL-10) response normally elicited by OA[Al$(OH)_3$] to one dominated by IFNγ.

Genetically restricted expression of OA-POL induced long-lived IgE inhibition.

The observation that the ability to display long-lived IgE inhibition is genetically restricted, led us to initiate an investigation of genetic factors potentially involved in the expression of this trait. This study examined the effect of OA-POL treatment in a number of MHC and Igh congenic mice, in order to investigate the relationship between genetic background and the ability of this chemically modified protein to elicit long-lived anergy in terms of IgE responsiveness. Each strain demonstrating OA-POL induced long-lived IgE inhibition (C57Bl/6 Igh^b, C57Bl/6 Igh^n, B10.D2) shared the characteristics of: (1) an extremely long-lived, T cell dependent ability to suppress allergen specific IgE responses in an isotype selective and antigen specific manner, (2) The IgE inhibition observed was very resilient to further booster immunizations. OA-POL treated mice never produced anti-OA IgE responses $>10\%$ of those produced by normal controls, despite many booster immunizations, (3) IgG_{2a} isotype responses in OA-POL treated groups are elevated 100 to over 1000 fold (relative to those seen in untreated/immunized or native OA treated immunized controls) in primary, secondary and tertiary responses.

These results demonstrated that the capacity of OA-POL to induce very long lived reciprocal changes in IgE and IgG_{2a} production is not solely dependent on MHC or Igh gene complexes nor on background genes, but appears to be

under multi-genic control where no single gene or gene complex is singularly responsible for this trait.

Relationship of the ability to display OA-POL induced long-term IgE inhibition and resistance versus susceptibility to *Leishmania major* infection.

Similarities exist between the present findings and those obtained following infection of mice with *Leishmania major. Leishmania* infection of resistant strains, such as C57Bl/6 and CBA, results in a limited lesion that remains local and is eventually cured, whereas, infection of susceptible strains such as BALB/c or DBA/2 results in progressive infection, systemic disease and death. The immune response of resistant strains is characterized by Th1-like activities, including strong parasite specific DTH, activation of macrophage killing pathways and inhibition of humoral responses. Contrarily, susceptible mice respond with a dominant Th2-like humoral response lacking strong DTH, a reaction that is not protective against infection by this intracellular pathogen (Scott, 1989; Locksley, 1991; Sher, 1992). Consistent with the findings in the OA-POL system (Figure 3), the ability to induce an IFNγ dominant protective response in this model is independent of MHC haplotype. BALB/c, BALB/b (H-2^b) and BALB/k (H-2^k) MHC congenic mice were equally susceptible to Leishmania infection (Liew, 1985). Moreover, B10.D2 mice were highly re-

sistant despite the presence of the H-2^d haplotype (Boom, 1990).

Taken collectively, there appears to be a parallel between a strain's ability to induce protective immunity to *Leishmania major* and the ability of this chemically modified allergen to induce long-lived, reciprocal regulation of anti-OA IgE and IgG_{2a} isotypes, both shown to be IFNγ dependent processes (Table 3). The apparent correlation between these two diverse experimental systems may indicate similarities in the mechanism(s) responsible for the induction of a dominant pattern of cytokine expression. The ability to manipulate the development of a dominant pattern of cytokine synthesis by altering the early balance between IFNγ and IL-4 in the OA-POL and *Leishmania major* models (Belosevic, 1989; Sadick, 1990; Scott, 1991; Chatelain, 1992) illustrates the central role that the balance between these cytokines has in the differentiation of a dominant cytokine synthesis pattern.

The results of these two systems are distinct from the finding that the MHC haplotype is the primary factor determining the pattern of cytokines elicited and the resulting humoral versus cell mediated immune response to human type IV collagen (Tite, 1987; Murray, 1989) or the recombinant malaria protein p190-3 (Suss, 1992). The reason for these differences remain open to speculation.

The amount of OA-POL required to induce long-lived IgE inhibition.

C57Bl/6 mice appear to be extremely sensitive to the effects of administration of this chemically modified allergen (Table 4). Treatment with a single i.p. injection of 20 ug OA-POL caused 10 fold reductions in IgE and 155-185 fold enhancement of IgG_{2a} similar to those routinely observed after three 80 ug injections. The inability of three 5 ug doses to have similar effects in the two experiments performed is puzzling. The small increases in allergen specific IgG_{2a} along with the 10 fold lower ability of this protocol to induce increased primary IgG1 (data not shown), compared to the regular 3 x 80 ug treatment, infers that spacing out the therapy with such low OA-POL doses reduces the ability of the immune system to mount a substantial response. The relative effectiveness of one 20 ug injection may be due to this dose being above a "threshold" level required to initiate long-term reciprocal regulation of IgE and IgG_{2a} synthesis.

In comparison, the inability of three 800 ug doses of OA-POL to significantly alter the lack of long-term IgE inhibition in a number of strains demonstrated that their failure to maintain the ability to inhibit IgE responses was not solely dependent on the requirement for higher doses of OA-POL in these mouse strains (Table 5).

The induction of IFNγ secreting CD4 T cells as a general mechanism mediating the inhibition of IgE responses in all murine strains shortly after OA-POL treatment.

Our findings in the short term model, that OA-POL inhibits allergen specific IgE and enhances IgG_{2a} responses in each strain tested, along with the demonstration that administration of anti-IFNγ mAb blocks OA-POL induced IgE inhibition in C57BL/6 mice (HayGlass, 1991c), implies that preferential induction of IFNγ production by allergen-specific CD4 T cells may be the general mechanism mediating the genetically unrestricted inhibition of IgE production and enhancement of IgG_{2a} responses observed when mice are challenged two weeks after treatment. The data from the long-term study are consistent with a model where OA-POL treatment of mouse strains which demonstrate long-lived IgE inhibition results in early enhanced induction/expansion of T cells expressing IFNγ dominated patterns of cytokine synthesis, leading to the generation of a population of stable memory cells retaining this characteristic. These OA-specific, IFNγ secreting memory CD4 T cells are presumably restimulated following OA[$Al(OH)_3$] immunization 70 weeks later, and dominate the OA specific response via the antagonistic effects of IFNγ on the induction/expansion and activity of IL-4/IL-10 secreting CD4 T cells and perhaps by directly inhibiting IgE (and promoting IgG_{2a}) production by allergen specific B cells.

The potential role of differential antigen presentation in the induction of distinct cytokine synthesis patterns by chemically modified versus native ovalbumin.

The consensus from both murine and human studies is that IFNγ or IFNγ inducing cytokines, such as IFNα and IL-12, direct the development of Th1-like responses and inhibit Th2 development, while IL-4 promotes Th2-like responses and inhibits the development of Th1 (reviewed, Scott, 1993b). The capacity of the balance between these cytokines to regulate a developing immune response suggests that therapeutic strategies which modulate this balance can change the nature of the resulting response.

The function of distinct APC populations during the initiation of immune responses is not well understood. Th1 cells proliferate optimally in response to antigen presented by macrophage, while Th2 proliferate optimally to antigen presented by resting B cells (Gajewski, 1991). Similarly, murine hepatic accessory cells stimulate proliferation of Th1 but not Th2 (Magilavy, 1989). These results suggest that preferential presentation of antigen by macrophage/liver adherent cells or by resting B cells during the initiation of an immune response may lead to the predominant expansion of Th1 or Th2 clones respectively.

Two notable characteristics of this chemically modified allergen are its 10-100 fold decrease in antigenicity (HayGlass, 1984a) and its massive size compared to native OA. These characteristics along with the different relative abilities of macrophage and B cells to optimally stimulate Th1 versus Th2 expansion respectively, lead us speculate that differences in the predominant type of APC responsible for the initial presentation of OA-POL compared to OA may lead to an early burst of IFNγ synthesis.

In this speculative model, the reduction in the ability of polymerized OA to be bound by allergen specific B cells, along with the large size of this molecule, bias presentation away from allergen specific B cells, toward a phagocytic macrophage like APC, that preferentially supports Th1 development or proliferation. This early burst of IFNγ would result in a balance in which the frequency of CD4 T cells capable of IFNγ secretion dominate over those that produce IL-4 or IL-10. The inability of anti-IFNγ administration during OA-POL treatment to significantly alter the increase in IFNγ producing CD4 T cells associated with this treatment, suggests that the initial enhancement in the frequency of IFNγ producing CD4 T cells is independent of IFNγ itself.

This hypothesis is strengthened by the recent demonstration that bone marrow derived, GM-CSF maintained macrophage, pulsed with *Leishmania major* antigens, are capable of inducing a protective Th1 response when

injected into the footpads of susceptible BALB/c mice, prior to infection with viable parasite (Doherty, 1993). Here, initial presentation of *Leishmania* antigens on macrophage overcomes the propensity of these mice to induce Th2 responses, instead initiating a protective Th1 response. Work to assess the contribution of B cells in the presentation of OA-POL, utilizing rabbit anti-mouse IgM treated, B cell deficient mice, is now underway. The addition of peritoneal macrophage to cultures of naive, TCR transgenic, CD4 T cells and dendritic cells caused the secondary response to be strongly biased toward IFNγ production (Macatonia, 1993a). This finding also supports the ability of predominant antigen presentation on macrophage to direct Th1 differentiation.

An alternate, but related possibility is that the modified physical nature of OA-POL may cause it to induce IL-12 and/or IFNα production by macrophage and/or B cell APC. Numerous studies have implicated these cytokines in the induction of very early IFNγ synthesis by NK cells, providing the IFNγ necessary to direct predominant Th1 development (Romagnani, 1992; Heinzel, 1993; Manetti, 1993; Sypek, 1993; Hsieh, in press). Presentation of OA epitopes simultaneous with cytokines which direct Th1 development, during the genesis of the response, may account for the early IFNγ independent enhancement of Th1 like cells. The activity of these cells would account for the domination of IFNγ in the balance of cytokine synthesis that subsequently develops. The incomplete inhibition of IgG_{2a} production after administration of

anit-IFNγ during OA-POL treatment may be due to the incomplete neutralization of *in vivo* IFNγ by this treatment, or alternatively it may indicate a role for IL-12 and/or IFNα, which have been shown to enhance IgG_{2a} production independent of IFNγ (Morris, 1993; Finkelman, 1991b). The potential role for IL-12 and/or IFNα in the induction of these responses is currently being investigated.

Factors involved in the divergent ability of OA-POL to induce long-lived IgE inhibition in C57Bl/6 compared to BALB/c mice.

The long-term relative balance of IFNγ versus IL-4 and IFNγ versus IL-10 producing CD4 T cells induced by OA-POL appears to be the primary difference between strains that display extremely long-lived inhibition of antigen-specific IgE responses and those which develop normal responses when immunized $\geq$ 10 weeks after treatment. The imbalance in the ratio of IFNγ to IL-4/IL-10 producing CD4 T cells found in BALB/c is reflected in the decreased efficacy of this modified allergen in that strain.

Factors which may contribute to such an imbalance include utilization of distinct antigen presentation pathways and/or differences in the nature of APC in C57Bl/6 compared to BALB/c mice. In support of this contention is a study demonstrating that distinct regulation of antigen presentation occurs in low compared to high IgE responder mice (Rizzo, 1991). Macrophages from

BALB/c mice had an enhanced ability to present antigen to Th-2 clones after exposure to IL-4 and IL-5, while the same treatment in low responder strains inhibited the capacity of macrophage to stimulate Th2. This implies that instead of inducing Th1 expansion, macrophages may help expand Th2 during an IL-4 dominated response in BALB/c, resulting in Th2 domination and IgE production. Thus, antigen presenting cells from BALB/c versus C57Bl/6 may differ in their capacity to stimulate Th1 versus Th2 responses.

It is also possible that APC from C57Bl/6 may produce higher levels of IL-12 and/or IFNα in response to OA-POL, or that NK and CTL from these two strains differ in their ability to produce IFNγ in response to IL-12. The correlation between weak NK activation early after *Leishmania major* infection and susceptibility to this parasite provides evidence that at least in that model, defective early activation of NK cells contributes to the development of a dominant non-protective Th2 response (Scharton, 1993).

A recent immunohistochemical study found that the first cell types found at the site of antigen/CFA or antigen[$Al(OH)_3$] injection are NK cells and macrophages (Bogen, 1993). These early arriving NK cells were shown to be producing IFNγ, possibly in response to IL-12 produced by the early arriving macrophage. These *in vivo* studies validate the idea that early activation of NK cells may occur in vivo, before detectable cytokine synthesis by T cell. Thus,

IFNγ may be present early enough during the genesis of the immune response to influence the subsequent differentiation of cytokine synthesis patterns. In the OA-POL model, a similar defect in the activation of NK in BALB/c mice may also contribute to the decreased efficacy of treatment in this strain.

These factors, or other as yet unidentified components may affect the initial expansion of IFNγ versus IL-4 and/or IL-10 producing CD4 T cells in C57Bl/6 compared to BALB/c mice. Weak initial expansion of IFNγ producing CD4 T cells (or the relatively strong induction of Th2 responses) in BALB/c mice may lead to the inhibited IgE and enhanced IgG_{2a} levels observed in the short term model, yet the cytokine balance may be insufficient to permanently bias the response towards Th1, the consequence being a lack of long-term IgE inhibition.

Alternatively, OA-POL may induce similar increases in IFNγ producing CD4 T cells in both strains but differences in the cross-regulation between Th1 and Th2 cytokines may exist. This is supported by the demonstration that addition of exogenous IFNγ to in vitro cultures of CD4 T cells decreased the production of IL-4 by 80% in cultures from low IgE responder mice, but failed to inhibit IL-4 synthesis in BALB/c cultures (DeKruyff, 1992). This study suggests that IFNγ may be less effective at inhibiting Th2 responses in BALB/c mice. A third possibility is that the IFNγ producing CD4 T cells induced in

BALB/c mice by OA-POL treatment are short lived or revert to the described Thm IL-2 producing cell. Any of these possibilities would result in the short term inhibition of IgE observed in this strain and would explain the lack of long-lived effects. The factors mentioned may also contribute to the many observations of enhanced Th2 activity in BALB/c mice (Scott, 1989; Morris, 1992; Silva, 1992; Swain, 1992; Suss, 1992; DeKruyff, 1993; personal unpublished observations). Analysis of cytokine synthesis patterns in BALB/c mice soon after OA-POL or OA treatment will help differentiate between these possibilities.

The implication that the lack of long-lived IgE inhibition in some strains has on the ability to utilize this model in a clinical situation is unclear. The fact that the ability to demonstrate strong IgE inhibition is genetically unrestricted in short term OA-POL studies, along with the ability of multiple courses of OA-POL treatment to abrogate established BALB/c IgE responses (Hayglass, manuscript in preparation), suggests that with sufficient treatment this model may be useful in treating human allergy. However, the 90-97% inhibition of IgE responses observed in OA-POL treated mice would probably not lead to a 90% decrease in symptoms. Furthermore, the dose of OA-POL per gram of body weight given to mice is markedly larger than the dose acceptable for the treatment of humans. Thus, the primary usefulness of OA-POL treatment at this stage is as an experimental model to investigate the factors involved in IgE

regulation. The advantage of studying the long-term effects of OA-POL treatment in this model is that disparity in the efficacy of OA-POL treatment in different murine strains becomes apparent. By better understanding the factors which affect the efficacy of this treatment, improved immunotherapeutic methods may be devised.

Regulation of IgG_{2a} responses.

Anti-IFNγ mAb administration coincident with OA-POL treatment decreased IgG_{2a} production by 60-90% when the mice were immunized 10 weeks later (Table 7), while similar treatment at the time of OA immunization (10 weeks after treatment) consistently failed to inhibit the increased IgG_{2a} response produced in OA-POL treated mice. These experiments suggested that production of IFNγ during the genesis of the immune response to OA-POL was responsible for the majority of the increase in IgG_{2a} observed in this long-term model.

The inability of anti-IFNγ to completely inhibit IgG_{2a} increases when administered during OA-POL treatment may reflect incomplete neutralization of in vivo IFNγ by this protocol, or it may reflect a contribution by the cytokines IFNα or IL-12, both reported to induce IgG_{2a} class switching independent of

IFNγ (Finkelman, 1991b; Morris, 1993). Additionally, Snapper et al. have suggested that the nature of the B cell antigen may also influence the regulation of isotype switching (Snapper, 1993, 1991). This implies that physical alterations caused by glutaraldehyde polymerization of OA may also contribute to the striking increase in this isotype after treatment. In support of this is the finding that transfer of CD4 T cell lines displaying Th1 cytokine synthesis profiles inhibited IgE responses in recipient mice but induced only modest increases in allergen specific IgG_{2a} in comparison to OA-POL treatment.

The data from cell transfer and in vivo depletion studies supports the contention that restimulation of OA-POL induced $B_{\gamma 2a}$ memory cells by native allergen $\geq$10 weeks after treatment is responsible for much of the IgG_{2a} produced at this time (rather than de novo induction of IgG_{2a} switching during restimulation). The variable decrease in anti-OA IgG_{2a} observed when T or CD4 T cell depleted populations are transferred to untreated recipients in this long-term model may represent decreased production of post-isotype-switch differentiation factors, such as IL-6, in the absence of transferred OA specific memory T cells. This is supported by the parallel decrease in the production of allergen specific IgG_1 observed in recipient mice receiving T or CD4 T cell depleted OA-POL treated spleen cell populations (data not shown). In further support of this notion is the finding that the transfer of 40 x 10^6 purified T cells (T cells were obtained by depletion of B cells and adherent cells by plating on

goat anti-mouse Ig coated petri dishes. The resulting population was 93% Thy 1.1 positive with less than 2% contamination with B cells, as assessed by flow cytometry) from mice treated with OA-POL 15 weeks earlier resulted in 95% inhibition of recipient IgE production, but only a 20-35 fold enhancement of IgG_{2a}. In comparison, transfer of unfractionated spleen cells resulted in 90% inhibition of IgE and 550-800 fold increases in IgG_{2a}. This finding strengthens the hypothesis that OA-POL treatment results in relatively early IFNγ dependent induction of an isotype switch to IgG_{2a} and production of $B_{\gamma 2a}$ memory cells.

Redirection of ongoing established cytokine synthesis patterns.

The ability of OA-POL to abrogate established IgE responses along with the increases in IgG_{2a} and enhanced ratio of IFNγ:IL-4 in bulk cultures from these mice (Xi Yang and K. T. HayGlass, manuscript in preparation), suggests that a shift from an IL-4 to an IFNγ dominated response is possible. Allergen specific PBMC derived from human atopics normally develop into Th2-like clones, while bacteria specific PBMC from the same donor normally develop into Th1 (Parronchi, 1991). The ability to redirect the cytokine synthesis of allergen specific PBMC derived from human atopics by the addition of IFNγ, IFNα or poly I-C to bulk culture prior to cloning, along with the ability to switch bacteria specific PBMC from the Th1 pattern by the addition of IL-4 to bulk cultures, suggests that even ongoing patterns of cytokine synthesis within the antigen

specific CD4 T cell repertoire can be manipulated by appropriate in vitro restimulation conditions (Romagnani, 1992; Maggi, 1992; Manetti, 1993). Whether this reflects direct inter-conversion of cytokine synthesis patterns within single cells or the selective outgrowth of rare cells expressing the opposite cytokine synthesis pattern remains to be resolved. These findings provide hope that established inappropriate in vivo cytokine synthesis patterns can be reversed via in vivo therapies aimed at preferentially stimulating the opposite response.

Maintenance of an IFNγ dominated cytokine synthesis pattern.

The balance between Th1 and Th2 activities in OA-POL treated mice is distinct from the virtual domination by Th1 or Th2 responses in Leishmania major resistant and susceptible mice, respectively. A number of experimental systems have been described where Th1-like or Th2-like responses dominate to the virtual exclusion of activities linked with the opposite subset, resulting in primarily cell mediated or humoral immunity (Parish, 1971; Scott, 1989; Tite, 1987; Murray, 1989). In contrast, this system appears to represent a more balanced situation where the amount of immunoglobulin produced between treated and untreated animals in secondary and tertiary responses is similar, the main difference being the distinct regulation of the IgE and IgG_{2a}

isotypes. Hence, the relatively minor shifts in the pattern of cytokine synthesis observed here compared to that found in the *Leishmania major* system (Moll, 1990; Morris, 1992) is compatible with the characterized *in vivo* effects of this modified allergen.

These studies demonstrate that such a balanced shift in cytokine synthesis can be maintained over time in spite of immunization with OA adsorbed to $Al(OH)_3$, a protocol which stimulates Th2-like responses (Yang, 1993). Therefore, in C57Bl/6 mice the early balance between the production of the antagonistic cytokines IFNγ and IL-4/L-10 *in vivo* dictates the resulting isotype pattern, which is maintained over time despite repeated exposures to native allergen. This suggests that the balance of cytokines induced during the genesis of a response against an allergen may become stable dictating the long-term IgE responsiveness of the individual upon repeated allergen restimulation. Although a switch between cytokine patterns is possible, once established, cytokine responses appear to resist conversion, as demonstrated by the relative ease that *de novo* IgE responses can be inhibited compared to established ongoing IgE responses, and the ability of OA-POL induced IgE inhibition to resist the IgE promoting effects of repeated OA(alum) immunization.

Memory CD4 T cells induced by OA-POL maintain their distinct cytokine synthesis pattern.

The classic definition of memory cells include the requirement for prior activation by antigen and a long lifespan. Studies by S. Swain et al. have suggested that memory cells obtained six weeks or longer after activation retain the ability to synthesize only very low levels of IL-4 and IFNγ (IL-2> >IL-3, IL-4, IFNγ), but differentiate into more efficient producers of these cytokines upon exposure to antigen (reviewed, Swain, 1992). In contrast, enhanced IFNγ synthesis has been noted in mouse memory CD4 T cell populations separated on the basis of high (memory) versus low (naive) CD44 expression (Budd, 1987). Enhanced IFNγ synthesis has also been observed in human memory populations separated on the basis of high expression of a panel of adhesion molecules, despite the fact that IL-2 levels were similar between naive and memory populations (Sanders, 1988).

The notion that OA-POL induced populations of memory cells retain their dominant Th1 cytokine synthesis pattern is supported by the ability to inhibit recipient IgE responses by transferring spleen cells from OA-POL treated C57Bl/6 mice at least 60 weeks after treatment, in the absence of prior restimulation (Figure 7). If the IFNγ dominated cytokine pattern of these cells was not maintained, the Th2 promoting effect of OA[Al(OH)$_3$] immunization

(Yang, 1993) should have neutralized the ability of these cells to mediate IgE suppression in recipient mice. The essentially permanent maintenance of reciprocally regulated IgE and IgG_{2a} responses after a single course of treatment despite at least nine booster immunizations in $Al(OH)_3$ over more than 250 days (data not shown) also attests to the maintenance of an IFNγ dominated response. Furthermore, the IFNγ dominated balance in CD4 T cells producing Th1 versus Th2 cytokines induced by OA-POL (Table 9) is maintained for at least 22 weeks post treatment, despite OA[$Al(OH)_3$] immunization. In BALB/c mice, OA-POL treatment induced significant increases in the frequency of cells able to produce IL-4 and IL-10 measured at least 22 weeks later, as assessed in a limiting dilution system.

These observations do not rule out that the IFNγ dominant cytokine pattern induced by OA-POL treatment fades in intensity after treatment. The retention of even a small bias toward IFNγ synthesis, upon OA restimulation, may be adequate to provide sufficient IFNγ to continue to shift subsequent responses toward Th-1 domination.

Cellular Immunotherapy.

We further investigated the importance of the ratio between cells producing

IFNγ versus IL-4/IL-10 by attempting to directly manipulate this balance via cellular immunotherapy. The ability of CD4 T cell lines that produced both high levels of IFNγ and IL-2 to inhibit IgE responses when transferred to normal recipients underscored the importance of the early balance between Th1 versus Th2 responses in determining the pattern of isotypes produced.

Although these experiments are preliminary results from one isolation of short term CD4 T cell lines, they demonstrate that *in vivo* IgE responses can be regulated via cellular immunotherapy. This work further supports the notion that early balance between the frequency of Th1-like and Th2-like cells is critical to the isotype pattern produced. Further studies combining the transfer of such CD4 T cell lines with the administration of recombinant IL-2, anti-IL-4, or anti-IL-10 mAb (or a combination of these) will determine if the efficacy of this treatment can be increased to a level capable of permanently inhibiting established IgE responses.

In summary, the results of this research project suggest that cytokine secretion patterns and consequently immunoglobulin isotype patterns are

amenable to manipulation *in vivo*. The study demonstrates that the physical form of antigen employed in therapy can influence the direction of the resulting immune response. The data also cautions that the long-term pattern of cytokines elicited in response to exposure to chemically modified allergen is under control by ill defined interactions between genetic loci.

OA-POL treatment represents a novel method to modulate cytokine expression *in vivo* to successfully select distinct patterns of cytokine synthesis and thereby control allergic responses. As such, this non-parasite model will be useful to study the mechanisms involved in the differentiation of dominant cytokine synthesis patterns. Collectively, the data suggest that the balance between IFNγ and IL-4/IL-10 elicited early during an immune response dictates the direction of secondary and tertiary responses provoked by multiple re-exposure to the antigen. The presence of a substantial frequency of IL-4 producing CD4 T cells in mice treated with chemically modified allergen demonstrates that the existence of such cells does not dictate that IgE will necessarily be produced. Instead the balance between the frequency of CD4 T cells producing IL-4 and those producing IFNγ appears to be an important factor (perhaps the determining factor) regulating the production of IgE in this animal immunotherapy model.

Successful induction of long-lived IgE inhibition was associated with a

virtually permanent shift in the pattern of cytokines produced towards a dominant Th1-like response, while in unsuccessful cases the balance was tilted toward a dominant Th2-like response. This model will permit further analysis of the mechanism(s) involved in the induction of divergent responses to OA-POL in C57Bl/6 compared to BALB/c mice. The results will lead to a better understanding of the mechanisms operative in successful versus unsuccessful immunotherapy and may result in the development of immunotherapeutic methods of improved efficacy.

The studies examining the effect of cellular immunotherapy on IgE responses form the basis to further explore the capacity of this novel form of atopic immunotherapy to manipulate the *in vivo* balance of cytokine synthesis and ensuing immunoglobulin isotype patterns.

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