THE UNIVERSITY OF MANITOBA

MODULATION OF SPECIFIC ANTIBODY AND CYTOKINE RESPONSES WITH A MULTI-EPITOPIC RECOMBINANT ALLERGEN

BY

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

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ABBREVIATIONS

Ab	: antibody
AIT	: allergen immunotherapy
$Al(OH)_3$	: aluminium hydroxide (or alum)
Amp	: ampicillin
AP	: alkaline phosphatase
APC	: antigen presenting cell
B6D2F1	: C57Bl/6J x DBA/2J
BFP	: bead-fusion protein of GST-rKBG8.3
BGG	: bovine IgG
bp	: base pair
BSA	: bovine serum albumin
BSF-1	: B cell stimulatory factor-1 (or IL-4)
CD	: cluster of differentiation
CD40L	: CD40 ligand
cDNA	: complementary deoxyribonucleic acid
cpm	: counts per minute
CTL	: cytotoxic T lymphocyte
CTLA-4	: cytotoxic T lymphocyte associated molecule-4
DTH	: delayed type hypersensitivity
DTT	: dithiothreitol
EAE	: experimental autoimmune encephalomyelitis
E. coli	: *Escherichia coli*
EDTA	: ethylenediamine-tetraacetic acid

ELISA : enzyme linked immunosorbent assay

Fcε : crystallized fragment of ε chain

FCS : fetal calf serum

FP : fusion protein

GITC : guanidine isothiocyanate

GM-CSF : granulocyte-macrophage colony-stimulating factor

GST : glutathione S-transferase

GVHD : graft vs host disease

^{3}H : tritium

HDM : house dust mite

IAC : chloroform and isoamyl alcohol

IDDM : insulin-dependent diabetes mellitus

IFN : interferon

Ig : immunoglobulin

IL : interleukin

IL-2R : IL-2 receptor

i.p. : intraperitoneal (or intraperitoneally)

IP_3 : inositol triphosphate

IPTG : isopropyl-β-thiogalactoside

i.v. : intravenous (or intravenously)

kAL : KBG crude extract allergen

KBG : Kentucky Bluegrass

kD : kilo dalton

LB medium : Luria-Bertani medium

LN : lymph node

log : logarithm

LPS : lipopolysaccharide

mAb : monoclonal antibody

MBP : myelin basic protein

2-ME : β-mercaptoethanol

MHC : major histocompatibility complex

MLC : mixed lymphocyte culture

mRNA : messenger RNA

mw : molecular weight

NK : natural killer

NRE-A : negative regulatory element-A

OD : optical density

OVA : ovalbumin

PBMC : peripheral blood mononuclear cell

PBS : phosphate buffered saline

PBST : PBS Tween 20 buffer

PCA : passive cutaneous anaphylaxis

pGEX2T-1 : pGEX2T-1 plasmid vector

PHA : phytohemagglutinin

pI : isoelectric point

PLA_2 : honey bee phospholipase A_2

Poa p : *Poa pratensis*

PWM : pokeweed mitogen

pWR590 : pWR590 plasmid vector

rAL : recombinant allergen

rpm : rotations per minute

RT-PCR : reverse transcription-polymerase chain reaction

s.c. : subcutaneous (or subcutaneously)

S-D rat : Sprague-Dawley rat

SDS-PAGE : sodium dodecyl sulphate polyacrylamide gel electrophoresis

SE : standard error

SP : spleen

TAE buffer: Tris-acetate and EDTA buffer

TCGF : T cell growth factor (IL-2)

TCR : T cell receptor

TGF-β : transforming growth factor-β

Th : helper T lymphocyte

TNF : tumor necrosis factor

Tris : Tris (Hydroxymethyl) Aminomethane

U : unit

UV : ultraviolet

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ABSTRACT

Allergens represent a class of antigens which upon inhalation, ingestion or contact with skin are capable of inducing the synthesis of IgE antibodies in genetically predisposed individuals. Kentucky blue grass pollen, one of the major factors leading to respiratory allergies, contains a multiplicity of antigenic and allergenic components. By using recombinant DNA techniques, three full length clones, rKBG60, rKBG31 and rKBG41, have been identified and designated as *Poa p* IX allergens.

A C-terminal fragment of rKBG60, rKBG8.3 (rAL) which contains multiple T and B cell epitopes of *Poa p* IX allergens was used in this study to compare *in vivo* immunological activity with KBG pollen extract (kAL). It was demonstrated that (1) rAL and kAL had similar capacity in eliciting IgE antibody response to rAL and kAL; (2) splenocytes of mice immunized *in vivo* with rAL or kAL, cultured *in vitro* in presence of rAL and kAL respectively, produced different levels of IL-2 and IL-4 (statistically insignificant). However, under the same conditions, IFN-γ production was significantly different after restimulation with rAL and kAL. Splenocytes of mice immunized with rAL or kAL, cultured *in vitro* with kAL restimulation, produced undetectable IFN-γ. In marked contrast, splenocytes restimulated with rAL *in vitro* produced significantly high level of IFN-γ.

In order to explore the potential of rAL in prevention and

immunotherapy of allergic disorders to KBG pollen allergen in a murine model, we vaccinated B6D2F1 mice with rKBG8.3 in different forms by i.v. and s.c. routes prior to immunization with rKBG8.3 in alum. This vaccination led to (1) marked reduction of IgE response to rAL which was long-lasting by i.v. route treatment, dose-dependent, and antigen-specific; (2) marked decrease of IL-2, IL-4 and slight decrease of IFN-γ synthesis, resulting in an increased ratio of IFN-γ:IL-4 at the levels of protein and mRNA.

Vaccination with rAL prior to immunization with kAL in alum dramatically enhanced IgG2a production in response to kAL.

Administration of rAL into primed mice modulated established immune responses by decreasing IL-2 production and increasing IgG2a and IgG1 Ab production.

Examination of the events following vaccination and prior to immunization demonstrated that administration of rAL resulted in (1) decrease of cytokine synthesis, specifically IL-2; (2) reduction of spleen cell proliferation which was reversed by addition of rmIL-2; (3) increase of IgG2a Ab production with undetectable IgE Ab production.

These results suggest that vaccination with rAL induces T cell unresponsiveness and consequently following immunization, a deviation from IL-4 dominant to IFN-γ dominant response which leads to suppression of IgE Ab production and enhancement of IgG2a Ab production. Collectively, these data indicate that multi-epitopic recombinant allergens may have potential for prevention of allergies.

INTRODUCTION

I. GENERAL BACKGROUND

In the last decade, there have been monumental progresses on the elucidation of cellular and molecular mechanisms that regulate immune responses in general and specific immunity in particular. Advances have been made in our knowledge of antigen presentation, T cell activation, differentiation and regulatory function, and mechanisms of peripheral tolerance. An overview of these advances is presented below.

1. Biology of Immune Response

1.1. Antigen Presentation

The initiation of immune response to foreign antigens (which include allergens) depends on the recognition of an antigen by cells of immune system. Antigen-presenting cells (APCs) can recognize and internalize antigens, and process them to form peptide fragments. These peptides, bound to cell-surface molecules of the major histocompatibility complex (MHC), are then presented to specific T cells (Braciale et al., 1987; Wallner and Gefter, 1994).

B cells, macrophages, dendritic cells, and Langerhans' cells are called "classical" APC (Schon-Hegrad et al., 1991), but recently eosinophils, and mast cells have been added to the list of

APCs (Saint-Remy, 1994). For example, dendritic cells function as "sentinels", because they reside in nonlymphoid organs where they pick up and process antigen and subsequently move to T cell areas of lymph node and spleen, where they provide optimal conditions for antigen delivery and for activation of naive T cells recirculating in these areas (Steinman, 1991; Lanzavecchia, 1993). Furthermore, the type of APC that is processing the antigen can directly influence the final outcome of an immune response (Saint-Remy, 1994). However, no independent relationship between type of APCs and cytokine response has been definitively established. In some cases, antigen presentation by dendritic cells and B cells might be more likely to stimulate IFN-γ production, which enhances IgG2a responses, than would antigen presentation by peritoneal macrophages. While in the others, macrophages, dendritic cells, and B cells can all induce either a Th1 or a Th2 response, given the proper cytokine environment (Finkelman et al., 1990; Paul et al., 1993; De Becker et al., 1994).

There are two types of MHC molecules: class I, expressed on all nucleated cells, and class II, which is expressed on macrophages, dendritic cells, B cells, and occasionally on other cell types. The MHC molecules are highly polymorphic cell-surface structures that bind peptide of 13-25 amino acids in length (Wallner and Gefter, 1994). Different allelic forms have distinct peptide binding specificities. The sequencing of peptides eluted from MHC molecules has revealed allele-specific motifs which correspond to critical anchor residues that fit into specific

pockets of MHC molecules (Rammensee et al., 1993).

Newly synthesized MHC molecules are exposed to peptides in distinct intracellular compartments. Peptides derived from degradation of cytosolic proteins are transported into endoplasmic reticulum where they bind to nascent class I molecules. Peptides generated along the endocytic pathway bind to newly synthesized class II molecules which are specifically targeted to this compartment (Neefjes and Momburg, 1993).

Although there are some exceptions, class I-class II discrimination based on selective sampling of peptides makes biological sense. In this way $CD8^+$ cytotoxic T lymphocytes (CTLs) which recognize class I molecules, will kill virus infected cells that synthesize viral proteins (Bevan, 1987), whereas $CD4^+$ Th cells, which recognize class II molecules, will stimulate selectively those cells that have captured antigen, for instance, antigen-specific B cells (Lanzavecchia, 1989).

1.2. Activation of Th Cells

Mature T lymphocytes that emerge from adult mammalian thymus migrate to peripheral lymphoid organs such as spleen and lymph node. There, these naive T cells encounter the peptides presented by APCs (Schwartz, 1990). The recognition of peptides by T cells is highly specific and mediated by T-cell receptors (TCR) (Beverly et al., 1992; Rothbard and Gefter, 1991). Ligation of TCR by specific peptide-MHC complexes is sufficient to trigger effector function in

already activated T cells. But for naive T cells and some activated T cells to be fully activated, additional signal (signal 2 or costimulatory signal) is required, which is provided by cell-surface molecules expressed on T cells and APCs (Schwartz, 1992) or by secreted growth factors such as lymphokines (Altman et al., 1990; Weaver and Unanue, 1990). It has been well established that costimulatory signal is antigen nonspecific and is delivered by CD28 or CTLA4 on T cells that interact with a ligand, B7, present on specialized (or classical) APCs. Ligation of CD28 induces increased transcription and stabilization of lymphokine mRNA in responding T cells (Schwartz, 1992). Full activation of T cells leads to synthesis of lymphokines, proliferation of T cells, and ultimately, activation and differentiation of B cells, resulting in the secretion of antigen-specific antibodies, or delayed type hypersensitivity (DTH; Wallner and Gefter, 1994).

1.3. Differentiation of Th Cells

Based on the profile of cytokine secretion, so far, at least three different subsets of Th cells have been described in both mouse and human system: Th1 cells which produce IL-2, IFN-γ and TNF-β; Th2 cells that produce IL-4, IL-5 and IL-10; and Th0 cells which produce both Th1- and Th2-type cytokines. IL-3, GM-CSF and TNF-α are variably produced by all subsets (**Table 1**). IL-3, IL-4 and IL-10 are growth factors for mast cells and IL-5 is a selective activating and differentiating factor for eosinophils (Mosmann and

Coffman, 1989; Romagnani, 1994).

Table 1. Cytokine production profiles of subsets of murine $CD4^+$ T-cell clones

Cytokine	Th0	Th1	Th2
IL-2	+	+	-
IFN-γ	++	++	-
TNF-γ		++	-
IL-4	++	-	++
IL-5	++	-	++
IL-9		-	++
IL-10	++	-	++
TNF-β		++	+
GM-CSF	+	++	+
IL-3	++	++	++

*** Paul, W. E., 1993**

An interesting question is therefore raised as to how Th0 cells differentiate into either Th1 or Th2 subset. Several factors are known to affect this differentiation (Saint-Remy, 1994). (i) The cytokine environment into which T cell differentiates is of primary importance. Thus, Th0 will preferentially differentiate into Th1 in the presence of IFN-γ, whereas Th2 will be formed in the presence of IL-4 (Maggi et al., 1992). The microenvironmental hormones can also promote the differentiation of Th cells or may favor a shift of already differentiated Th cells from one cytokine profile to another. For example, glucocorticoids enhance Th2

activity and synergize with IL-4, whereas dehydroepiandrostenone sulphate enhances Th1 activity (Daynes et al., 1991). (ii) As stated above, types of APC may condition how Th0 differentiates. (iii) The amount of antigen presented to T cells also alters the profile of cytokines produced by T cells. For example, T-cell clones specific for phospholipase A2 of bee venom (PLA_2) produce mainly IL-4 when exposed to low or intermediate doses of allergen; increasing the amount of allergen switches the same T-cell clones mostly to the production of IFN-γ (Carballido et al., 1992). (iv) Both the MHC class II haplotype and the structure of peptide bound to it influence the selection of either Th1 or Th2 cells (Soloway et al., 1991; Romagnani, 1994).

1.4. Regulatory Function of $CD8^+$ T Cells

Most studies of cytokine production have focused on the activities of $CD4^+$ T cells. $CD8^+$ T cells have, however, been shown to make a number of cytokines and there is a considerable literature concerning the activities of $CD8^+$ suppressor cells. Recent studies have clearly established that $CD8^+$ T cells can have a regulatory role in certain circumstances and may not necessarily function purely as cytotoxic killers and that differential cytokine production may be largely responsible for such immune regulation (Croft et al., 1994). IL-4 can also induce mature $CD8^+$ T cells to develop Th2-type effector functions and lose cytotoxic activity (Seder and Le Gros, 1995). Some $CD8^+$ T cells secrete IL-4 and

express CD40L (CD40 Ligand) following stimulation of TCR and also provide help for B cells. Such cells may play a role in antibody responses to antigenic challenges that are restricted only by class I MHC antigens (Cronin II et al., 1995).

1.5. Antibody Isotype Regulation by Th1 and Th2 Cells

One important difference between helper function of the two Th subsets is in their ability to stimulate the production of certain immunoglobulin (Ig) isotypes, most importantly IgE and IgG2a Ab.

Th2 clones but not Th1 are capable of inducing significant IgE response, IgE production stimulated by Th2 clones can be substantially or totally inhibited by either IFN-γ or anti-IL-4 antibody (Coffman et al., 1988). The unique dependence of IgE response on IL-4, and the ability of relatively low concentration of IFN-γ to inhibit this activity of IL-4 (Coffman and Carty, 1986), suggest that Th1 cells may act as isotype-specific suppressor cells for IgE, quite possibly under conditions in which they act as helper for responses of other isotypes (Mosmann and Coffman, 1989).

Th1 clones, in contrast, induce substantially more IgG2a than Th2 clones. Both *in vivo* and *in vitro* studies suggest that IFN-γ is important for high levels of IgG2a production, but other factors may be also involved, since neither the addition of anti-IFN-γ antibody to Th1-stimulated cultures nor the addition of IFN-γ to Th2-stimulated cultures causes much change in IgG2a response

(Stevens et al., 1988; Coffman et al., 1988).

Another important observation made with Th-stimulated cultures is that IL-4 is much less important for the production of IgG1 than was predicted on the basis of its strong IgG1-enhancing activity in LPS-stimulated cultures (Vitetta et al., 1985). The addition of IL-4 can enhance the ability of some Th1 clones to induce IgG1 response, but many other Th1 clones induce substantial IgG1 response in the absence of IL-4 (Stevens et al., 1988; Coffman et al., 1988). This is consistent with observations that anti-IL-4 antibody causes little or no inhibition of IgG1 response either *in vitro* or *in vivo* and suggests that substantial IgG1 response can be induced by an IL-4 independent mechanism (Mosmann and Coffman, 1989; Zhang and Mohapatra, 1993).

2. Modulation of Immune Response

Many diseases are associated with abnormally elevated immune responses. Among these are autoimmune diseases and allergic diseases. Efforts have been made to explore the possibility of modulating immune responses specifically without significantly undermining the ability of the host to respond to other antigens.

The direct modification or polymerization of antigens in modulation of immune responses has been turned out to be promising. For example, heat-aggregation of a protein can increase its immunogenicity (Weigle, 1973), whereas deaggregation of an antigen essentially abolishes its immunogenicity, furthermore, the

deaggregated protein has also been shown, under some circumstances, to be tolerogenic (De Wit et al., 1992). Glutaraldehyde polymerized ovalbumin (OVA) preferentially elicits Th1-like cytokine synthesis patterns *ex vivo* (Yang, X. et al., 1993). Another method for reducing immunogenicity of protein antigens has been the covalent coupling onto the antigen a number of polyethylene glycol (PEG) molecules (Sehon, 1991). Therefore, the chemical modification of an antigen can alter its immunogenicity. However, high doses of aqueous protein antigens without modification can also induce immunological tolerance (Burstein and Abbas, 1993).

The mechanisms underlying these immune suppression or modulation include clonal deletion, anergy, immune deviation and active suppression. More than one of the mechanisms may play a role in tolerance induction at the same time. This may depend on the way antigen is administered and the type or chemical structure of the antigen involved (Jacobs et al., 1994).

3. Mechanisms of Peripheral Tolerance

Immunological tolerance, also referred to as specific immunological unresponsiveness, has been defined as "the phenomenon whereby antigen interacts with the lympoid system to impair its capacity to respond to that antigen" (Nossal, 1983; 1989).

Tolerance can be classified into two categories, central and peripheral tolerance. In thymus, deletion of immature T cells that express self-antigen-reactive receptors acts as a safeguard against

autoimmunity (central tolerance). In peripheral lymphoid tissues, there is a requirement for establishment of tolerance (peripheral tolerance) because (i) deletion in thymus does not eliminate 100% autoreactive T cells, and (ii) there exist many antigens in periphery which are not represented in thymus (Strasser, 1995).

All tolerance phenomena would henceforth have to be examined from the viewpoint of whether responsiveness had been induced in T cells, B cell, or both (Nossal, 1989). Weigle's group have found that (i) serum protein could induce both T and B cell tolerance, but tolerance in T cell was induced more rapidly and at much lower antigen concentrations than that in B cell; (ii) given that B cells were harder to tolerize, it seemed possible that authentic self-tolerance to some antigens, for example, those present in serum and extracellular fluids at low concentrations, might reside exclusively in T cells (Weigle, 1973).

The induction of peripheral T cell tolerance to specific antigens is a well-known phenomenon. Many strategies have been used to induce immunological tolerance. The procedures may be divided conceptually into two general modes, according to whether they are applied prior to induction of the specific antibody response (preventive mode) or subsequent to it (curative mode). The preventive mode of treatment (or vaccination) is administered to a nonimmune, naive animal, whereas the curative (or therapeutic) mode is administered to an animal already sensitized to the antigen (Dintzis, HM and Dintzis RZ, 1992).

Tolerance induction has been achieved by oral feeding of

antigen (Melamed and Friedman, 1993; Nelson and Weiner, 1994), or by administration of antigen via subcutaneous (s.c.) (Breiner et al., 1993), intraperitoneal (i.p.) (Schols et al., 1995; De Wit et al., 1992; Romball and Weigle, 1993; Burstein and Abbas, 1993; Chu et al., 1995), and intravenous (i.v.) (Jacobs et al., 1994; Peterson et al., 1993) routes.

T cell tolerance can be induced by (a) death of T cells that are specific for a particular MHC-peptide combination (clonal deletion which involves apoptosis); or (b) T cell survival in a form that is unresponsive or hyporesponsive to activating stimuli (anergy); or (c) T cell survival in a form that responds strongly to a particular stimulus, but in a way that differs from the standard response (immune deviation; Finkelman, 1995). Other mechanisms may be also operating, such as active suppression which involves participation of antigen-specific suppressor cells (Sercarz and Krzych, 1991).

3.1. Clonal Deletion

In peripheral lymphoid tissues, physiological death of TCR-activated, mature T cells probably serves to delete autoreactive T cells with specificity for antigens that are not presented in thymus. This provides termination of an immune response after the battle against a pathogen has been won (Strasser, 1995).

Activation-induced death or apoptosis (programmed cell death) was first observed in T-cell hybridomas. These cells undergo growth

arrest, functional activation (secretion of IL-2) and apoptosis upon stimulation with antigens, mitogens or antibodies specific for TCR/CD3, Thy-1 or Ly-6. Signal transduction initiated by all these stimuli requires a functional TCR/CD3 complex and *de novo* macromolecular synthesis (Ashwell et al., 1987). Death induced by TCR activation also occurs *in vitro* in normal and leukemic T-cell lines, in normal T lymphoblasts (Dhein et al., 1995), and *in vivo* in normal antigen-reactive Th1 and Th2 cells undergoing oral administration of antigen (Chen et al., 1995).

Recent studies have provided evidence that activation of the cell-surface receptor known as Fas, APO-1 or CD95 is the weapon involved in activation-induced apoptosis *in vitro*. CD95 is a member of the nerve growth factor receptor superfamily, TCR-induced apoptosis can occur through a CD95 ligand-mediated autocrine suicide (Dhein et al., 1995; Brunner et al., 1995; Ju et al., 1995).

Since TCR stimulation triggers not only proliferation and functional activation but also cell death, how can a T-cell-dependent immune response be generated? The answer may lie in the observation that although T cells upregulate CD95 expression within 24 hours of TCR stimulation, they become sensitive to CD95-mediated apoptosis several days later (Owen-Schaub et al., 1992; Klas et al., 1993). Maybe this phase is all the time that they have to carry out their immune functions (Strasser, 1995).

3.2. Anergy

T-cell unresponsiveness or anergy was originally induced in $CD4^+$ helper T-cell clones as a consequence of their stimulation with antigen-MHC complexes, i.e., with peptide antigens presented by other T cells, fixed APCs, purified MHC molecules, mitogens, or cross-linking anti-CD3 antibodies, in the absence of costimulatory signals provided by APCs (Lamb et al., 1983; Jenkins and Schwartz, 1987; Quill and Schwartz, 1987; Mueller and Jenkins, 1995). *In vivo* studies have demonstrated that peptides and antigens delivered with adjuvants cause stimulation, whereas delivered without adjuvants result in specific T-cell anergy (Scheffer, 1993); oral administration of high doses of antigens also results in a state of clonal anergy (Melamed and Friedman, 1993).

These observations led to the proposal of a "two-signal" model of T-cell activation which holds that signals sent to T cells through TCR and costimulatory receptors promote T-cell responsiveness, whereas TCR signaling alone induces subsequent functional unresponsiveness (Mueller et al., 1989; Schwartz, 1992). In the absence of costimulation, antigen-MHC complex recognition induces proximal biochemical signaling events, such as tyrosine kinase activation and calcium mobilization (Mueller and Jenkins, 1995), elicits partial IL-2 receptor (IL-2R) and lymphokine (for example, IFN-γ) production, but little or no IL-2. Instead of dividing, the cells are induced into a state of anergy (Schwartz,

1990). T-cell unresponsiveness is also induced following exposure to a calcium ionophore and is blocked by cyclosporin A and protein synthesis inhibitors (Mueller and Jenkins, 1995).

Among the multiple costimulatory pathways identified, interaction of CD28 and CTLA-4 on T cells with either of two ligands, B7-1 or B7-2, on APC is the most important costimulatory pathway for the response to antigens (Linsley and Ledbetter, 1993), CD28 occupancy by its ligands or by anti-CD28 antibodies, greatly augments the amount of lymphokine that the T cell makes in response to TCR stimulation (June et al., 1989) and prevents the induction of T-cell unresponsiveness (Harding et al., 1992). Furthermore, B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways, thus interaction of B7-1 and B7-2 with shared counterreceptors CD28 and CTLA-4 results in very different outcomes in clinical disease by influencing commitment of precursors to a Th1 or Th2 lineage. Anti-B7-1 drives naive MBP-specific Thp (Th precursor) cells along a Th2 pathway while anti-B7-2 favors Th1 development. Consistent with these *in vitro* differentiation results, blocking B7 molecules *in vivo* did not inhibit generation of antigen-specific T cells, but affected cytokine profile of the responding T cells (Kuchroo et al., 1995). Therefore CD28 and CTLA-4 deliver opposing signals that appear to be integrated by the T cells in determining the response to activation (Krummel and Allison, 1995). Furthermore, Kearney et al. have shown that antigen-driven proliferation and phenotype conversion of naive T cells is dependent on CD28-derived signals

and is inhibited by CTLA-4 (Kearney et al., 1995).

The capacity of unresponsive or anergic T cells to produce IL-2 and express IL-2R or to proliferate was found to be blunted, regardless of the level of antigen presentation and costimulation provided by APCs during rechallenge (Mueller and Jenkins, 1995). In anergic T cells, however, IL-2 is not the only lymphokine whose activity is modulated, IL-3 and IFN-γ are also decreased (Mueller et al., 1989; Schwartz, 1990; Jenkins et al., 1990). It has also been observed that, in T-cell clones that are capable of producing both IL-2 and IL-4 (Th0) (Mueller et al., 1991; Gajewski et al., 1994), and in Th2 clones (Sloan-Lancaster et al., 1994), IL-4 gene remains inducible even after the induction of clonal anergy. It is consistent with the observation of *in vitro* studies that induction of anergy *in vivo* turns out similar results, downregulation of Th1 response, but not Th2 response (De Wit et al., 1992; Karpus et al., 1994). However, there are some reports which demonstrate the induction of tolerance in both Th1 and Th2 subsets (Briner et al., 1993; Romball and Weigle, 1993).

The anergic state, once induced *in vitro*, routinely lasts for several weeks and can be reversed by stimulating the cells with high concentration of IL-2 (Essery and Feldman, 1988; Beverly et al., 1992; Melamed and Friedman, 1993). CD2 is also related to the reversal of T cell anergy (Bell and Imboden, 1995). This reversible situation raises a possibility that the anergic state is maintained by a stable negative regulatory factor or factors that are diluted out with multiple rounds of division. Thus it is conceivable that

anergic cells could be rescued *in vivo* by IL-2 from neighboring cells that are responding to other antigens (Schwartz, 1990).

Anergic T cells exhibit a defect in antigen-induced transcription of IL-2 gene (Kang et al., 1992). Recently, the mechanism of negative IL-2 gene regulation in anergic T cells has been proved (Becker et al., 1995). High amounts of binding activity to the negative regulatory element A (NRE-A) of IL-2 promotor were detected in nuclear extracts from human T cells shortly after induction of anergy. However, most of the signaling events at the surface of anergized T cells are normal (Schwartz, 1990). Furthermore, anergic T cells are not necessarily completely non-responsive. Due to partial activation, effector functions may be exerted in the absence of proliferation. Partial activation was even demonstrated *in vivo* that, when mice were injected with high doses of TNP-conjugated aqueous antigen, secretion of IL-2 and IFN-γ, but not IL-4 was reduced (Van Reijsen et al., 1994). Anergic T cells can also function as suppressor cells *in vitro*, causing inhibition of antigen-specific and allospecific T cell proliferation (Lombardi et al., 1994). Four possible mechanisms could contribute to this T cell-mediated suppression. (i) Anergic T cells, although unable to secrete IL-2, could be more lytic of the APC, thus depriving the responsive T cells of the opportunity to interact with ligand (Go et al., 1993). (ii) IL-2 was consumed because T cell anergy is accompanied by increased levels of IL-2R expression (Lamb et al., 1987) and of responsiveness to added IL-2 (Lamb et al., 1983). (iii) Anergic T cells do secrete a cytokine or

cytokines that inhibit the potentially reactive T cells. Three candidate cytokines for such an effect are IL-4, IL-10, and TGF-β (Martinez et al., 1990; Ding et al., 1993; Kehrl et al., 1986). (iv) Anergic T cells exert their suppressive effects in a passive manner by competing with responsive cells for access to the APC membrane (Lombardi et al., 1994).

3.3. Immune Deviation

Clonal deletion and anergy seem to be the most complete forms of tolerance, but they may be limited by thymic replacement of deleted or anergic antigen-specific T cells. Immune deviation, on the other hand, can be self-perpetuating because cytokine responses of fully differentiated T cells can influence the differentiation of newly generated, antigen-stimulated T cells (Burstein and Abbas, 1993; Finkelman, 1995).

Immune deviation was first described in 1960s (Asherson and Stone, 1965), but mechanistic understanding of the process was limited before the description of $CD4^+$ T cell subsets that can be distinguished by the cytokines they produce (Mosmann and Coffman, 1989). Although individual T cells or T cell clones can secrete other combinations of cytokines (Firestein et al., 1989), $CD4^+$ T cell responses in disease states often are predominantly Th1- or Th2-like (Heinzel et al., 1989; Else et al., 1994; Garside and Mowat, 1995; Liblau et al., 1995), depending on the environment in which they are activated (Romagnani, 1994). Several factors,

including the dose of antigen, site of antigen presentation, the type of APC, the major MHC class II haplotype, costimulatory molecules, influence the differentiation of naive $CD4^+$ T cells into specific Th subsets. However the best characterized factors affecting the development of Th subsets are cytokines themselves (Paul and Seder, 1994; Liblau et al., 1995).

IL-12, IFN-α, IFN-γ, and IFN-β all promote secretion of IFN-γ and inhibit type 2 cytokine secretion (with the exception that IL-12 stimulates IL-10 secretion) (Gajewski and Fitch, 1988; Finkelman et al., 1991; Hsieh et al., 1993). IFN-γ itself inhibits type 2 cytokine secretion and contributes to the anti-type 2 effect of IL-12 (Finkelman, 1995). IL-4 stimulates $CD4^+$ T cells to differentiate into cells that secrete IL-4 and other type 2 cytokines, and inhibits type 1 cytokine secretion by differentiating $CD4^+$ T cells (Seder et al., 1992). IL-10 inhibits macrophage antigen presentation but has more of an inhibitory effect on Th1 responses than Th2 responses, possibly because it suppresses macrophage IL-12 secretion and NK cell IFN-γ secretion (Ding et al., 1993; Tripp et al., 1993). TGF-β suppresses the development of both Th1- and Th2-related effector responses and appears to ultimately promote the development of a type 1 or type 2 response in different situations (Swain et al., 1991; Barral-Netto et al., 1992; Sad and Mosmann, 1994; Luger and Schwartz, 1995).

Polarized cytokine production has been implicated in disease states. Specific cytokine patterns are associated with cure or exacerbation of both infectious and immune-mediated diseases

(Liblau et al., 1995; Finkelman, 1995). For example, T-cell-mediated organ-specific autoimmune diseases of experimental autoimmune encephalomyelitis (EAE) and insulin-dependent diabetes mellitus (IDDM) are the models of Th1-mediated autoimmune diseases, where Th2 cells may play a protective role (Liblau et al., 1995). In atopic allergy (Mosmann and Coffman, 1989), parasitic infections (Sher and Coffman, 1992), graft versus host disease (GVHD) (Umland et al., 1992), immunopathologic responses correlate with the presence of Th2 responses, where protective responses correlate with the presence of Th1 cells.

Association of specific immune-mediated disorders with cytokine patterns raises the possibility that immunization with a disease-related antigen in a way that would promote the development of an opposing set of cytokines might prevent disease development (Racke et al., 1994; Finkelman, 1995).

Although effector $CD4^+$ T cells seem to be irreversibly committed to one given Th subset, the generation of Th1 or Th2 effector cells from naive or memory $CD4^+$ T cells appears to be dependent on the cytokine milieu at the time of their antigenic challenge. Therefore, therapeutically changing cytokine profile of autoreactive $CD4^+$ T cells, at the population level, might be feasible. The fact that immunotherapy in allergic patients can alter the Th1-Th2 balance of allergen-specific T cells suggests that this is indeed the case (Liblau, 1995).

II. SPECIFIC BACKGROUND

1. Allergy and Allergens

Atopic allergy is a genetically determined disorder characterized by an increased ability of B lymphocytes to form IgE antibody to certain groups of ubiquitous antigens that can activate the immune system after inhalation or digestion, and perhaps after penetration through the skin. The antigens which induce allergic responses are called allergens (Romagnani, 1994; the term of antigen and allergen is used interchangeably in this study). Allergens represent a class of antigens which are capable of inducing synthesis of IgE antibodies at extremely low doses. The known allergens include, but are not limited to, weed pollens, grass pollens, tree pollens, mites, animal danders, fungi, mold spores, insects, foods, drugs, latex, chemicals, etc. (WHO/IUS Allergen Nomenclature Subcommitte, 1995).

2. A Synopsis of Allergic Response

Allergic response may be characterized by a typical biphasic response consisting of an immediate reaction to an allergen, followed by a late-phase response. However, on many occasions only an isolated early or on fewer occasions an isolated late phase response may be seen (Wallner and Gefter, 1994).

Early allergic reactions are elicited by IgE-mediated activation of polymorphic granulocytes. Allergic specific IgE antibodies bind to Fc_ϵ receptors on basophils and mast cells, and upon interaction with the allergen, cause degranulation and release of inflammatory mediators which include histamines, prostaglandins, and leukotrienes (Norman, 1975; Lewis and Austen, 1981). Mononuclear cells also produce various cytokines, MIP1 (macrophage inflammatory protein 1), etc. which cause or potentiate IgE-independent degranulation of mast cells and basophils (Kuna et al, 1992; Langdon and MacDonald, 1992), and chemotactic factors, which enhance infiltration of lymphocytes to inflammed tissue (Matsushima and Oppenheim, 1989). These account for immediate hypersensitivity reaction during an allergic response (Norman, 1975).

Late phase response occurs almost certainly in relationship to a combination of biochemical mediators and the presence of cytokines, which is largely inflammatory and is characterized by the infiltration of eosinophils, neutrophils, macrophages, lymphocytes, and basophils into reaction site, resulting in release of secondary mediators (Oertel and Kaliner, 1981; Naclerio et al., 1985; Kay et al., 1991; O'Hehir et al., 1991).

It is known that lymphokines secreted by T cells have been recognized as an integral component of allergic responses (Hiratani et al., 1981). They are required to initiate, maintain, and regulate all immune responses as well as inflammatory and allergic responses.

3. Cytokine Regulation on Allergic Response

To a great extent, Th cells play a pivotal role in early and late phases of allergic responses through the cytokines (or lymphokines) they secrete. Cytokines secreted by Th1 and Th2 cells differentially activate, inhibit, or promote growth of cells involved in allergic reactions, and reciprocally regulate each other's function (Wallner and Gefter, 1994).

3.1. IL-2

The discovery of IL-2, which was originally described as T-cell growth factor (TCGF), had a major impact in immunology because it enables growth of T cells in culture and consequently the study of their function (Ruscetti, 1990).

IL-2 is produced by T cells in response to activation by antigens or mitogens. It initiates proliferation of T cells via binding to specific, high-affinity IL-2Rs which are not expressed on resting T cells, but which rapidly appear after stimulation. Upon elimination of the stimulus, transcription of IL-2 and IL-2R genes is downregulated, followed by cessation of T-cell proliferation (Waldmann, 1993; Luger and Schwartz, 1995).

IL-2 has the capacity to induce NK cells and T cells to produce IFN-γ (Kasahara et al., 1983; Handa et al., 1983). The role of IFN-γ in downregulation of IgE antibody production has been well

recognized, while the role of IL-2 in the induction of IgE antibody remains controversial. IL-2 is a growth factor for both Th1 and Th2 cells. Some reports have shown that IL-2 and IL-4 act in a synergistic manner in enhancement of IgE antibody production *in vitro* (Maggi et al., 1989), whereas others indicate that these two lymphokines inhibit each other's activities (Miyajima et al., 1991; Spiegelberg et al., 1991; Nakanishi et al., 1995). Thus, these two cytokines are sometimes mutually inhibitory or cooperative related to a cross-talk between IL-2 and IL-4 via IL-2R β or γ chains on B cells (Takeshita et al., 1992; Kondo et al., 1993; Russel et al., 1993; Nakanishi et al., 1995).

3.2. IL-4

IL-4 is recognized as an important mediator of allergic responses in mouse system, as well as in human system, being critical for activation and proliferation of Th2 cells, and being a necessary cofactor for isotype switch of B cells to IgE antibody secretion (Vercelli et al., 1989; Gascan et al., 1991; Wallner and Gefter, 1994).

It is well established that the ability of IL-4 to induce isotype switching to IgG1 and IgE depends at least partially on transcriptional activation on the unrearranged $\gamma 1$ and ε C_H genes before switch recombination (Coffman et al., 1993).

IL-4, in addition to its role in IgE-isotype switching, stimulates macrophages to secrete IL-1 during allergic responses.

Because IL-1 is a proinflammatory lymphokine, its elevated level during allergic reaction enhances inflammatory responses and cellular infiltrates during late-phase reactions (Powrie and Coffman, 1993).

The major source of IL-4 is Th2 cells, there are also recent evidences that keratinocytes under certain circumstances may express IL-4 (Aragane et al., 1994) and non-B/non-T cells are the dominant source of IL-4 and IL-6 in the spleens of immunized animals (Aoki et al., 1995). CD1-specific splenic $CD4^+$ $NK1.1^+$ T cells also produce IL-4 promptly on *in vivo* stimulation (Yoshimoto et al., 1995).

IL-4 is required for development of Th2 $CD4^+$ T cells which provide help for antibody responses (Le Gros et al., 1990; Swain et al., 1990; Chatelain et al., 1992; Seder et al., 1992), regulates growth and differentiation of B cells and mast cells (Coffman et al., 1986). IL-4 is also a strong inhibitor of Th1 activation and Th1-specific cytokine production, functioning by directly blocking IFN-γ synthesis from peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutin (PHA), pokeweed mitogen (PWM), IL-2, or during mixed lymphocyte culture (MLC) (Peleman et al., 1989; Wagner et al., 1989). In addition, IFN-γ secretion from purified $CD4^+$ T cells stimulated with anti-CD3 antibodies has been shown to be inhibited by IL-4 (Brinkmann et al., 1993). However, IL-4 exerts differential effects on enhancing IFN-γ secretion and on inhibiting development of IFN-γ-producing lymphocytes (Noble and Kemeny, 1995).

Activation of B cells by IL-4 is different in human and mouse. For example, it is known that IL-4 activation of resting human B cells results in inositol triphosphate (IP_3) release and an increase in cytosolic calcium, followed a few minutes later by elevation of cAMP (Finney et al., 1989). This sequence does not occur in mouse B cells (O'Garra et al., 1987). Furthermore, IgE production in human B cells requires the presence of T cells (Callard and Turner, 1990). No structural differences in the cloned mouse and human IL-4Rs have been reported to explain this, but there is some evidence for a second IL-4-binding protein in human cells (Foxwell et al., 1989). Moreover, the regions covered by amino acid positions 91-128 for human and mouse IL-4 share very little homology (Yokota et al., 1988).

3.3. IFN-γ

IFN-γ is produced by activated Th1 cells (both $CD4^+$ and $CD8^+$). In contrast to IL-4, IFN-γ strongly stimulates Th1 activities, but downregulates Th2 cellular functions, including Th2-mediated IgE synthesis.

IFN-γ promotes production of immunoglobulin by activated murine and human B cells stimulated with IL-2 (Leibson et al., 1984) and causes human B cells treated with antibodies to Ig to enter S phase of cell cycle (Romagnani et al., 1986). It has been shown that IgG2a production is enhanced by IFN-γ in LPS-stimulated mouse splenic B cells (Snapper et al., 1988).

Conversely, IFN-γ specifically suppresses proliferation of Th2 cells, but not that of Th1 cells, which suggests that Th cell subsets might differ in their activation of IFN-γ signaling pathway. The lack of IFN-γ receptor β chain in Th1 cells prevents IFN-γ signaling in Th1 cells and achieves an IFN-γ resistant state (Pernis et al., 1995).

IFN-γ inhibits actions of IL-4 (or named previously B cell stimulatory factor-1, BSF-1) on resting B cells, including IL-4 induction of class II MHC molecule expression (Mond et al., 1986) and costimulation of proliferation (Rabin et al., 1986). IFN-γ also suppresses enhancement by IL-4 of IgG1 and IgE synthesis in B cells stimulated with LPS (Coffman and Carty, 1986).

3.4. Other Cytokines

A number of other cytokines in addition to IL-2, IL-4 and IFN-γ have been shown to regulate development of Th1 and Th2 cells. The macrophage-derived cytokines IL-12 and IFN-α are particularly effective at inducing both secretion of IFN-γ and development of Th1 cells (Romagnani, 1992; Brinkmann et al., 1993; Scott, 1993).

The inhibitory activity of IL-12 on Th2 cellular functions, including Th2-mediated IgE synthesis, is counteracted by IL-10, allowing upregulation of Th2 functions and increasing Th2 cytokine effects on immune system and allergic state (MacNelt et al., 1990; Fiorentino et al., 1991). Th2-type cytokines, except IL-4, i.e., IL-3, IL-5, IL-10 stimulate B cells, mast cells, basophils, and

eosinophils, thus enhancing allergic responses (Powrie and Coffman, 1993; McKenzie et al., 1993). It was also reported that another T cell-derived cytokine, IL-13, induces naive human but not mouse B cells to switch to IgE-producing cells, independent of IL-4 (Punnonen et al., 1993; Fasler et al., 1995; Brinkmann and Kristofic, 1995).

4. Antibody Responses in Allergic Disorders

IgE has a central role in the pathogenesis of human allergic disorders, which causes mediator release from mast cells after interaction with allergens. In allergic patients allergen-specific IgG Abs are frequently found in addition to IgE Ab. Prolonged exposure of predisposed individuals to allergen induces an initial IgG1 Ab response which is followed by a marked shift toward IgG4 Ab production (Johansson et al., 1995; Batard et al., 1993). IgG4 Ab has been of particular interest as a result of its possible role in blocking allergen from binding to IgE Ab. The parallel antigen recognition observed with IgE and IgG4 antibodies specific for parasite and grass pollen support this hypothesis (Hussain and Ottesen, 1986; Batard et al., 1993). In murine system, IgE Ab of immunized mice displayed similar cross-reactivity to certain allergens as IgE Ab of allergic patients (Vrtala et al., 1995). However, the overlap of Ab production between murine and human is not complete. The studies of immune modulation by using glutaraldehyde-polymerized allergen have shown that the modified

allergen has the capacity to induce pronounced inhibition of IgE responses and concomitant increase of IgG2a responses, and IgG1 responses were not correlated with IgE responses. These data suggest that IgG2a may function as protective Ab in response to allergens (Hayglass, 1992).

5. Allergen Immunotherapy (AIT)

The current practice for treatment and prophylaxis of allergic diseases consists of (i) allergen avoidance, (ii) pharmacologic management, (iii) allergen immunotherapy (AIT) (Rasp, 1993). Although avoidance of allergen is one of the effective means by which allergy to animal and mite may be prevented, it is frequently difficult or impossible to achieve, particular to the air-borne particles such as pollens, molds, etc..

Commonly used drugs for allergy therapy include steroids, bronchodilators, antihistamines and other antagonists of vasoactive mediators released from mast cells and basophils, which control symptoms by interfering with mediator release or with inflammation produced by the mediators (Mohapatra and Sehon, 1992).

Immunotherapy is a specific form of controled allergen administration that changes immunoreactivity to allergen leading to clinical improvement (Rasp, 1993). Since allergy reflects an inappropriate immunological reaction, there has been a longstanding interest in therapeutic approaches related to immunology. The initiation and, largely empirical, development of allergen

immunotherapy introduced in 1911 (Noon et al., 1911) and currently practised involves repeated injections of specific allergen extracts over periods of months or years, which prevents allergic symptoms in many patients and offers the advantage of being antigen-specific (Scheffer, 1993). However, the inconvenience to patients due to the lengthy course of therapy and its high cost, and the finite risk of severe asthmatic or anaphylactic reactions, occasionally even fatal, limit the utility of this treatment (Mohapatra and Sehon, 1992; Mohapatra et al., 1995).

Allergen immunotherapy is recognized as an effective treatment of patients with severe allergic rhinitis (Varney et al., 1991), sensitive to bee stings (Lichtenstein et al., 1974; Hunt et al., 1978; Muller et al., 1979), and probably allergic asthma (Scheffer, 1993). However the specific immunologic mechanisms by which allergen immunotherapy achieves its effectiveness has not been fully elucidated and remains controversial (Secrist et al., 1995; Jutel et al., 1995).

Earlier studies have demonstrated that immunotherapy induces a rise in IgG blocking antibodies, particularly IgG4 (Bousquet et al., 1991; Reid et al., 1986), whereas other studies have demonstrated that $CD8^+$ suppressor cells are generated during immunotherapy which can modulate allergen-specific IgE production (Rocklin et al., 1980). These effects are not always observed in patients on immunotherapy, which suggests that other mechanisms are operating.

Since natural exposure to allergen occurs by inhalation,

digestion or through skin, whereas treatment with immunotherapy involves subcutaneous administration of allergen. It has been postulated that the route of allergen exposure may significantly affect the cytokine profiles that develop in allergen-specific T cells (Saloga et al., 1993; Secrist et al., 1995).

In addition, because the amount of allergen administered during immunotherapy is much greater than the amount naturally inhaled, it is hypothesized that higher antigen concentrations would reduce IL-4 synthesis in allergen-specific $CD4^+$ T cells, possibly by altering the type of APCs or monokines involved (Parish and Liew, 1972; Hayglass and Stefura, 1991; DeKruyff et al., 1992; Manetti et al., 1993).

The successful conventional immunotherapy has been demonstrated to correlate with a decreased allergen-specifc T cell response in recent studies, in which clinical improvement was not correlated with changes in allergen-specific IgE levels (Creticos, 1992; Jutel et al., 1995). The mechanism in AIT has been attributed to T-cell anergy. As a consequence of anergy, certain T-cell lymphokines which are important in propagating the allergic cascade are not produced (Wallner and Gefter, 1994). The correlation of successful immunotherapy with decreased T-cell reactivity presents an opportunity to design a treatment for allergies, which directly targets specific T cells. In experimental settings, antigen-specific inactivation of T cells and downregulation of lymphokines has been achieved through the intervention of T-cell activation at the initiation step of immune response, during presentation of the

allergen (Wallner and Gefter, 1994).

Recent studies provide evidence that production of IgE antibody that is believed to be decisive in the induction of immediate hypersensitivity, is largely dependent on the ratio between IFN-γ and IL-4 cytokines (Jutel et al., 1995). Immunotherapy to grass pollen and bee venom has been associated with the increase in the ratio of IFN-γ:IL-4 response in human to the allergens (Secrist et al., 1993; Varney et al., 1993; Jutel et al., 1995). This is also achieved in a murine system with chemically modified allergen (Gieni et al., 1993). These results support the concept that the cytokine profiles of allergen-specific memory $CD4^+$ T cells are mutable and can be manipulated by *in vivo* therapies.

The study on modulation of house dust mite (HDM) immune responsiveness in mouse has demonstrated the induction of T-cell anergy with specific-peptide, which displayed a potential to reduce secretion of lymphokines (IL-2, IL-3/GM-CSF and IFN-γ) when restimulated *in vitro* with sepcific allergen. The mechanisms underlying T-cell anergy are accompanied by a transient downregulation of TCR and CD28, and mediated by a shift in the cytokine profile from that of "IL-4 dominant" to "IFN-γ dominant" functional phenotype of $CD4^+$ T-cells (O'Hehir et al., 1993).

6. Recombinant Allergens

Crude allergenic extracts used in conventional allergen

immunotherapy isolated from natural sources are complex mixtures of numerous proteins and other molecules (Scheiner and Kraft, 1995). The major drawbacks that undermine the efficacy of allergen immunotherapy using extracts are that (i) the composition of crude allergenic extracts differs greatly in allergenic as well as nonallergenic materials, and is difficult to standardize. Some extracts may contain major allergens in insufficient concentrations, and may be contaminated with unwanted components to which the patient is not allergic. Furthermore, important allergens may be lost during extraction. In addition, biochemical and immunochemical procedures of protein purification are relatively costly and time-consuming (Scheiner and Kraft, 1995); (ii) during allergen immunotherapy, patients receive large numbers of proteins to which they are not allergic. In this way, they might even become sensitized to new allergens (Scheiner and Kraft, 1995); (iii) the effects of conventional immunotherapy are temporary and variable, may provoke a transient increase in IgE antibody synthesis that may produce untoward side effects (Norman, 1993). For all these reasons, interest has been focused on development of the effective alternatives.

Various strategies of 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.

In 1975, Takatsu, Ishizaka, and King found that urea-denatured *Amb a* I (antigen E) downregulated IgE production in mice sensitized

to this antigen (Takatsu et al., 1975). This preparation was then administered to a small group of patients allergic to ragweed, and prevented seasonal rise in ragweed IgE antibodies and only caused a minimal increase in IgG antibodies to antigen E (Norman et al., 1980). Norman et al. have achieved desensitization with modified allergens such as glutaraldehyde-treated ragweed extracts, or allergoid, in the immunotherapy of ragweed hay fever (Norman et al., 1982). Litwin and co-workers have developed a peptide fragment (P-1) from PLA_2 (Litwin et al., 1988), the principal allergen of honey bee venom, and subsequently a peptide preparation from ragweed (Litwin et al., 1991). These studies involved the use of pepsin-digested allergens that were subjected to gel filtration to separate the peptides $\leq$ 10 kD in size. These peptide fragments relieved symptoms in the allergic patients.

However, since the products resulting from above methods include a heterogeneous mixture of molecules in a variety of forms, it would be difficult to prepare in a reproducible manner that would yield standardized activity (Norman, 1993).

For all these reasons, recombinant allergens synthesized by recombinant DNA/RNA technology have been recently developed. During last few years, an astonishing number of proteins with allergenic characteristics from pollens, foods, arthropods, vertebrates, priotists, have been produced by recombinant methods (Scheiner and Kraft, 1995). Recombinant allergens were produced by cDNA cloning method that comprises isolation of mRNA from the allergen source, and reverse transcription of mRNA into cDNA. After cloning in

suitable vector systems, allergen-encoding cDNAs can be identified by hybridization, or by antibody screening of cDNA libraries with sera from allergic patients or appropriate monoclonal antibodies (mAbs). The nucleotide sequences of the inserted cDNAs can be used to deduce amino acid sequences of the cDNA-encoded proteins (Mohapatra et al., 1990).

This gene cloning technology is being fervently exploited for (i) expression of the cDNAs of allergens in *Escherichia coli* (*E. coli*) and other hosts for their production in high yield; (ii) determination of primary structures of rALs and their epitopes; (iii) determination of cross-reactivities among different rALs; (iv) definition of biological and immunological functions of these proteins, and (v) attempts at developing new therapeutic modalities with the aid of appropriately modified rALs and their epitopes (Mohapatra and Sehon, 1992).

Based on experimental results that epitopes of rALs, rather than their entire molecules, may prove to be effective for therapeutic purposes, efforts have been made to determine and synthesize their B- and T-cell epitopes. Each allergen seen by the immune system is recognized by its three-dimentional structure by immunoglobulin (B-cell epitopes), or, after ingestion and processing by APCs, as small peptide fragments bonded with MHC class II molecules by T cells (T-cell epitopes) (Scheiner and Kraft, 1995).

The availability of B-cell epitopes may be useful not only for diagnosis of allergies but also for development of therapies which

may result in eliminiation of B; cells responsible for production of IgE antibodies, or blocking of the allergen-binding sites of these antibodies thus preventing their cross-linking by the multivalent allergen or its degradation products resulting from its processing by the patient's appropriate cells (Mohapatra, 1992).

Since Th cells have a crucial role in control of IgE synthesis, administration of peptides corresponding to T-cell epitopes leads to T-cell unresponsiveness to the complete antigens and to the specific peptides (Briner et al., 1993), and may switch off production of IgE antibody. It turned out that most, if not all, allergens contain multiple T-cell epitopes, immunodominant epitopes may be sufficient to tolerize Th2 cells, activate Th1 cells, or tolerize and activate both (Perez et al., 1990; Hoyne et al., 1993; Bungy Poor Fard et al., 1993; Spiegelberg et al., 1994; Scheiner and Kraft, 1995).

Although AIT cannot be envisaged at present, major allergens produced by cDNA technology allow the application of tailor-made allergen preparation in AIT. The recombinant T cell epitope-containing polypeptides, which harbor multiple T cell reactive regions but have significantly reduced reactivity with allergic human IgE, constitute a novel potential approach for desensitization to important allergens (Rogers et al., 1994). Moreover, rALs could be polymerized or modified more easily in order to reduce IgE-binding capacity but still retain T-cell receptor activity. In addition, cDNAs encoding for allergens can be inserted into nonpathogenic microorganisms to construct live

vaccines. AIT with T-cell-reactive peptides derived from allergens seems to provide another form of treatment to induce peripheral T-cell nonresponsiveness and may thereby represent an important component of the next generation of allergen-specific immunotherapy (Scheiner and Kraft, 1995).

7. Recombinant Allergens of Kentucky Blue Grass Pollen

Kentucky blue grass (KBG) pollen, one of the major factors leading to respiratory allergies in North America, Europe and Australia, revealed a multiplicity of antigenic and allergenic components with a wide range of molecular sizes and charges (Eramoddoullah et al., 1977; Chakradarty et al., 1981). KBG pollen has been shown previously to contain *Poa p* I and *Poa p* IV allergens (Lin et al., 1988).

By usign cDNA cloning, a new group of isoallergens from this grass pollen have been identified. Initially, intact translatable mRNA was isolated from KBG pollen and a cDNA library was constructed in λgt11 expression vector. Screening of the library with sera from KBG-allergic patients led to identification of several IgE antibody-binding clones, including three full length clones rKBG60, rKBG31, and rKBG41, and some partial clones (Mohapatra et al., 1990; Silvanovich et al., 1991). Sequence analysis revealed that the above three full-length clones share over 95% homology to one another, only minor similarity to other known allergens, and no homologies to other known proteins or

genes. The predicted molecular mass for the cloned proteins range from 28.3 to 37.8 kD with pI values of 9.6-10.2. This group of newly defined allergens were designated as *Poa p* IX allergens in accordance with the International Allergen Nomenclature system.

The recombinant KBG allergens were initially cloned in pWR590-1 vector, which led to expression of the allergenic molecules as water-insoluble fusion proteins (Mohapatra et al., 1990; Yang et al., 1991; Olsen et al., 1991), then subcloned in a pGEX-2T-1 vector (pGEX system uses 25.5 kD GST as carrier protein; Smith and Johnson, 1988). The latter system expressed water-soluble recombinant allergen fusion protein (Olsen and Mohapatra, 1992), which facilitates production of rALs in virtually unlimited quantities that is essential for development of diagnosis, immunological characterization, prevention and therapy of allergies.

Utilizing overlapping peptides, B cell epitopes for the *Poa p* IX allergen were examined in rKBG60. The results indicate that rKBG60 protein possesses at least 10 antibody binding epitopes (Zhang et al., 1992). The T-cell epitopes of this rAL were also examined by T-cell proliferation assay using peripheral blood monocytes and T-cell lines and clones. It was demonstrated that this rAL had a number of epitopes and that the B-cell and T-cell epitopes appeared to overlap. Moreover, the epitopes recognized by individuals differed (Mohapatra et al., 1995).

The C-terminal fragment of rKBG60, rKBG8.3 (201 amino acid, 20 kD), which constitutes the conserved region of *Poa p* IX allergens

and includes most of T- and B-cell epitopes, provides a candidate to elucidate the potential of multi-epitopic recombinant allergen proteins in diagnosis and therapy of the specific allergies as a substitute of the crude extract.

III. RATIONALE AND SPECIFIC GOALS

As stated above, previous studies in our laboratory included molecular cloning and expression in *E. coli* of KBG pollen allergens which were designated as *Poa p* IX allergens, and their immunological characterization on antibody responses. The C-terminal fragment, rKBG8.3 (201 amino acid, 20 kD of mw), constitutes the conserved domain of *Poa p* IX allergens and includes multiple T- and B-cell epitopes.

On the basis of (i) the above progress in our laboratory, (ii) the advances in molecular characterization of allergens, (iii) allergen immunotherapy attenuating Th2-like responses, and (iv) the disadvantages of conventional immunotherapy, it has been suggested that recombinant allergens singly or in combination with other recombinant allergens may effectively substitute the crude extract in allergen immunotherapy, allergy diagnosis.

It has been demonstrated that (1) a significant proportion (20-30%) of individuals are sensitized to a few major offending aeroallergens (Eriksson and Holmen, 1994), (2) development of sensitization to allergens occurs within the first two years of life (Barres et al., 1995), (3) specific immunotherapy with one

aeroallergen prevented general sensitization to other allergens (Des Roches et al., 1995). These studies support the notion of early intervention of allergic diseases in childhood.

Our main hypothesis is that early interventions in form of prophylaxis and specific immunotherapy could induce Th1-like responses instead of default Th2-like responses, or in other words, induce a state of immune deviation, i.e., shifting responses from Th2-like to Th1-like. In this study we attempted to test this notion by using the recombinant allergen, rKBG8.3 in a mouse model.

The major objectives of this study were:

1. (a) Synthesis of a recombinant allergen, rKBG8.3, and analysis of its immunogenicity in B6D2F1 mice;

(b) Comparison of rAL (rKBG8.3) versus kAL (KBG extract allergen) on antibody and cytokine responses.

2. (a) Modulation of specific antibody responses with different forms of rAL;

(b) Modulation of antibody responses to kAL with rAL;

(c) Modulation of antibody and cytokine responses with soluble form of rAL by different routes;

(d) Modulation of established immune responses with rAL.

MATERIALS AND METHODS

I. IMMUNOLOGICAL TECHNIQUES

1. Animals

Female 6-8 weeks old B6D2F1 (C57BL/6J x DBA/2J) mice were purchased from Charles River, St. Constant, PQ, Canada. Mice used were 7-10 weeks old at the initiation of the experiments.

Male Sprague-Dawley (S-D) rats were bred at the University of Manitoba breeding facility, and the rats used for passive cutaneous anaphylaxis (PCA) were of 300-400 grams.

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

2. Antigen Preparation

2.1. Preparation of KBG Pollen Allergen (kAL)

The KBG pollen protein extract was prepared according to the method as described before (Ekramoddoullah et al., 1980). The mixture of allergens present in the extract is collectively referred as kAL. Briefly, the dried pollen (Hollister-Stier Laboratory, Mississauga, Ontario, Canada) was first defatted with ether, and pollen protein

was extracted by stirring the pollen in distilled water overnight. The water-soluble component was further dialysed through Spectra/pore membrane tubing (mw cutoff = 6000-8000 d; Spectrum Medical Industries Inc., Los Angeles, CA). Protein concentration was determined by Bio-Rad protein assay and SDS-PAGE.

2.2. Preparation of Recombinant Allergen

Recombinant protein was isolated and purified by appropriate modification of the original protocol (Smith and Johnson, 1988; Olsen and Mohapatra, 1992). JM105 bacterial cells which contain recombinant plasmid pGEX 2T-1 were cultured in 10 ml LB (Luria-Bertani broth: Bacto-tryptone 10 g, Bacto-yeast extract 5 g, NaCl 10 g, per liter, pH 7.5) containing 100 μg/ml of ampicillin (Amp) for 16-24 h at 37°C, then transferred to 200 ml LB (containig 100 μg/ml Amp) and incubated for 16-24 h. The overnight cultures were diluted 1 in 5 into 1 liter of LB (containig 100 μg/ml of Amp) and incubated with consistent shaking at 37°C for 1-2 h before addition of isopropyl thiogalactopyranoside (IPTG) at 1 mM to induce protein expression. After 5-7 h of incubation with IPTG, cells were harvested by centrifugation at 6,000 rpm for 20 min at 4°C, then washed with 0.2 M Na-phosphate buffer, pH 7.3, 0.15 M NaCl (HPBS), the final pellet was resuspended in 20 ml HPBS containing 1% Triton X-100 and quickly frozen at -70°C.

The frozen bacterial cells were then thawed at 37°C, sonicated for 30-40 sec, 3-4 times, the lysates were cleared by

centrifugation at 10,000 rpm for 40 min at 4°C. Approximately 20 ml of the supernatants were mixed with glutathione agarose beads (Sulphur Linkage, Sigma Chemical Co., St. Louis, MO), which were preswollen with HPBS, at the ratio of 5:1 (v:v) to allow specific binding of the GST moiety of the FP (or GST itself) to the glutathione on the beads. After incubation on a roller for 30 min at 4°C, the beads were washed 6 times each with 50 ml of HPBS and once with 50 ml of 50 mM Tris-HCl, pH 8.0. The concentration of fusion protein on the beads was measured by SDS-PAGE. The freshly prepared bead-rKBG8.3 fusion protein was designated as BFP and used for injection.

To prepare GST-rKBG8.3 fusion protein (FP) and GST, the bound materials were eluted from 2 ml of the beads soaked in 3 ml of elution buffer (50 mM Tris-HCl, pH 8.0 and 5 mM reduced glutathione (Sigma Chemicals Co, St. Louis, MO)). The elution was repeated 2-3 times. The concentration of the eluted proteins was measured by Bio-Rad protein assay and SDS-PAGE. The eluted materials were aliquoted and stored at -20°C. The yield of the FP ranged from 5 to 10 mg per liter of cultured bacteria.

For the preparation of rKBG8.3, the FP was cleaved with thrombin (Sigma Chemical Co.) when it was bound to the glutathione-agarose beads. Briefly, the washed beads were resuspended in thrombin cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.5 mM $CaCl_2$). Thrombin was added at 1 U/ml for 1 h incubation at room temperature and the cleaved protein was obtained from the elution of the beads. The elution containing cleaved rKBG8.3 was

transferred into dialysis tubing with the molecular weight cutoff around 12,000-14,000 daltons (Spectrum Medical Industries Inc., CA), dialysed against PBS overnight at 4°C. The dialysis buffer was replaced twice. The concentration of cleaved rKBG8.3 was measured by Bio-Rad protein assay and SDS-PAGE. In the context of this thesis, rAL was mainly referred to thrombin-cleaved rKBG8.3.

2.3. Ovalbumin (OVA)

OVA (5x crystallized; Sigma Chemical Co., St. Louis, MO) was dissolved in PBS (0.1 M, pH 7.4) at 1 mg/ml, stored at -20°C, and used for antibody assay and immunization.

2.4. Bio-Rad Protein Micro Assay

Protein concentration was determined by Bio-Rad Protein Micro Assay according to the Manufacturer's instruction (Bio-Rad Laboratories, Mississauga, ON Canada) by optimal modification. Briefly, bovine IgG (BGG; Sigma Chemicals Co.) as protein standard was serially diluted from 1 to 16 μg/ml in distilled H_2O, 0.8 ml of diluted standards and appropriately diluted samples were placed in test tubes (distilled H_20 as blank control), followed by addition of 0.2 ml Dye Reagent Concentrate (Bio-Rad Laboratories). The mixtures were vortexed and incubated at room temperature for 30 min, 0.2 ml of the mixtures were then transferred to microtitre plate (Corning Science Products, Rochester, NY), then read OD_{595} value in an

automatic ELISA reader with Softmax Software (Molecular Devices Corporation, Menlo Park, CA). The standard curve was plotted by measured OD values versus concentration of the standard, unknown concentration of samples was obtained from the standard curve.

2.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a discontinuous system according to Laemmli (Laemmli, 1970) using a Bio-Rad mini slab gel apparatus to examine the expression of the recombinant proteins. Samples were prepared in SDS-PAGE loading buffer (0.0625 M Tris-HCl, pH 6.8, 2.5% SDS, 6% glycerol, 5% 2-ME, 1.55% DTT, 0.002% bromophenol blue) by boiling for 5 min, then loaded onto 12% polyacrylamide gel and separated by electrophoresis at 180-200 v for 40-60 min, followed by staining with 0.25% Coomassie brilliant blue in 10% acetic acid and 45% methanol for 1 h, destaining in 40% methanol and 10% acetic acid solution and drying at room temperature. Molecular weights of the components were estimated with reference to the mobilities of prestained protein standards (Bio-Rad Laboratories, Mississauga, ON).

3. Immunization and Treatment

3.1. Alum Precipitation of Antigen

Antigen in alum (as adjuvant) was prepared as follows. Two ml of 10% $AlK(SO_4)_2$ were added into antigen preparation in 2 ml PBS with 2 drops of 0.2% phenol red, during stirring followed by dropwise addition of 0.5 N NaOH until the solution just got slightly pink (pH 7.4). The alum ($Al(OH)_3$) absorbed antigen was pelleted by centrifugation at 2,000 rpm for 5 min, washed with PBS twice. The final precipitate was resuspended in PBS to 10 ml which leads to the concentration of alum at 2 mg/ml for injection.

3.2. Protocol of Immunization and Treatment

Immunization included injection of antigen absorbed onto 1 mg alum (0.5 ml for each mouse) by intraperitoneal route (i.p.) or subcuteneous route (s.c.). Boosters were given later with same dose of the antigen and alum.

For the immunomodulation, groups of 4 mice each were injected intravenously (i.v., through tail vein) or s.c. with different doses of BFP, FP or rAL in PBS, control groups were injected with PBS only.

To modulate *de novo* immune responses, mice were vaccinated with BFP, FP or rAL which were administered 6 days prior to immunization with rAL, kAL, or OVA in alum. Mouse sera were usually collected by bleeding tail artery around day 20 after immunization, and day 6 after booster unless otherwise stated.

To modulate established immune responses, mice were primed first with 2 μg rAL in alum i.p. around 148-170 days before rAL

treatment, and were challenged 6 days after rAL treatment. Mice were bled 143 days after priming and 5 days after booster.

The mouse sera were stored at -20°C until analysed for antibody production by PCA and ELISA.

4. Antibody Assay

4.1. Antigen-Specific IgE Ab

Individual mouse sera were evaluated for the level of anti-rAL, anti-kAL, anti-GST, and anti-OVA IgE by 48 h passive cutaneous anaphylaxis (PCA) as described before (Zhang and Mohapatra, 1993). Briefly, hooded male S-D rats were sensitized by intradermal injection on the back with 0.1 ml of serial dilutions of murine sera. Forty-eight h later, the rats were challenged by i.v. injection of 200 μg of rAL, GST, or 1 mg of kAL, OVA in 1 ml PBS containing 0.5% Evan's blue (PCA titers sometimes vary one two-fold dilution on challenge with rAL or kAL). PCA titer was calculated as the highest dilution giving a clearly visible skin reaction (>0.5 cm in diameter).

4.2. Total IgE Ab

Total IgE level in sera was determined by ELISA using two rat mAbs specific for nonoverlapping epitopes on murine IgE (PharMingen, San Diego, CA). Purified mAb (clone R35-72) was used as capture

antibody and diluted to 2 μg/ml in coating buffer (0.1 M $NaHCO_3$, pH 8.2). Fifty μl of the diluted mAb was added to microtiter plates (Corning Science Products), incubated overnight at 4°C. After washing with PBST (PBS containing 0.05% Tween 20), excess protein binding sites were then blocked with 3% bovine serum albumin (BSA; ICN Biochemicals, Montreal, Canada) in PBS for 2 h at room temperature. The plates were washed again, the diluted serum samples and mouse IgE standard in 3% BSA were added at 100 μl/well, and incubated overnight at 4°C. After washing, biotinylated anti-mouse IgE mAb (clone R35-92) was added at 100 μl/well (2 μg/ml) for 45 min incubation at room temperature. Streptavidin-alkaline phosphatase (S-AP, diluted to 1:1000; Sigma Chemical Co., St. Louis, MO) was added after washing and further incubated for 30 min at room temperature. Finally the plates were washed and the enzyme substrate (1 mg/ml of p-nitrophenyl phosphate (pNPP; Sigma Chemical Co.) in substrate buffer (1 M diethanolamine, 0.25 mM $MgCl_2$, pH 9.8)), was added into the plates and developed at 37°C for 1 h. The plates were read in the ELISA reader. The concentration of total IgE in the sera was calculated from the standard curve by plotting the $OD_{405-690}$ value versus the standard IgE concentration in the curve.

4.3. Antigen-Specific IgG1 and IgG2a Ab

Antigen-specific IgG1 and IgG2a antibodies were determined by ELISA. Briefly, 96 well microtitre plates were coated with 100

μl/well of antigen (0.1 μg of rKBG8.3, GST, or 1 μg of kAL, OVA for each well respectively) in carbonate-bicarbonate buffer (0.05 M, pH 9.6) overnight at 4°C. The plates were washed with PBST and then blocked with 200 μl/well of 1% BSA in PBS (pH 7.4) for 1 h at 37°C. After washing, the serum samples in the dilution buffer (0.5% Tween 20, 0.5% BSA in PBS) were added at 100 μl/well for 1 h incubation at 37°C, and washed, then detected with AP conjugated rabbit anti-mouse IgG1 (1:1000 dilution) or IgG2a (1:500 dilution; Zymed, South San Francisco, CA) for 1 h at 37°C, followed by adding pNPP at 100 μl/well of 1 mg/ml in substrate buffer for enzymatic color development. Extensive washing was applied to the plates between each steps. The plates were read by ELISA reader after 30 min incubation at 37°C. Antibody titers were determined by 0.5 units of $OD_{405-690}$ value in the highest dilution (0.5 units of OD value were usually three-fold above that of the normal serum used as control).

II. CELLULAR BIOLOGICAL TECHNIQUES

1. Preparation of Single Cell Suspension

Individual mouse spleens and pooled mesenteric lymph nodes were collected aseptically, and single cell suspensions were prepared by using glass homogenizer. Debris was removed by passing cell suspensions through nylon membranes into centrifuge tubes. Cells were spun down at 2,000 rpm for 5 min and washed once with RPMI 1640 medium (Gibco, Grand Island, NY) containing 5% fetal calf

serum (FCS). Cell pellets were resuspended in RPMI 1640 culture medium containing 10% FCS. The number and percentage of viable cells were determined by staining cell preparations with 0.04% trypan blue and counted using a hemocytometer.

2. Protocol of Spleen and Lymph Node Cell Culture

Mice were sacrificed at various times following immunization, vaccination or *in vivo* treatment. Individual spleen cell suspensions or pooled lymph node cell suspensions (within the group of mice by same treatment) were cultured at the number of 10 x 10^6/ml (2 ml/well) alone or with different concentration of rAL, kAL in 24-well tissue culture plates (Corning Science Products) at 37°C, 5% CO_2 in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) which was designated as complete culture medium. Culture supernatants were harvested at different times and stored at -20°C, the harvested cells were stored at -70°C to minimize RNase activity, until expression of cytokine protein and mRNA was analysed.

3. Proliferation Assay

Groups of mice were immunized s.c. with 2 μg of rAL in 1 mg alum, or treated s.c. with 500 μg of soluble rAL in PBS. Individual spleens were collected aseptically on day 11 after immunization or

treatment for proliferation assay. The cells at the number of 1 x 10^6/well were stimulated with 50 μg/ml rAL alone or with 50 μg/ml rAL and 50 U/ml of rmIL-2 in 96-well flat-bottomed microtiter plates at a final volume of 0.2 ml in complete medium. All points were done in triplicate. After 56 h, 1 μCi [^{3}H]thymidine (ICN Radiochemicals, Irvine, CA) was added into each well and cultured for an additional 16 h, at which time the cells were harvested onto glass fiber membrane (Cambridge Technology Inc. Watertown, MA) using a cell harvester (Cambridge Technology Inc.). The incorporation of [^{3}H]thymidine into DNA was quantitated by liquid scintillation counting (Packard Tri-Carb 2200CA, Packard Instrument Company, Downer Grove, IL).

4. Cytokine Protein Measurement By ELISA

Cytokine protein production of IL-2, IFN-γ and IL-4 from culture supernatants was measured by using sandwich ELISA reagents (Pharmingen, San Diego, CA) according to the Manufacturer's Instruction. Ninety-six well ELISA plates were coated with 0.1 μg/well of purified capture mAbs of anti-mouse IL-2 (rat IgG2a, clone JES6-1A12), or anti-mouse IFN-γ (rat IgG1, clone R4-6A2), or anti-mouse IL-4 (rat IgG2b, clone BVD4-1D11). After overnight incubation at 4°C, the plates were washed with PBST and blocked by 3% BSA for 2 h at room temperature. After washing, the culture supernatants and cytokine standards of rIL-2 (from baculovirus-infected Sf9 cells), or rIFN-γ (from baculovirus-infected T.ni

cells), or rIL-4 (from baculovirus-infected Sf9 cells) serially diluted in 3% BSA were added to the plates for overnight incubation at 4°C. The plates were washed again and 0.1 μg/well of corresponding biotinylated mAbs of anti-mouse IL-2 (rat IgG2b, clone JES6-5H4), or anti-mouse IFN-γ (rat IgG1, clone XMG 1.2), or anti-mouse IL-4 (rat IgG1, clone BVD6-24G2) were added and incubated for 45 min at room temperature. The plates were washed with PBST and the cytokines were detected with S-AP (Sigma) for 30 min at room temperature, followed by adding pNPP (dissolved in the substrate buffer) for 1-2 h development at 37°C after extensive washing. The plates were read at $OD_{405-690}$ in ELISA reader, and the standard curves were plotted by concentration of recombinant cytokines versus OD value. In each of the cytokine assay, supernatants from the cells cultured without addition of antigens were used as negative or background control.

III. MOLECULAR BIOLOGICAL TECHNIQUES

1. Isolation of Total Cellular RNA

Total RNA was isolated from *in vitro* cultured spleen cells and lymph node cells, in the presence or absence of specific antigen. Guanidinium-isothiocyanate/phenol method (Maniatis et al, 1982) was used to prepare total RNA in this study. Prewarmed (at 60°C) 4.0 M guanidine isothiocyanate (GITC; BRL, Gathersburg, MD) buffer (4 M GITC, 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate, 0.1 M

2-ME) of 0.3 ml were added to the harvested cells pelleted at the number of 20 x 10^6 in 1.5 ml centrifuge tubes. Cell suspensions were then sheared evenly by using 18-gauge needle fitted 1 ml syringes. Equal volumes of acidic phenol (around pH 5; BRL) and a mixture of chloroform and isoamyl alcohol (IAC, 24:1) were mixed into the sheared samples, which were heated at 60°C for 5 min, and subsequently cooled on ice for 15-30 min, then centrifuged at 14,000 rpm for 20 min at 4°C. The aqueous phase was transferred to another tube, reextracted with phenol and IAC. The aqueous phase was collected and pelleted by ethanol at -70°C overnight. The total RNAs were pelleted by centrifugation at 4°C, 14,000 rpm for 1 h, then the pellets were washed with 70% ethanol twice, dried and resuspended in 50 μl of diethylpyrocarbonate-treated H_2O, frozen at -70°C.

2. Reverse Transcription (RT) of Cytokine mRNA

Poly(A) mRNA in total RNA was reversely transcribed into first strand cDNA. For each reaction to 10 μl of total RNA, a mixture of 0.35 μg of oligo$(dT)_{12-18}$ primers (BRL, Life Technologies Inc., Gaithersburg, MD), 200 U of reverse transcriptase (BRL), 0.2 mM dNTP (BRL), 25 U RNA guard (BRL) were added in the reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$) to a total volume of 50 μl. This mixture was then incubated at 23°C for 10 min, 42°C for 120 min and 95°C for 5 min to synthesize first strand cDNA.

3. Polymerase Chain Reaction (PCR)

Specific cytokine cDNA was amplified by PCR, primers for amplification of β-actin and cytokine sequences were purchased from Clontech Laboratories Inc. (**Table 2**). An annealing temperature of 58°C was used for the primer sets of β-actin, IFN-γ, 60°C for IL-2, IL-4 (Allen et al., 1993). For each reaction, 2 μl of cDNA mixture were added to 0.2 mM dNTP, 0.25 μM each of sense and anti-sense

Table 2. Sequences of primers used in this study

Gene	Location	Sequence
β-actin	nucleotides 25-564	5'-GTGGGCCGCTCTAGGCACCAA-3'
		5'-CTCTTTGATGTCACGCACGATTTC-3'
IL-2	nucleotides 225-511	5'-GAGCAGGATGGAGAATTACAGG-3'
		5'-TCCTCAGAAAGTCCACCACAGT-3'
IFN-γ	nucleotides 113-539	5'-TGGCTGTTTCTGGCTGTTACTG-3'
		5'-AATCAGCAGCGACTCCTTTTCC-3'
IL-4	nucleotide 854 of exon 1	5'-GTCATCCTGCTCTTCTTTCTCG-3'
	- nucleotide 125 of exon 4	5'-GATGCTCTTTAGGCTTTCCAGG-3'
	expected size = 378 bp	

* Allen et al., 1993

primers (specific for the sequence of interest) in PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, 0.1 mg BSA) to a total volume of 23.5 μl. This mixture was covered with 50 μl of mineral oil, incubated at 95°C for 1 min and then held at annealing temperature while 0.75 U (1.5 μl) of Taq DNA polymerase (BRL) were added. This mixture was then cycled through 95°C for 1 min, annealing temperature for 2 min and 72°C for 3 min (35 cycles, 10 min extension was used in the last cycle) in a Perkin Elmer Cetus thermal cycler.

4. Electrophoresis of PCR Products on Agarose Gel

PCR products were assessed by electrophoresis on agarose gel. Agarose gel at 1% was prepared by dissolving 1 g agarose (BRL) in 100 ml TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0), followed by adding ethidium bromide to a final concentration of 0.5 μg/ml, immediately pouring onto a gel tray. After the gel became solid, the samples mixed in DNA loading buffer (10% glycerol, 7% sucrose, 0.025% bromophenol blue) were loaded into the wells. The electrophoresis was performed at 80-100 v for 30-40 min. Gels were photographed under UV light after electrophoresis. The positives of the photographs were scanned on a densitometer and expressed as OD value x scanned area.

IV. STATISTICAL ANALYSIS

PCA and antibody titers were logarithm (log) transformed and expressed as geometric means ± standard error (SE). Cytokine and total IgE concentrations were calculated as arithmetic means ± SE and plotted using a linear scale. Production of antibodies and cytokines was compared between control and treated groups of mice using unpaired two-tailed Student's *t* tests. The significant difference between two groups was expressed as * ($p \leq 0.05$) or ** ($p \leq 0.001$).

RESULTS

I. CHARACTERIZATION OF IMMUNE RESPONSE TO rAL AND kAL

It was shown previously by using antibody to the recombinant allergens that KBG pollen possessed a group of allergenic proteins corresponding to the cloned allergens (Mohapatra et al., 1990; Silvanovich et al., 1991), that the most of the antibody binding sites were located in the C-terminal region of *Poa p* IX allergens, rKBG8.3 (Zhang et al., 1992). However, these studies were based on *in vitro* experiments. In order to address the question whether recombinant allergens could replace the crude extract in *in vivo* diagnosis and prevention or therapy of allergies, we compared the ability of rAL and kAL to induce antibody and cytokine synthesis.

1. Preparation of Allergens

The expression, purification of rAL, rKBG8.3 is presented in **Figure 1**. Among ten Amp-resistant colonies, the bacterial lysates from two of them were found to highly express GST-rKBG8.3 fusion protein (#5, lane 5 and #10, lane 10), #5 was chosen for rAL production.

After sonication, the expressed rAL appeared in the supernatant (**Figure 2**, lane 2 and 3) which indicated that rKBG8.3 fusion protein was soluble. Since the expression of the recombinant protein by plasmid vector pGEX 2T-1 is associated with Lac Z gene, IPTG in our system had effect on the induction of the recombinant protein (**Figure 2**, lane 6 and 7).

In **Figure 3**, purification of thrombin-cleaved rKBG8.3 with molecular weight around 20 kD is shown. The fusion protein was cleaved almost completely by thrombin, with major amount of GST, trace of uncleaved fusion protein and cleaved rKBG8.3 left on the beads.

With a similar procedure of FP preparation, GST was purified as illustrated in **Figure 4**, most of the GST protein appeared in the glutathione elution, although some GST still bound to beads.

In the above experiments, molecular weight of the cleaved recombinant KBG protein was about 20 kD which is consistent with the length of the protein deduced from the sequence of the cDNA insert.

The SDS-PAGE of KBG pollen extract is shown in **Figure 5**, which contained all previously identified groups of grass pollen allergens (Olsen et al., 1991; Olsen and Mohapatra, 1994) and was used in this study.

2. Antibody Response to rAL vis-a-vis kAL

2.1. Antibody Response to the Fusion Protein of rAL

Immunization with GST-rKBG8.3 fusion protein (FP) in alum led to induction of antibodies to both the corresponding rKBG8.3 allergen and GST. The FP induced relatively low titers of antibody production during primary response to GST (**Table 3**). The anti-GST antibody production indicated that GST may also be potentially

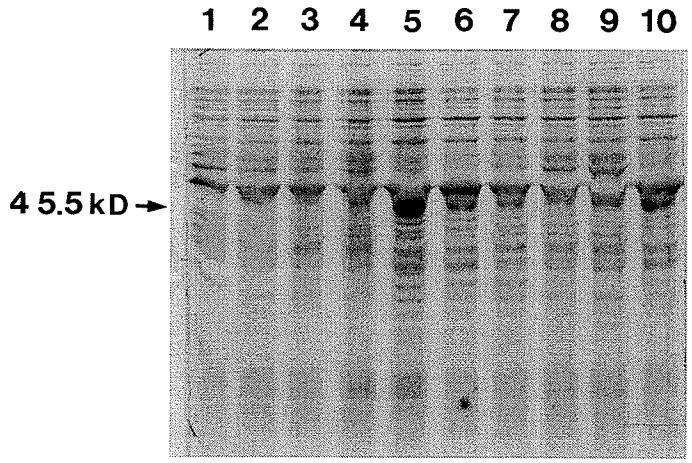

Figure 1. Expression of GST-rKBG8.3 fusion protein. The bacterial lysates of Amp-resistant colonies (#1-10, lane 1-10) after IPTG induction were examined using SDS-PAGE. GST-rKBG8.3 fusion protein with molecular weight around 45.5 kD is shown by an arrowhead.

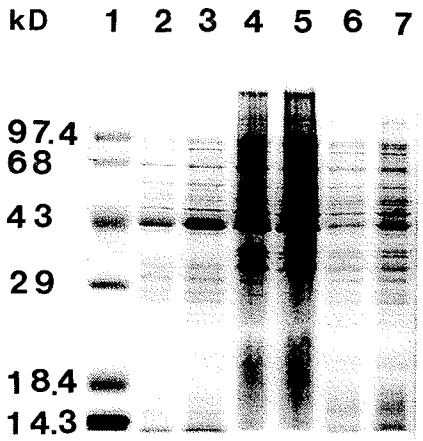

Figure 2. Expression of GST-rKBG8.3 fusion protein in a soluble form and induced by IPTG. Lane 1, molecular weight marker. Lane 2 and 3, supernatants collected after sonication of cultured bacteria #5, samples loaded 2 and 4 μl respectively. Lane 4 and 5, pellets after sonication of bacteria #5, samples loaded 2 and 4 μl. Lane 6 and 7, culture of bacteria #5 without IPTG induction.

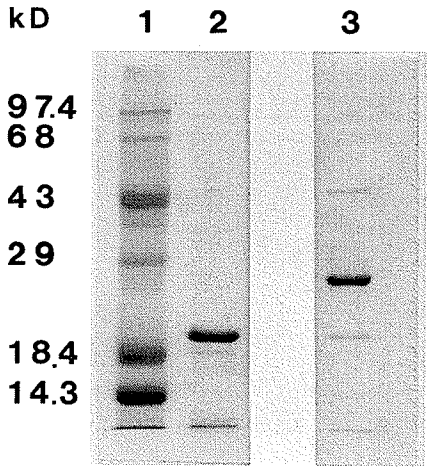

Figure 3. rKBG8.3 obtained by thrombin cleavage of fusion protein bound on the agarose beads. Lane 1, molecular weight marker. Lane 2, rKBG8.3 in elution after thrombin cleavage of fusion protein. Lane 3, beads after thrombin cleavage.

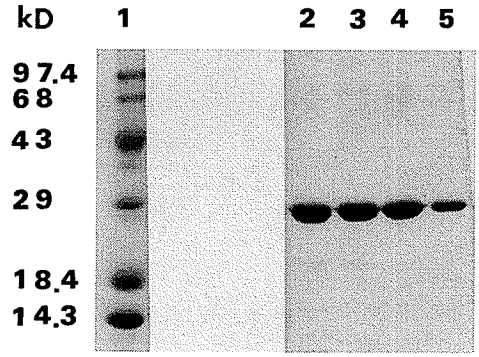

Figure 4. Purification of GST protein. Lane 1, molecular weight marker. Lane 2, beads after binding with GST protein in the sonicated supernatant. Lane 3 and 4, GST protein in the first and second elution obtained by addition of glutathione onto GST bound beads. Lane 5, beads after elution with glutathione.

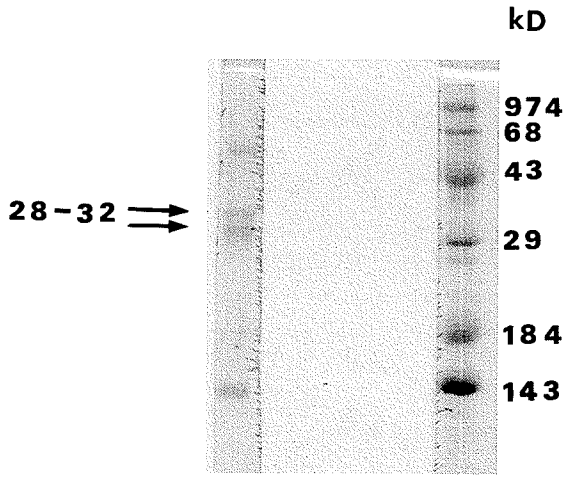

Figure 5. SDS-PAGE of KBG pollen extract. 10% discontinuous gel showed the electrophoresis spectrum of the pollen proteins. Molecular weight standards of 28-32 kD are indicated as the location of the major *Poa p* IX allergens.

allergenic in the fusion protein because it elicited specific IgE response.

The possibility of cross-reactivity between rKBG8.3 and GST was ruled out by employing antibodies to cleaved rKBG8.3 and GST. In PCA assay of IgE detection, rats sensitized with antisera to GST-rKBG8.3 fusion protein responded to challenge with kAL and GST as shown in **Table 3**. However, antisera to rKBG8.3 or to GST reacted with the corresponding antigens respectively (**Figure 6A**). Similar patterns of IgG1 and IgG2a responses in ELISA were shown in **Figure 6B** and **6C**. These results indicated that there was no apparent cross-reactivity between rKBG8.3 and GST.

Table 3. Titers of Ab production to rKBG8.3 and GST induced by immunization with GST-rKBG8.3 fusion protein

	IgE	IgG1	IgG2a
Anti-rKBG8.3			
1° response[a]	640	160,000	300
2° response[b]	1,280	260,000	20,000
Anti-GST			
1° response	<20	200	<50
2° response	160	25,000	300

[a] **primary response on day 14.**

[b] **secondary response on day 7.**

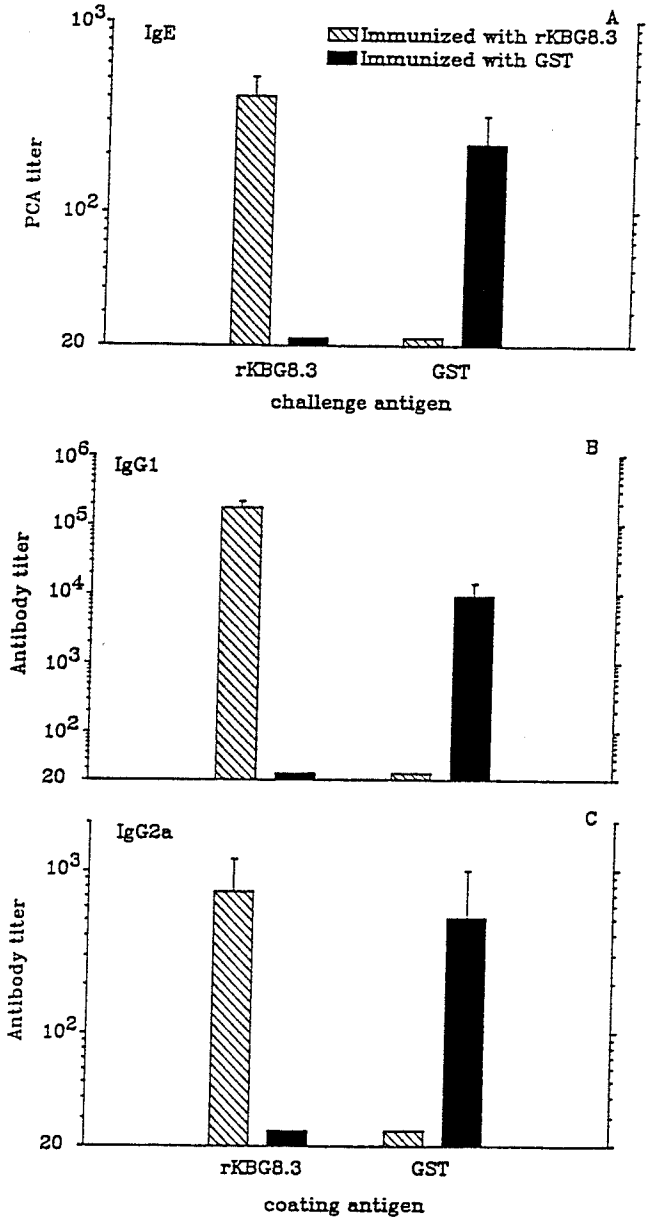

Figure 6. Non-overlapping specificity between antibodies to kAL and GST. Mice were immunized with 2 μg of rKBG8.3 or GST in alum. In the PCA assay of IgE Ab, sensitized rats were challenged with 1 mg of kAL or 0.2 mg of GST (A); 0.1 μg of rKBG8.3 or GST were used for coating plates in ELISA of IgG1 (B) and IgG2a (C) Abs. Abs induced by immunization with rKBG8.3 or GST strongly reacted with corresponding antigen.

2.2. Antibody Response to Cleaved rKBG8.3

Immunization with rKBG8.3 in alum elicited high titers of IgE and IgG1 Abs, strong antigen-specific IgE Ab response peaked 14 days after immunization, IgG2a response, however, was poor at day 14. Production of IgE, IgG1 and IgG2a Abs persisted at high level up to 143 days there was no apparent reduction in the titers (**Figure 7**).

2.3. Antibody Production Induced by rAL versus kAL

Antisera from the mice immunized with rAL or kAL were analysed by PCA and ELISA for their ability to react with rAL or kAL (**Figure 8**). Corresponding to the *in vitro* experiment (Olsen and Mohapatra, 1994), antibodies of different isotypes produced by the mice immunized with kAL bound equally to itself (kAL) as well as to rKBG8.3 (**Figure 8A**; $p>0.05$). Isotype distribution of antibodies produced in response to rKBG8.3 was different. rKBG8.3 induced IgE antibody which bound equally to rKBG8.3 and kAL ($p>0.05$), but IgG1 and IgG2a antibodies induced by rKBG8.3 bound preferentially to rKBG8.3 and less to kAL (**Figure 8B**; $p<0.05$). These results together suggest that IgE antibody response in mice to rKBG8.3 or to its natural counterpart is similar.

3. Cytokine Response to rAL vis-a-vis kAL

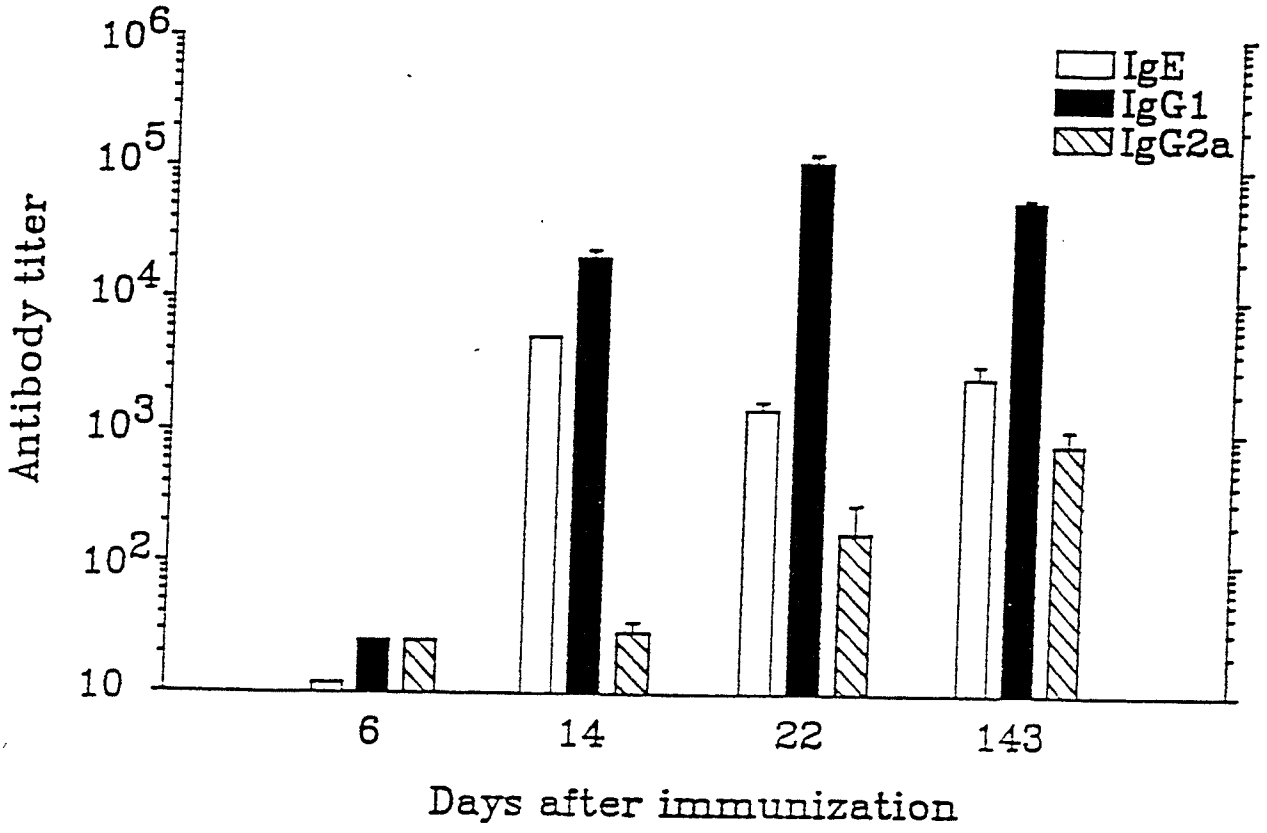

Figure 7. Antibody production to rKBG8.3 immunization. Mice were immunized (i.p.) with 2 μg of rKBG8.3 in alum. Specific antibody titers were measured from the sera collected at different times after immunization. rKBG8.3 elicited strong IgE and IgG1 responses. Ab titers are expressed as geometric mean ± SE (n=8).

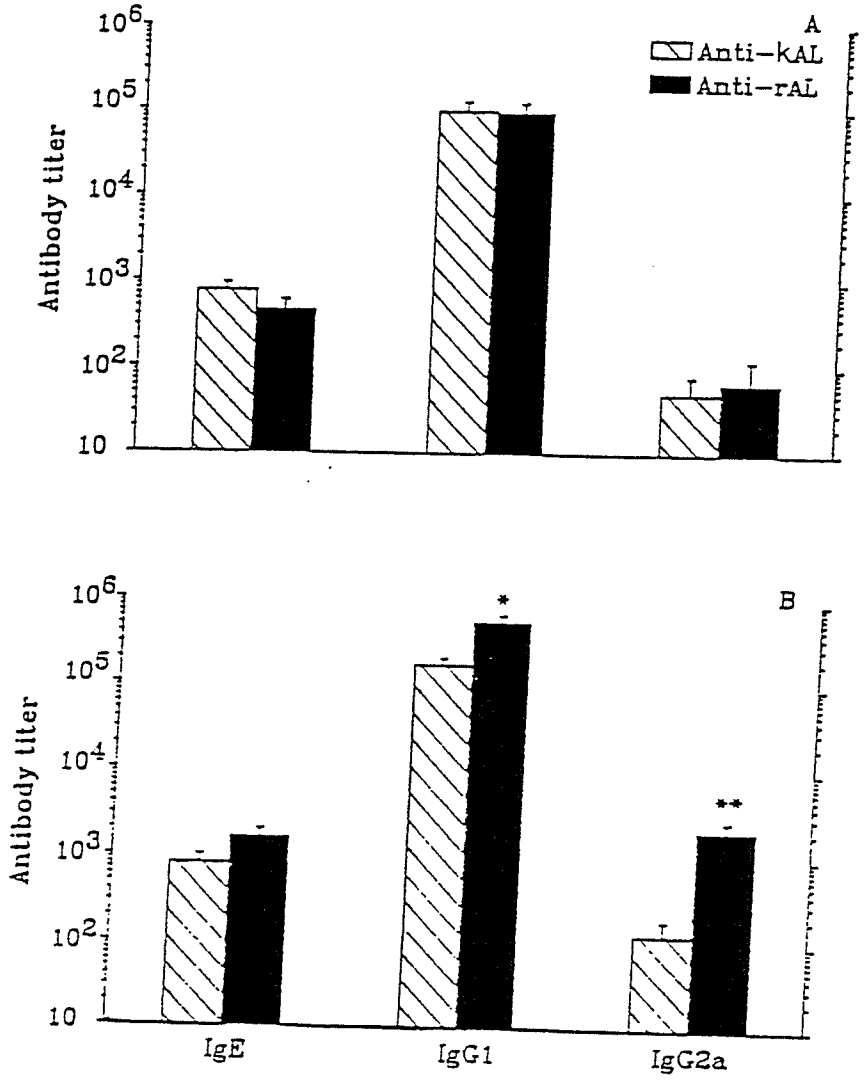

Figure 8. Antibody responses to kAL vs rAL. The mice were immunized (i.p.) with 10 µg of KBG extract (A), or 2 µg of rKBG8.3 (B) in alum, 4 mice in each group. Individual mouse sera were analysed for IgE antibody production by PCA with challenge of KBG extract or rKBG8.3, for IgG1 and IgG2a antibody production by ELISA with coating KBG extract or rKBG8.3. Antibody titers are presented as geometric mean ± SE. This experiment was repeated once, similar results were obtained.

In order to characterize antigen-driven cytokine synthesis, we immunized (s.c.) mice with 2 μg of rKBG8.3 or 10 μg of KBG extract in alum. Five days later, mouse spleen and lymph node cells were cultured for measurement of cytokine protein production by ELISA. The detection level of cytokine protein by ELISA was about 10-12 pg/ml for IL-2, 20 pg/ml for IFN-γ, and 2-4 pg/ml for IL-4.

3.1. Optimal Culture Conditions for Cytokine Assay from the Mice Immunized with rAL

Kinetic study of cytokine secretion in *in vitro* cultures from the mice immunized with 2 μg of rKBG8.3 demonstrated that IL-2, IFN-γ and IL-4 were readily detectable in culture supernatants 7 h following antigen-specific stimulation and were maximal or near-maximal at 24 h, decreased after 24 h culture (**Figure 9**).

For *in vitro* restimulation, rKBG8.3 at 4 μg/ml consistently induced cytokine secretion and 200 μg/ml was the concentration that could be used for highest cytokine secretion (except IFN-γ) compared to 4 and 20 μg/ml (**Figure 10**). It is shown in **Figure 11** that 10×10^6 cells/ml gave maximal *in vitro* cytokine synthesis.

Therefore, except where specified, all the experiments involving cytokine gene expression in this study examined the cytokine profile of spleen and lymph node cells obtained from the mice immunized with rAL 5 days prior to assay and cultured *in vitro* at 10×10^6 cells/ml with 200 μg/ml of rKBG8.3 for 24 h.

It was demonstrated by RT-PCR that expression of cytokine mRNA

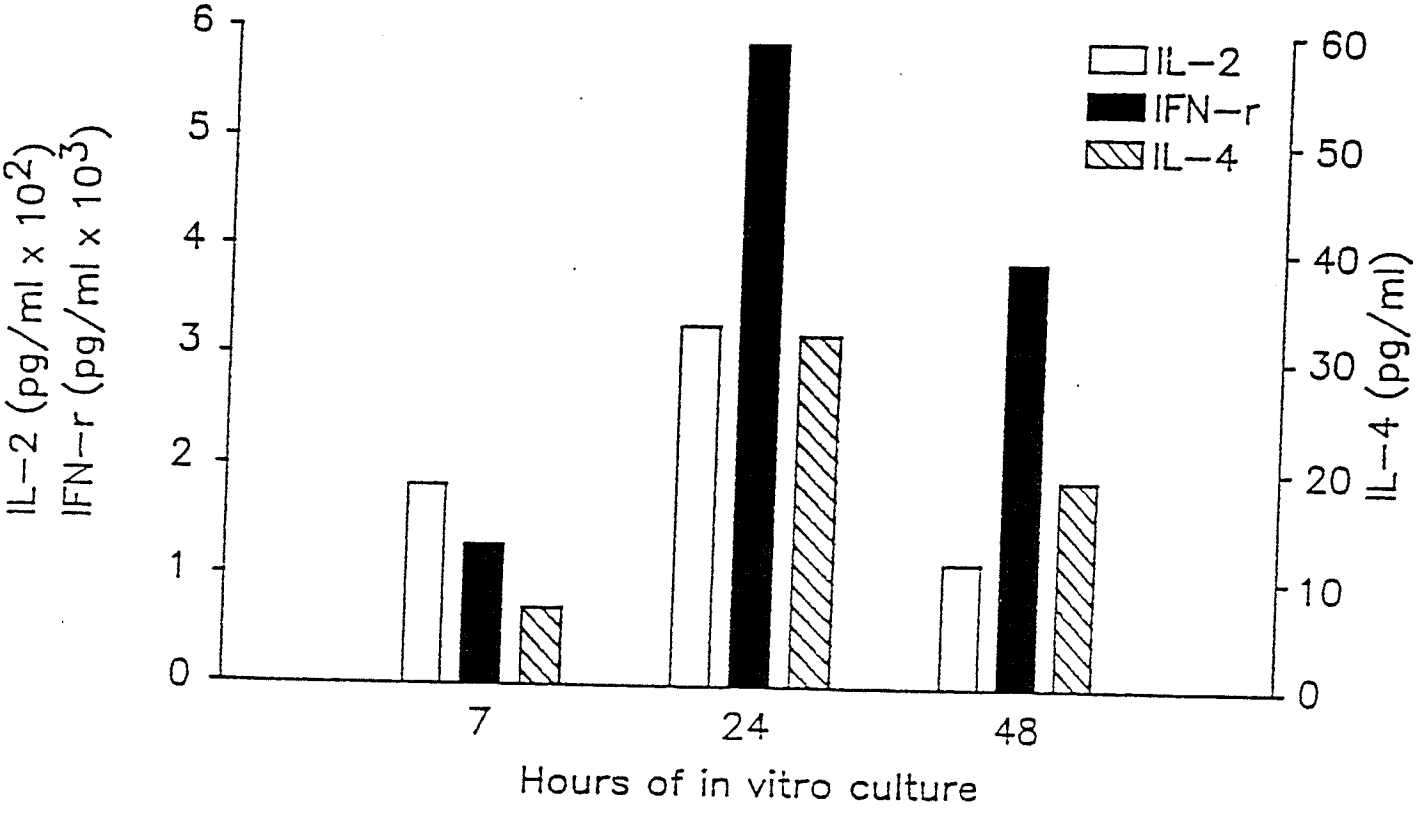

Figure 9. Kinetics of antigen-dependent cytokine synthesis in vitro. Four mice were immunized (s.c.) with 2 µg of rKBG8.3 in alum. Five days later, mouse spleens were collected. The pooled spleen cells at 10 x 10^6/ml were restimulated in vitro with 200 µg/ml of rKBG8.3. The culture supernatants were harvested at different times and cytokine production was measured by ELISA.

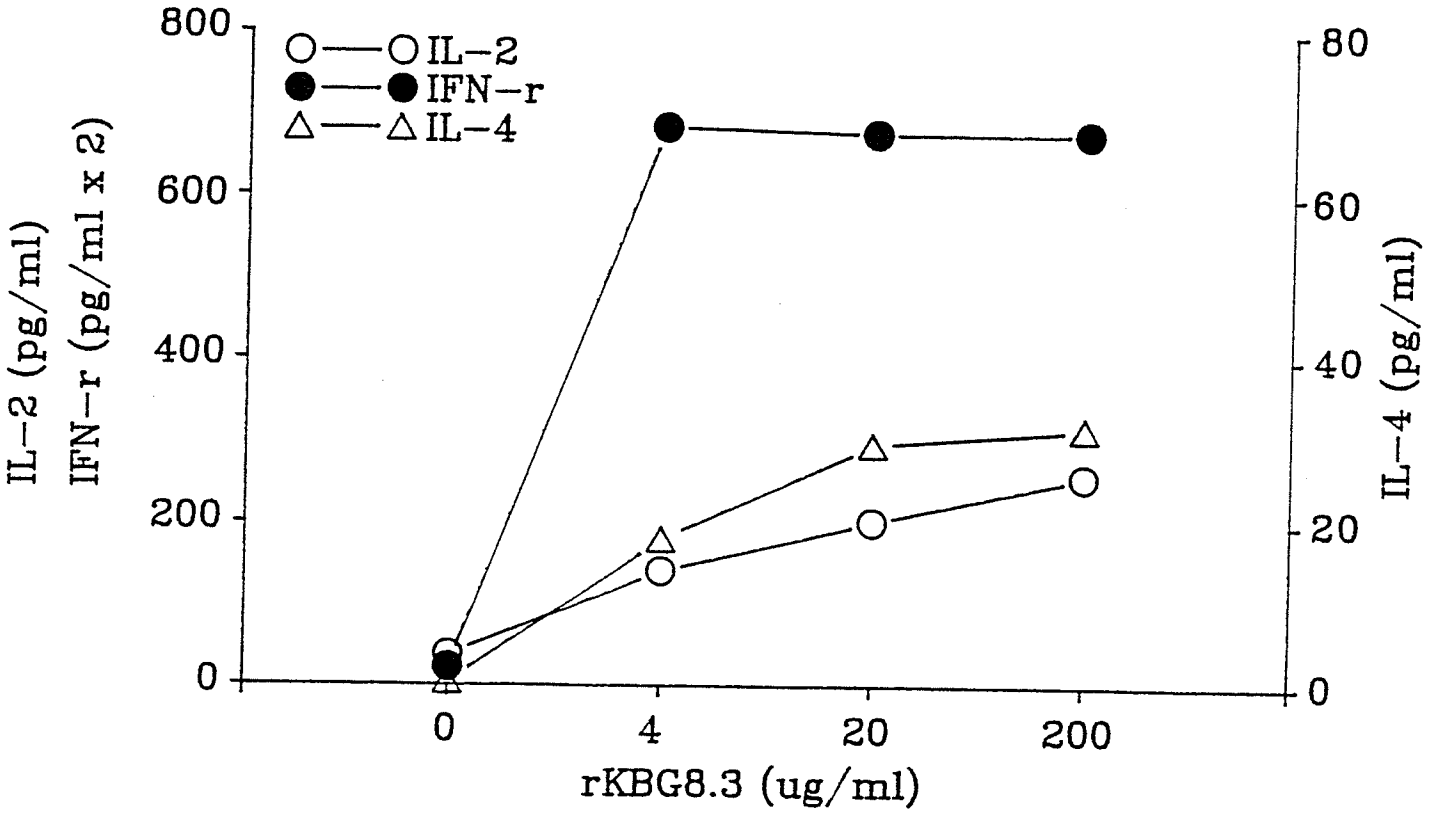

Figure 10. Optimal antigen concentration for <u>in vitro</u> restimulation. Four mice were immunized (s.c.) with 2 µg of rKBG8.3 in alum. Five days later, mouse spleens were collected. The pooled spleen cells at 10×10^6/ml were cultured <u>in vitro</u> in the presence of different concentration of rKBG8.3. Cytokine synthesis was measured by ELISA from the supernatants after 24 h culture.

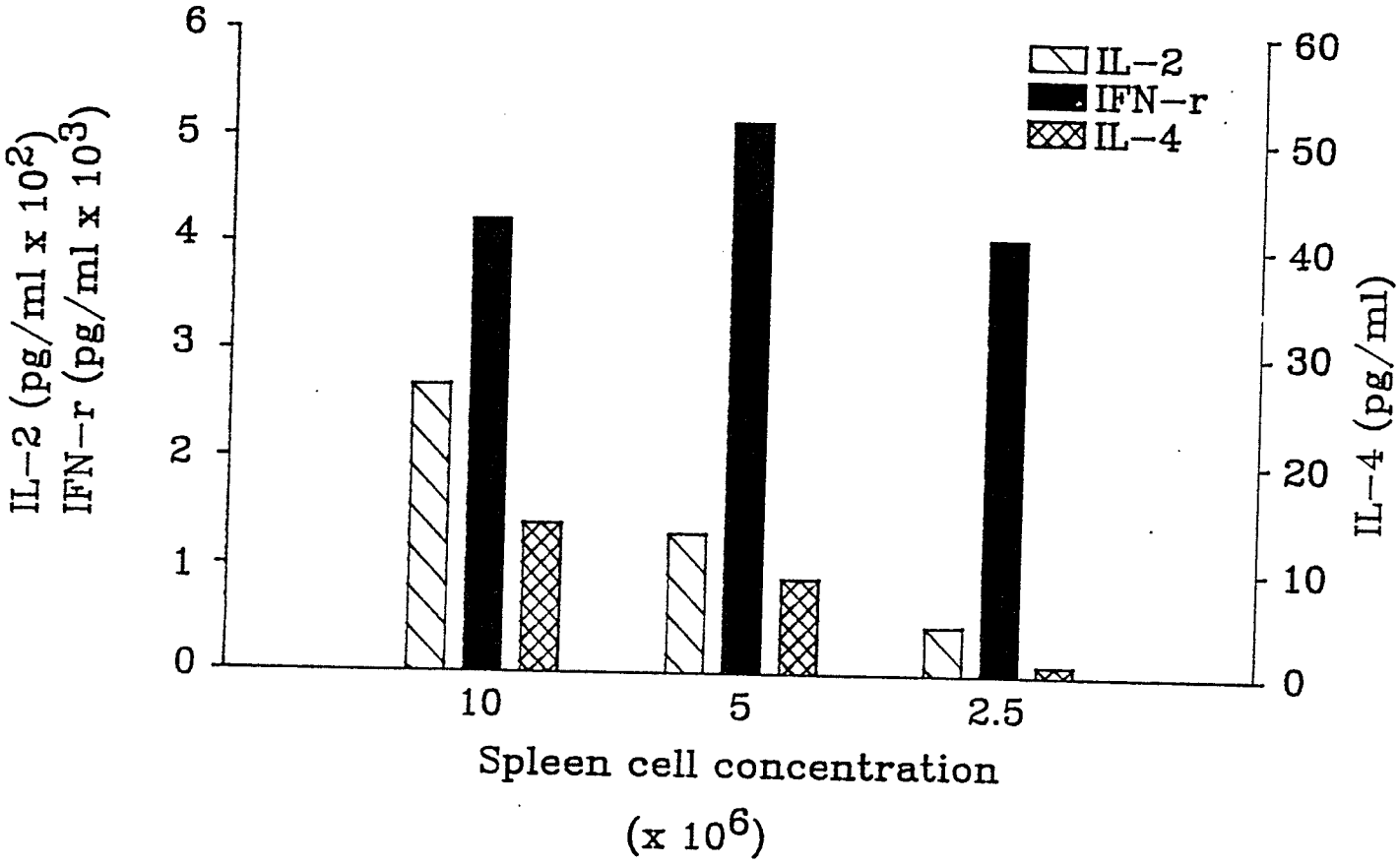

Figure 11. Optimal cell concentration for <u>in vitro</u> cytokine production. Four mice were immunized (s.c.) with 2 µg of rKBG8.3 plus alum. Five days later, mouse spleens were collected. The pooled spleen cells were cultured at different cell concentration in the presence of optimal concentration of rKBG8.3 (200 µg/ml). The supernatants were collected after 24 h culture for cytokine assay.

could be detected in the spleen cells and lymph node cells cultured *in vitro* for 7 h (data not shown) and 24 h (**Figure 12**), although the expression level in the cells with 7 h culture was not as high as that with 24 h culture. Cytokine expression could also be measured in the cells cultured in the presence or absence of antigen although in the latter situation the cells, from which we were unable to detect cytokine protein, exhibited low level of cytokine mRNA expression.

3.2. Optimal Culture Conditions for Cytokine Assay from the Mice Immunized with kAL

In order to optimize culture conditions with KBG extract, mice were immunized (s.c.) with 10 μg of KBG extract in alum. Mouse spleens were collected 5 days after. The pooled spleen cells were cultured in the presence of 4, 20, 200 μg/ml of KBG extract at 10×10^6 cells/ml (**Figure 13**), and at the number of 2.5, 5, 10×10^6 cells/ml with 200 μg/ml of KBG extract (**Figure 14**). The culture supernatants were collected after 24 h incubation for cytokine protein assay. The *in vitro* culture conditions with 200 μg/ml of KBG extract at 10×10^6 cells/ml giving the maximal cytokine production were selected in this study for most of the experiments of examining cytokine synthesis from the mice immunized with kAL.

3.3. Cytokine Protein Synthesis Induced by rAL versus kAL

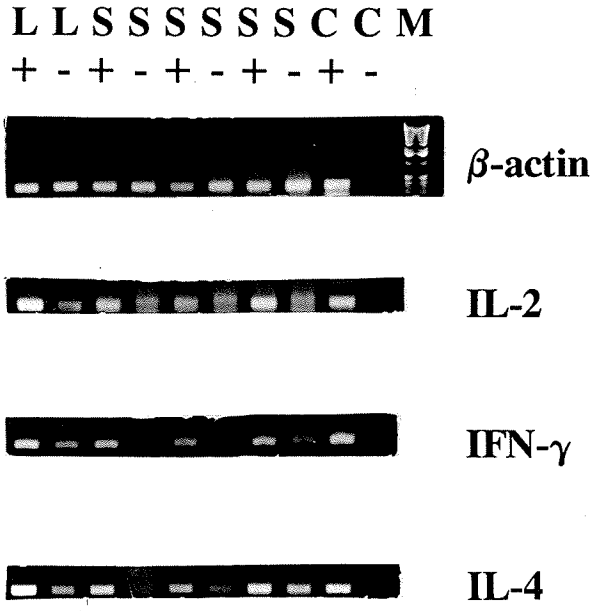

Figure 12. Cytokine mRNA expression demonstrated by RT-PCR. Mice were immunized (i.p.) with 2 μg of rKBG8.3 in alum. Five days later, mouse spleens and lymph nodes were collected. The individual spleen cells and pooled lymph node cells were cultured *in vitro* in the presence or absence of rKBG8.3. M, DNA ladder for molecular weight. C -, negative control without cDNA in PCR reaction. C +, positive control with specific cDNA. S, spleen cells. L, lymph node cells. +, in the presence of rKBG8.3. -, in the absence of rKBG8.3.

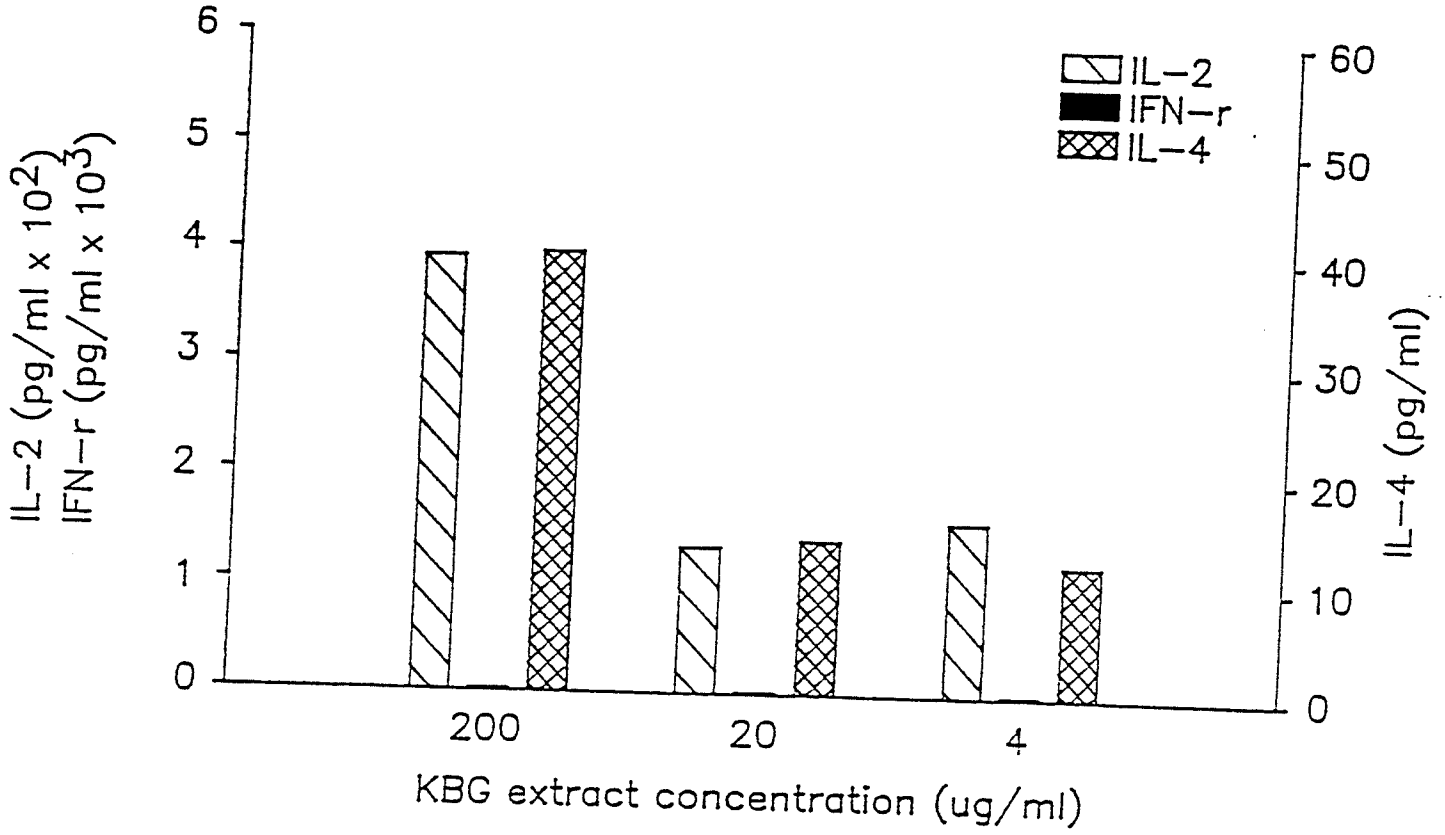

Figure 13. Optimal concentration of KBG extract for in vitro restimulation. Mice were immunized (s.c.) with 10 μg of KBG extract in alum. The mouse spleens were collected 5 days later. The pooled spleen cells at 10 x 10^6/ml were cultured in vitro in the presence of different concentration of KBG extract. Cytokine synthesis in the supernatants was measured after 24 h culture.

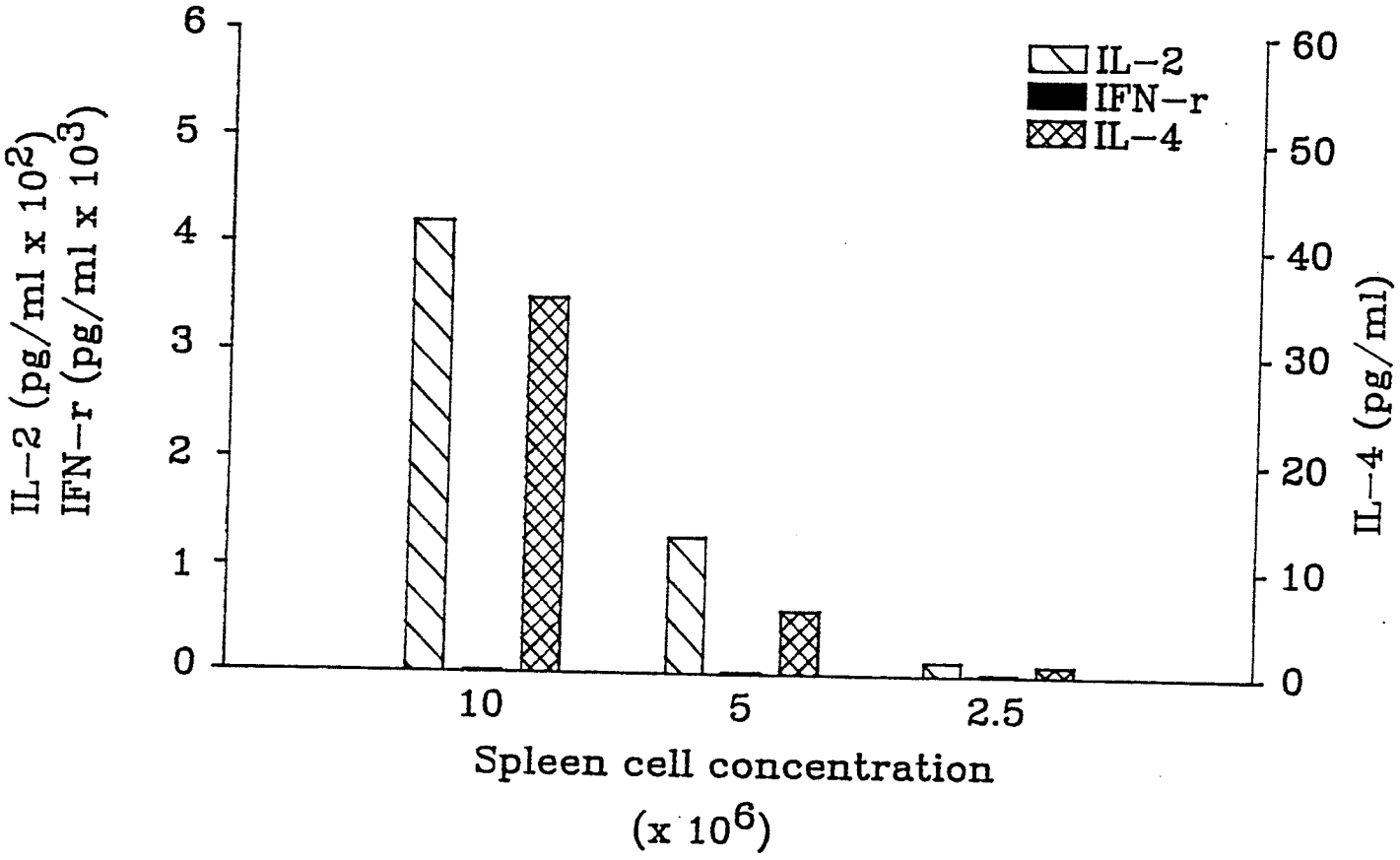

Figure 14. Optimal cell concentration for <u>in vitro</u> restimulation. Mice were immunized (s.c.) with 10 µg of KBG extract. The mouse spleens were collected 5 days later. The pooled spleen cells were cultured in the presence of optimal concentration of KBG extract (200 µg/ml) at different cell numbers. Cytokine production was measured from 24 h culture supernatants.

To compare the patterns of cytokine synthesis in the mice immunized with rAL or kAL, two parellel groups of mice were set, one group immunized with rKBG8.3, the other group immunized with KBG extract. Five days later, the mice were sacrificed and the spleens were collected. Individual spleen cells were cultured *in vitro* with 200 μg/ml of rKBG8.3 or KBG extract. After 24 h culture, the supernatants were harvested for cytokine analysis. The mice immunized with rKBG8.3 produced high level of IL-2, IFN-γ and IL-4 in response to *in vitro* restimulation with rKBG8.3. These cells produced a bit lower level of IL-2 and IL-4, but undetectable IFN-γ in response to restimulation with KBG extract (**Figure 15A**). In contrast, the mice immunized with KBG extract produced high level of IL-2 and IL-4, undetectable IFN-γ to restimulation with KBG extract, a bit lower level of IL-2 and IL-4, but high level of IFN-γ to restimulation with rKBG8.3 (**Figure 15B**). Thus, rKBG8.3 preferentially elicits IFN-γ synthesis in addition to IL-2 and IL-4.

Taken together, this section of the results demonstrated that that KBG pollen extract (kAL) possessed allergenic proteins which may be equivalent of rKBG8.3 (rAL) in the capacity to induce IgE antibody, IL-2 and IL-4 responses *in vivo*. These results also suggest that rKBG8.3 may substitute KBG extract for *in vivo* diagnosis to KBG allergy.

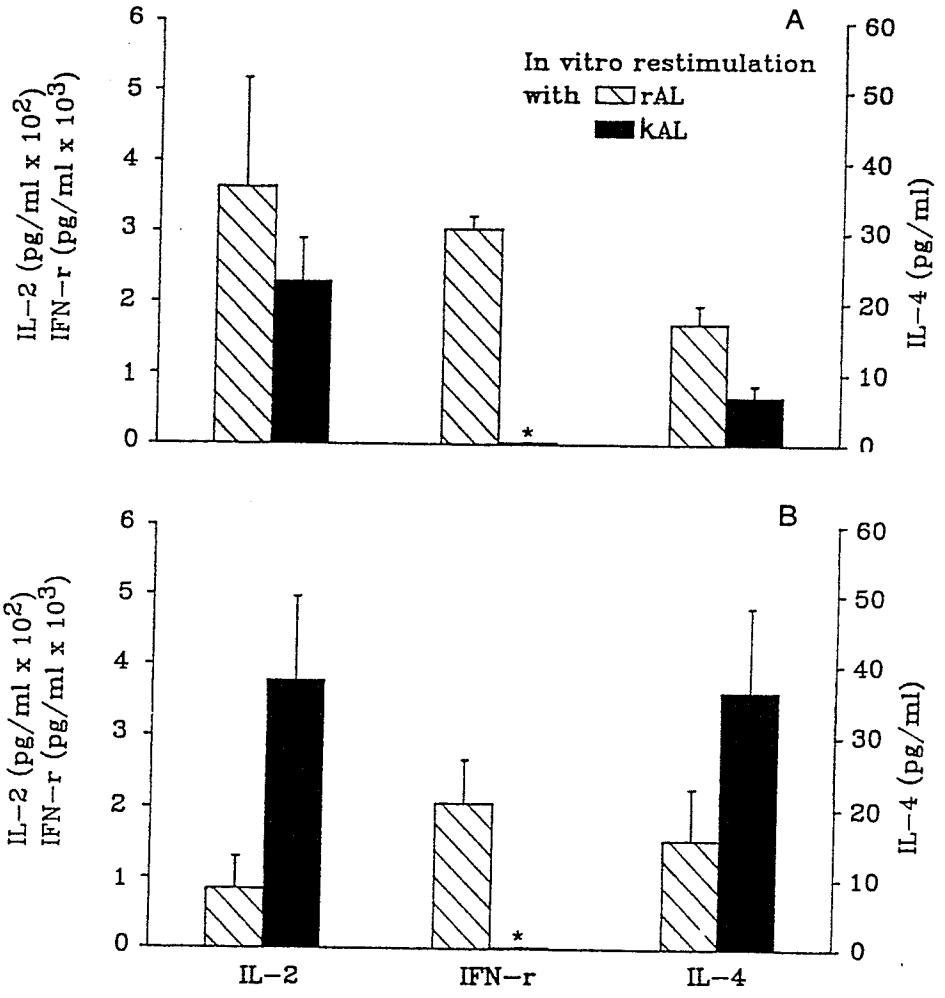

Figure 15. Cytokine production induced by rAL versus kAL. Mice were immunized (s.c.) with 2 μg of rKBG8.3 (A), or 10 μg of KBG extract (B) in alum, 4 mice in each group. Five days later spleens from these mice were collected. Individual spleen cells were cultured in the presence of 200 μg/ml of rAL or kAL for 24 h. Cytokine production was measured by ELISA and expressed as arithmetic mean ± SE. This experiment was repeated once, similar results were obtained.

II. IMMUNE MODULATION BY VACCINATION WITH rAL

Functional specialization of T cells provides an enormous opportunity for immune modulation on allergic response. One may be able to exploit the reciprocal regulation of Th1 and Th2 to induce protective responses to suppress IgE antibody production (Lanzavecchia, 1993). Furthermore, selection of the appropriate T cell population is an antigen-driven process that occurs during the early stages of immune response in naive host (Holt, 1994). Based on these hypotheses, therefore, we attempted to induce protective Th1 response to an allergen by vaccination with rAL in a mouse model.

1. Modulation of Antibody and Cytokine Responses by i.v. Vaccination

It has been known that administration of particular antigen by intravenous route before immunization is a potential way to induce antigen-specific peripheral T-cell tolerance. But less is known about the underlying mechanisms. In this section, first, we induced tolerance by i.v. administration with rKBG8.3, then we utilized this model to investigate the mechanisms at the level of cytokine production.

1.1. rAL Vaccination Elicits Reciprocal IgE and IgG2a Responses

In order to examine the potential of vaccination, mice were injected i.v. with soluble rKBG8.3 in PBS. Control mice were injected with PBS only. After 4 days both control and treated groups were immunized with rKBG8.3 in alum. Individual mouse sera were collected around day 20 after primary immunization and day 6 after secondary immunization. In treated mice, specific IgE antibody titers were undetectable at the lowest dilution of 1:20 ($p<0.001$), production of rKBG8.3-specific IgG1 antibody reduced 78% ($p<0.05$), but IgG2a antibodies increased 94% ($p=0.06$) after primary immunization (**Figure 16A**). IgE, IgG1 and IgG2a antibodies showed similar patterns after secondary immunization (**Figure 16B**) as those after primary immunization in treated mice. It is concluded from these results that rAL vaccination reciprocally altered the isotype distribution pattern of IgE and IgG2a specific to rAL.

1.2. Suppression of IgE Response Is a Long Term Effect

To examine long term effect of the vaccination, IgE antibody was measured from the individual mouse sera collected on day 7 after primary immunization, day 7, 21, 31 after secondary immunization, day 7 and 21 after tertiary immunization. Production of specific IgE antibody reduced >91% in the treated mice on day 7 after secondary immunization. Inhibition of IgE antibody production lasted 87 days around 85-95% (**Figure 17**) when the experiment was terminated, eventhough there were three immunizations after rKBG8.3 treatment. Therefore it is concluded that vaacination could induce

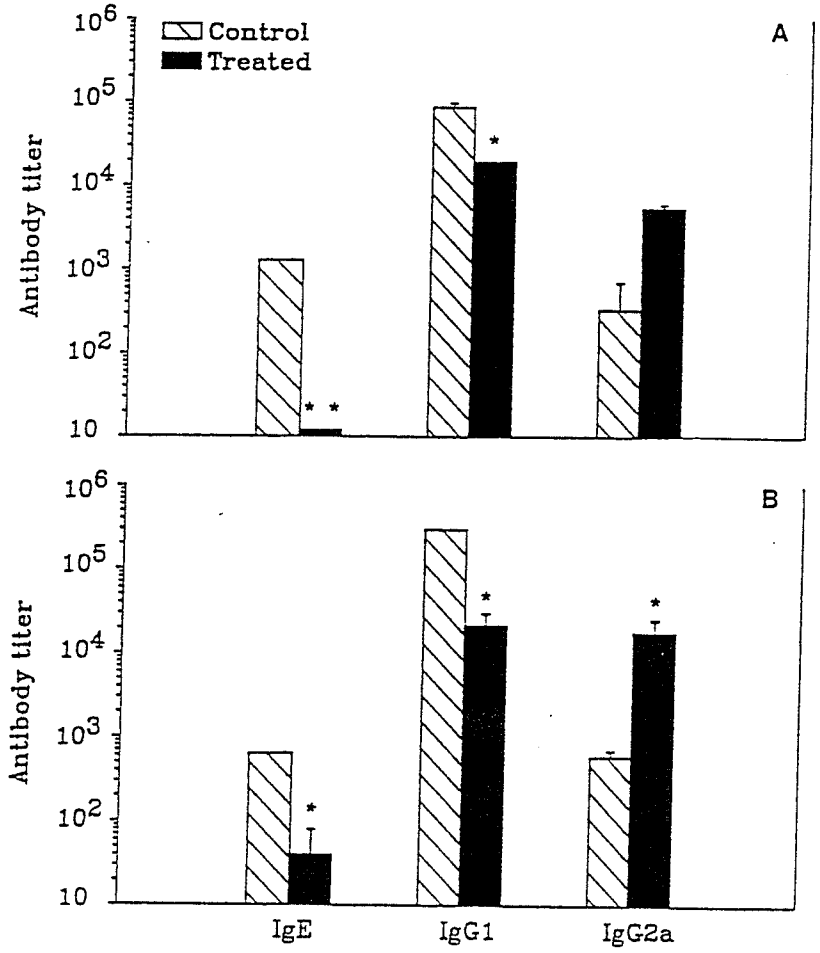

Figure 16. Reciprocal regulation of IgE and IgG2a production with i.v. treatment of rKBG8.3. Mice were injected i.v. with 500 µg per day of rKBG8.3 in PBS continuously for two days, control mice were injected with PBS, 4 mice in each group. All the mice were then immunized (primary) with 10 µg of rKBG8.3 in alum 4 days later (A). Secondary immunization was given around 30 days after primary immunization (B). Antibody titers were determined from individual mouse sera 20 days after primary immunization and 6 days after secondary immunization, and presented as geometric mean ± SE. This experiment was repeated once, similar results were obtained.

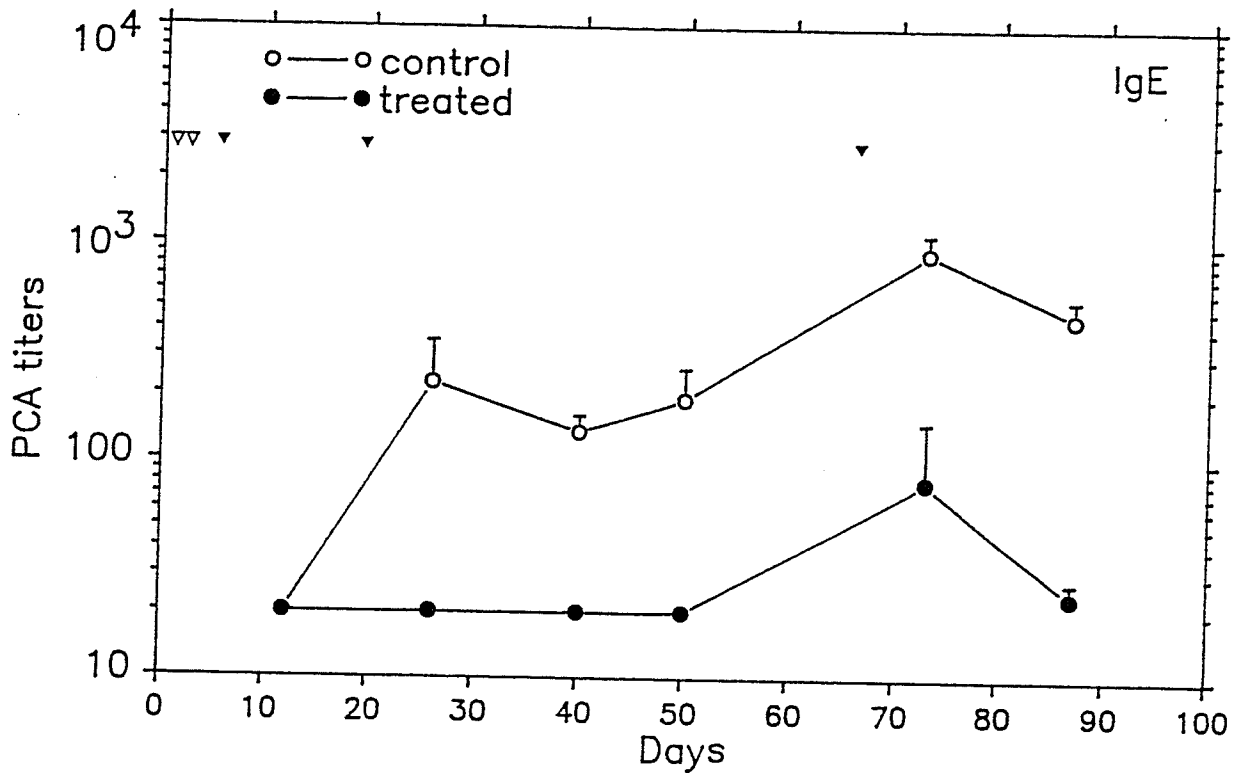

Figure 17. Long-term suppression of specific IgE response in rKBG8.3 treated mice by i.v. route. B6D2F1 mice were injected i.v. with 500 µg of rKBG8.3 on day 1 and day 2 (treated) or with PBS (control), then immunized on day 5, boostered on day 19, 66 with 10 µg of rKBG8.3 in alum, 4 mice in each group. Production of specific IgE antibody was measured from the individual mouse sera collected on day 7 after primary immunization, day 7, 21 and 31 after secondary immunization, day 7 and 21 after tertiary immunization. (▽), time of treatment. (▼) time of immunization. Antibody titers were presented as geometric mean ± SE. This experiment was repeated once, similar results were obtained.

long term suppression of IgE antibody.

1.3. Inhibition of IL-2, IL-4 and IFN-γ Production

It is well known that IFN-γ and IL-4 are critical in IgE and IgG2a regulation. In order to investigate the relationship between isotype specific antibody and cytokine production in response to antigen stimulation, cytokine patterns between vaccinated and control mice were compared. Mice were injected with soluble rKBG8.3 (vaccinated) or PBS (control) i.v. prior to immunization with rKBG8.3 in alum. Then the cells from individual mouse spleen and pooled mesenteric lymph nodes were cultured *in vitro* alone or with rKBG8.3. Production of IL-2, IL-4 and IFN-γ was determined by sandwich ELISA from 24 h culture supernatants. All the cytokines tested were reduced in the cells, from the vaccinated mice on day 6 after primary immunization, restimulated with rAL (**Figure 18**; $p<0.05$). Production of IL-2 reduced markedly in culture supernatants of spleen cells (22 fold) and of lymph node cells (172 fold). Production of IL-4 was undetectable (<4 pg/ml). Whereas production of IFN-γ reduced 7 fold in spleen and 4 fold in lymph node cell culture. In secondary response, production of IL-2 and IFN-γ was slightly reduced, whereas IL-4 was not reduced in treated mice (**Figure 19**; $p>0.05$).

1.4. Increase of IFN-γ:IL-4 Ratio

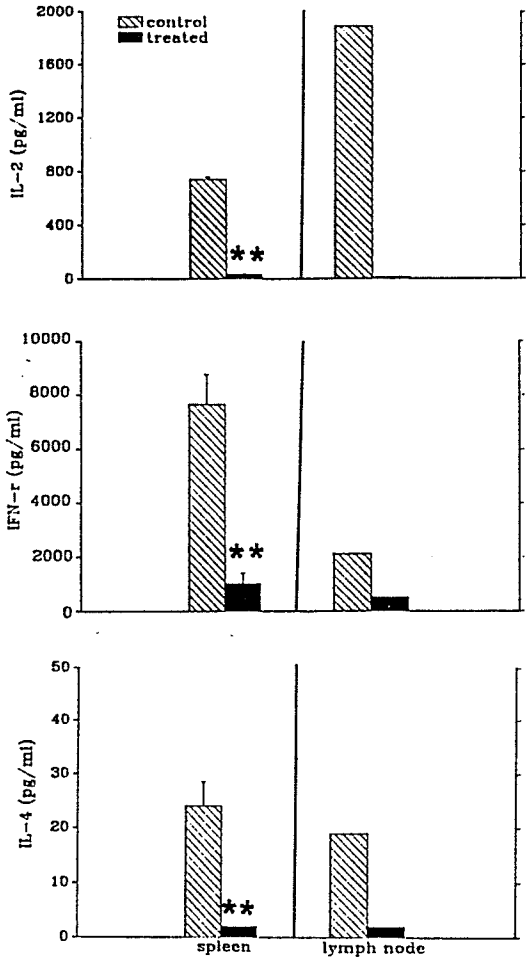

Figure 18. rKBG8.3 treatment (i.v.) prior to immunization decreases production of Th1- (IL-2 and IFN-γ) and Th2-like (IL-4) cytokines. B6D2F1 mice were injected with 500 μg of rKBG8.3 or PBS on day 1 and 2, and immunized (i.p.) with 10 μg of rKBG8.3 in alum 4 days later, 4 mice in each group. All the mice were sacrificed 5 days after immunization for spleen and lymph node cell culture. Cytokine synthesis was measured from the supernatants of individual spleen and pooled lymph node cells after 24 h culture in the presence of 200 μg/ml of rKBG8.3. Cytokine production in individual spleen cell culture was presented as arithmetic mean ± SE. This experiment was repeated once, similar results were obtained.

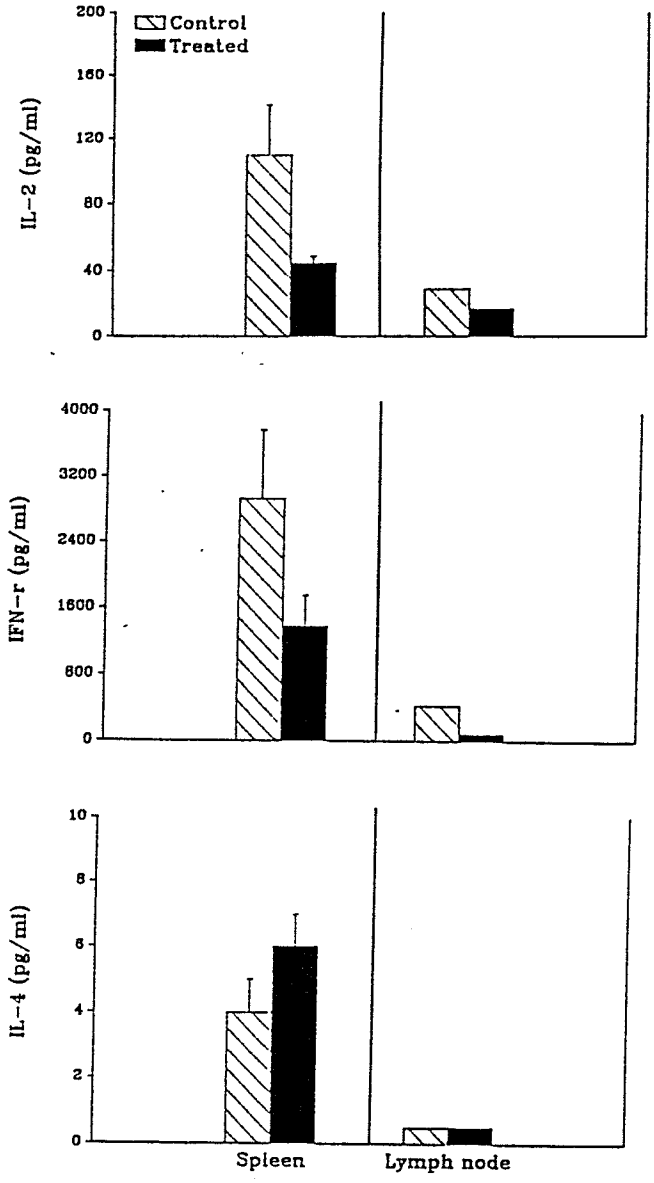

Figure 19. Inhibition of cytokine synthesis in secondary response. The treatment and in vitro culture were same as in Figure 18 except that cytokine synthesis was measured from the mice 5 days after secondary immunization (~ 30 days interval between primary and secondary immunization). Cytokine production was presented as arithmetic mean ± SE in individual spleen cell culture.

Interestingly, although synthesis of IL-2, IFN-γ and IL-4 was reduced, comparison of IFN-γ:IL-4 ratios revealed that vaccination with rAL increased the ratio in rAL treated mice as opposed to control mice in spleen cell cultures. Similar patterns appeared in lymph node cell cultures. However, the IL-2:IL-4 ratio was decreased in the treated mice. In **Table 4**, results from two repeated experiments were summarized.

Taken together, these results suggest that treatment with rAL may induce a state of antigen-specific T cell anergy as shown by the inability of the cells to specifically produce cytokines particularly IL-2, subsequently result in a shift from IL-4- to IFN-γ-dominant response as seen by increased ratio of IFN-γ:IL-4. These alterations in cytokine synthesis patterns may in turn suppress IgE and enhance IgG2a production in vaccinated mice.

Table 4. The ratio of IFN-γ:IL-4 and IL-2:IL-4 in i.v. treated mice

		IFN-γ:IL-4		IL-2:IL-4	
		SP[a]	LN[b]	SP	LN
Expt. 1	Control	318.5	111.8	30.88	99.42
	Treated	512.5	259	16.5	5.5
Expt. 2	Control	777.3	609.5	46.3	406
	Treated	2020	910	16	9

[a] spleen
[b] lymph node

2. Modulation of Antibody and Cytokine Responses by s.c. Treatment

Studies in the preceding section demonstrated that i.v. vaccination with rAL induced a shift in cytokine response from default Th2-like to Th1-like. Furthermore, it also induced a long term suppression of IgE production. One major drawback of the vaccination by i.v. is that it is not practicable in human because of its potential to induce rapid systemic reactions and possibly anaphylactic responses. Current immunization in human involves s.c. injection. We therefore vaccinated the mice with rAL by s.c. route, then examined antibody and cytokine responses to rAL.

2.1. Treatment with Particulate versus Soluble Form of FP

According to the biology of immune response, different forms of antigen may target different APCs which in turn could present antigen to different subsets of T cells and thus influence the pattern of immune response. To compare the effect of insoluble and soluble forms of recombinant fusion protein on modulation of IgE antibody production, we treated the mice with different doses of BFP and FP in HPBS by s.c. route before immunization with rKBG8.3 in alum, control mice were injected with glutathione agarose beads saturated in HPBS (**Table 5**). Antigen-specific IgE Ab was measured by PCA. Kinetic study of IgE Ab production in the mice treated with BFP and FP demonstrated that BFP and FP treatment strongly inhibited IgE production in primary response ($p \leq 0.001$).

Furthermore, these treatment inhibited IgE response significantly in secondary ($p \leq 0.001$) and slightly in tertiary responses (**Figure 20**). The inhibition of IgE Ab production revealed dose-dependent pattern, treatment with BFP revealed stronger inhibition of IgE production than with FP in most of the doses used (**Figure 21**).

To evaluate the effect of BFP and FP treatment on IgG1 and IgG2a Ab production, antigen-specific IgG1 and IgG2a Abs were determined by ELISA. Production of IgG1 Ab in different doses of treatment with BFP and FP exhibited slight changes compared to control mice. However, IgG2a Ab revealed a pattern opposed to IgE production ($p<0.001$; **Figure 22**).

Since GST existed as a moiety in BFP and FP, to evaluate its effect on immune responses, we measured anti-GST Abs in these mice treated with BFP and FP. Anti-GST IgE Ab could not be detected in all the mice, low level of IgG1 Ab production could be measured in BFP treated mice, IgG2a Ab production also in low level was measured in FP treated mice (**Figure 23**).

These results demonstrated that both particulate and soluble forms of GST-rKBG FP could inhibit specific IgE production and enhance IgG2a production although to different extent.

2.2. Dose Effect and Antigen Specificity

In the preceding studies, GST in FP and BFP was antigenic and also induced antibody production although to a low level. In order to eliminate the possible interference of GST, the potential of

Table 5. Protocol of *in vivo* treatment by s.c. route

Group of Mice	Treatment	Dose
1	Beads	-
2	BFP	500 μg x 3
3	BFP	300 μg x 3
4	BFP	100 μg x 3
5	FP	500 μg x 3
6	FP	300 μg x 3
7	FP	100 μg x 3

cleaved rKBG8.3 alone in modulating specific immune responses to rAL was examined.

To carry out the vaccination, mice were injected s.c. with cleaved rKBG8.3 prior to immunization with the same rAL plus alum. Individual mouse sera were collected on day 20 after first immunization for antibody assay. As shown in **Figure 24A**, the effects of rAL treatment were isotype selective. Production of specific IgE and IgG2a antibodies were reciprocally altered by cleaved rAL treatment via s.c. route. Production of specific IgE antibody was markedly suppressed (>99%, $p<0.001$). Production of IgG1 antibody was also suppressed (62%, $p>0.05$). In contrast, specific IgG2a antibody synthesis was markedly elevated

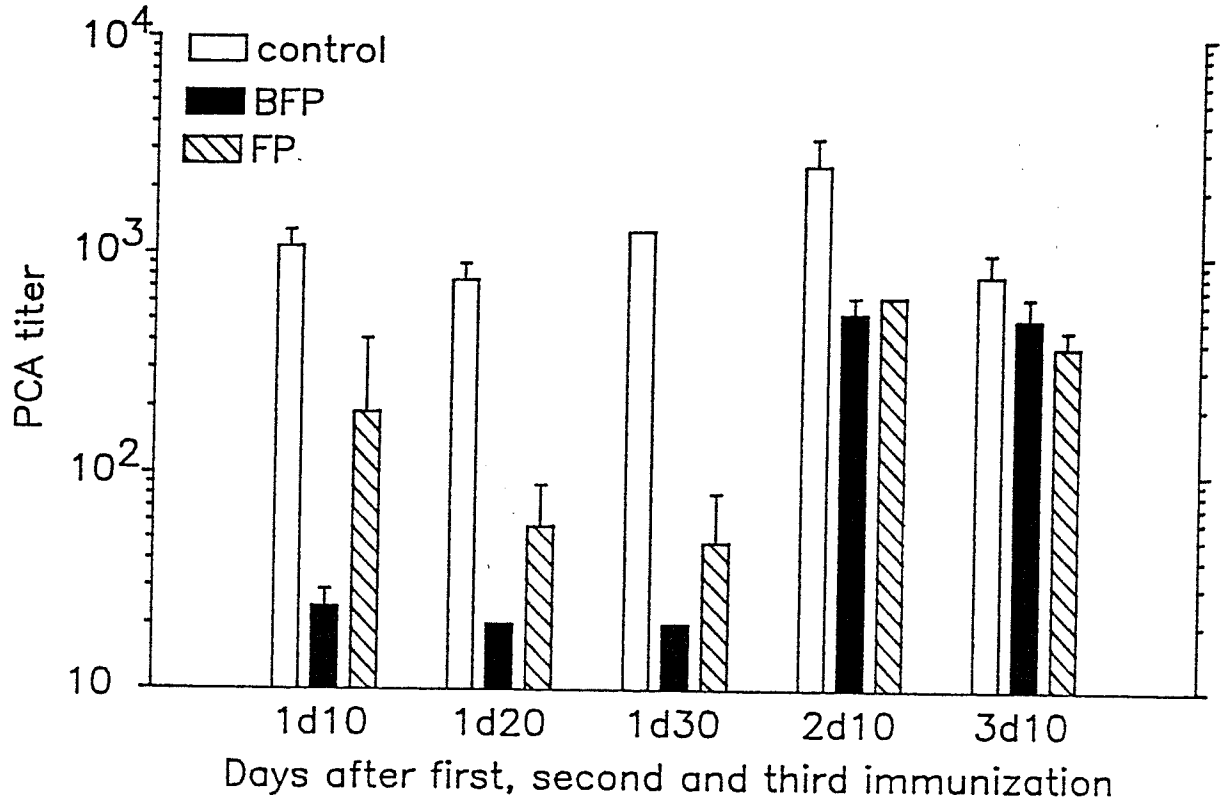

Figure 20. Inhibition of specific IgE production with BFP and FP treatment. Mice were injected with 500 μg per day each of BFP or FP s.c. for 3 days, control mice injected with the beads in HPBS, 4 mice in each group. All the mice were immunized with 2 μg of rKBG8.3 in alum 5 days later (primary immunization). Secondary and tertiary immunizations were given at an interval of 30 days. KBG extract-specific IgE antibody titers were determined by PCA on day 10, 20, 30 after primary immunization (1d), on day 10 after secondary immunization (2d), and on day 10 after tertiary immunization (3d). IgE titers were presented as geometric mean ± SE.

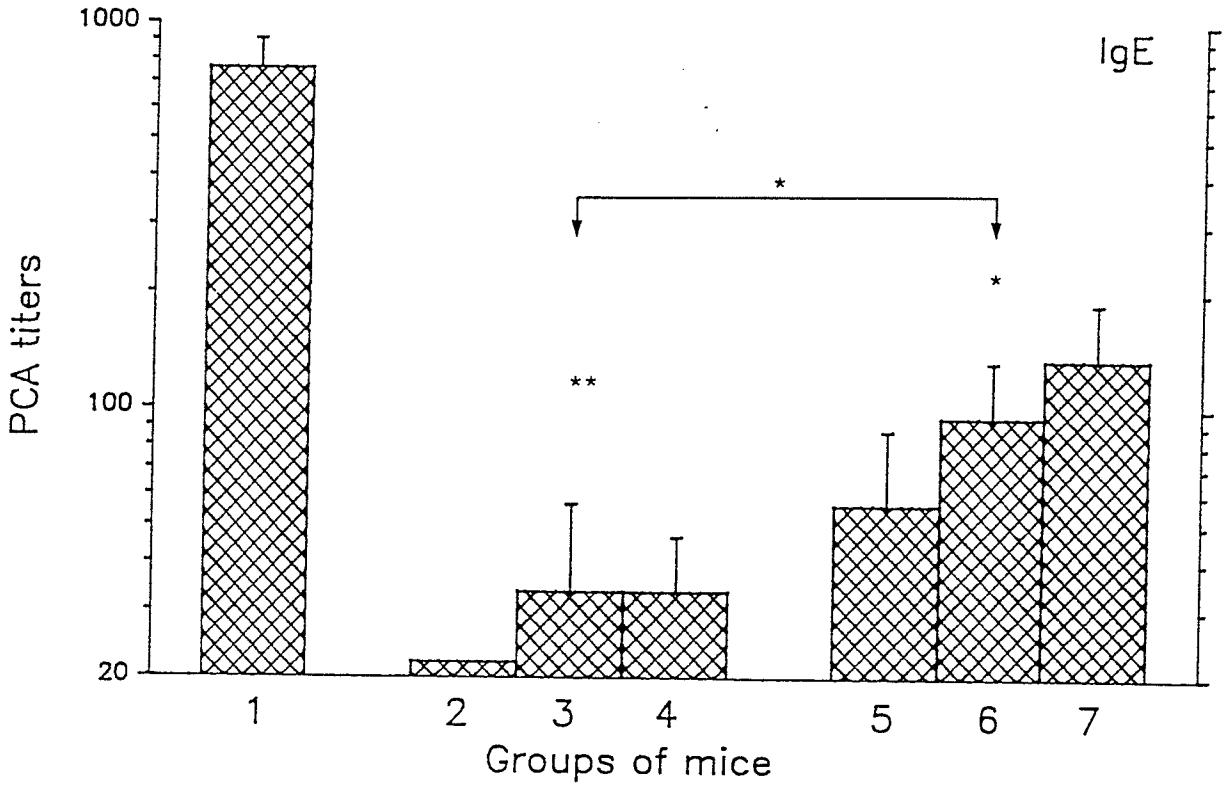

Figure 21. Dose dependent inhibition of specific IgE production with BFP and FP treatment. Mice were injected s.c. with 500, 300, 100 µg per day each of BFP or FP for three days before immunization with rKBG8.3 in alum (i.p.), 4 mice in each group. Fourteen days after immunization, KBG extract-specific IgE antibody titers were measured by PCA and presented as geometric mean ± SE.

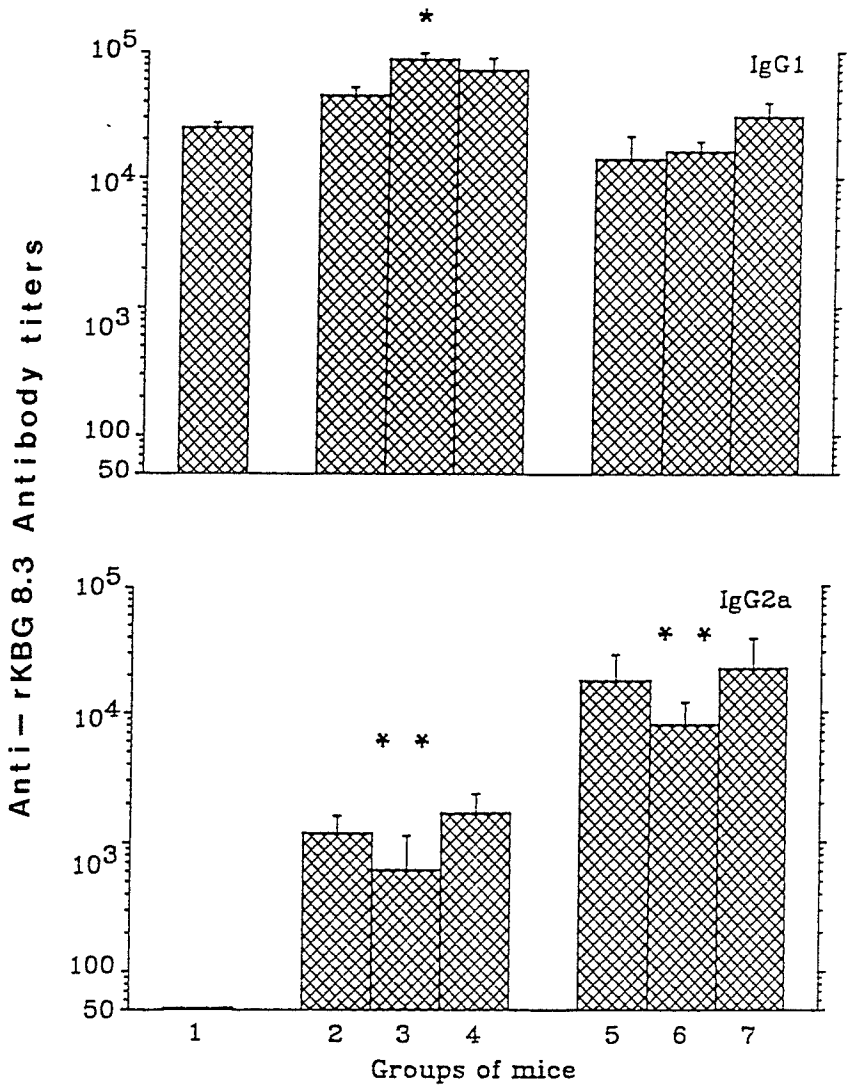

Figure 22. rKBG8.3-specific IgG1 and IgG2a production in the mice with BFP and FP treatment. Antibody titers were measured from the mice treated and immunized same as described in Figure 21.

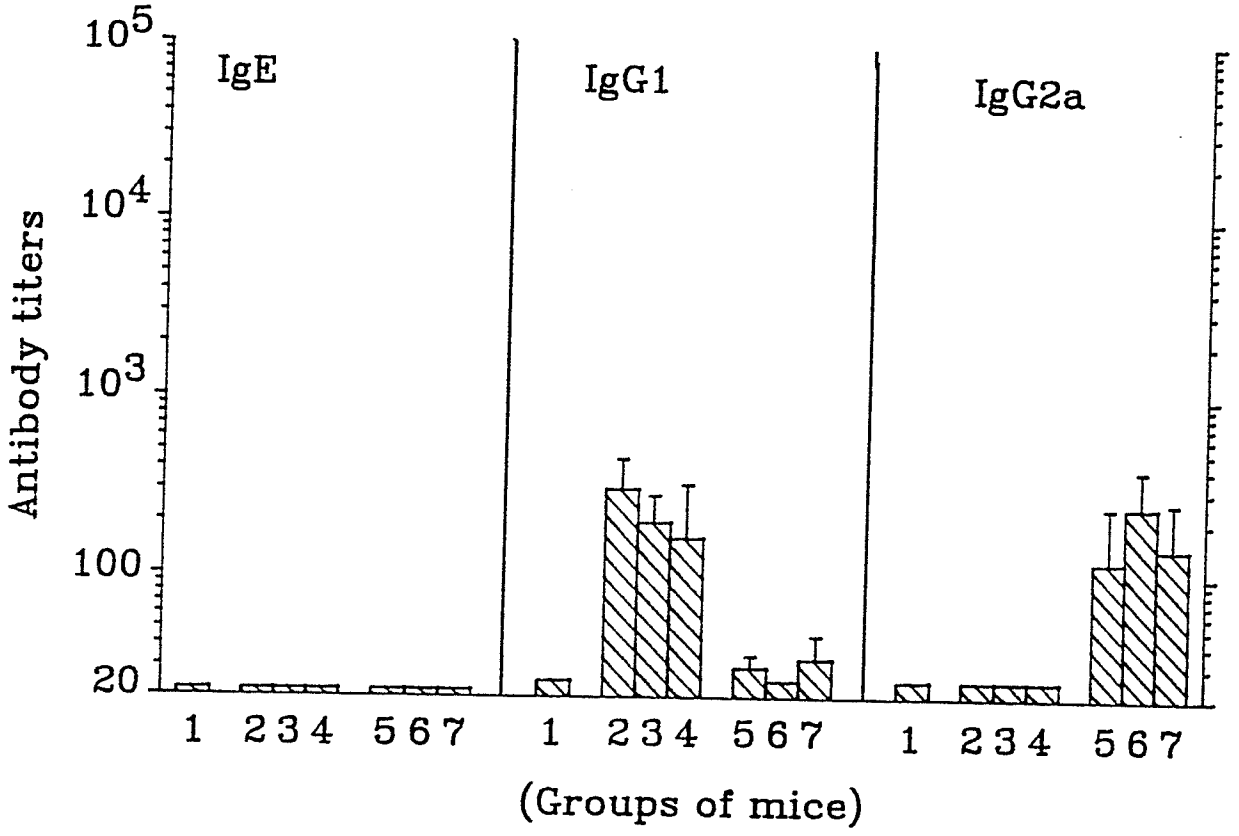

Figure 23. Anti-GST antibody production in the mice treated with BFP and FP (as described in Figure 21).

(97%, $p<0.001$) following treatment. Secondary IgE, IgG1 and IgG2a antibody responses showed a similar pattern ($p<0.05$; **Figure 24B**) to those of primary response in treated mice. These results demonstrated that vaccination by s.c. route induced suppression of IgE response and enhancement of IgG2a response.

The suppression of IgE Ab production and the enhancement of IgG2a Ab production were dependent on the doses of treatment (**Figure 25**). No apparent change was seen in IgG1 Ab production at different doses of rAL used for vaccination. These results demonstrated that antigen dose played a very important role in shifting the profiles of antibody isotypes.

Treatment with rAL followed by immunization with OVA did not affect OVA-specific IgE, IgG1 and IgG2a Ab production (**Figure 26**). These results indicated that reciprocal modulation of IgE and IgG2a responses by rAL treatment was antigen-specific.

2.3. Inhibition of IL-2, IL-4 and IFN-γ Protein Synthesis

To examine the effect of vaccination on T cell response, cytokine production of IL-2, IFN-γ and IL-4 was measured by ELISA *ex vivo* following treatment and immunization. Mice were administered rKBG8.3 in PBS by s.c. route 6 days prior to immunization. The mice were sacrificed 5 days *post* immunization, after which time short-term cultures of spleen and lymph node cells were used to evaluate the pattern and the level of cytokine response. Individual spleen cells and pooled lymph node cells were cultured *in vitro* alone or

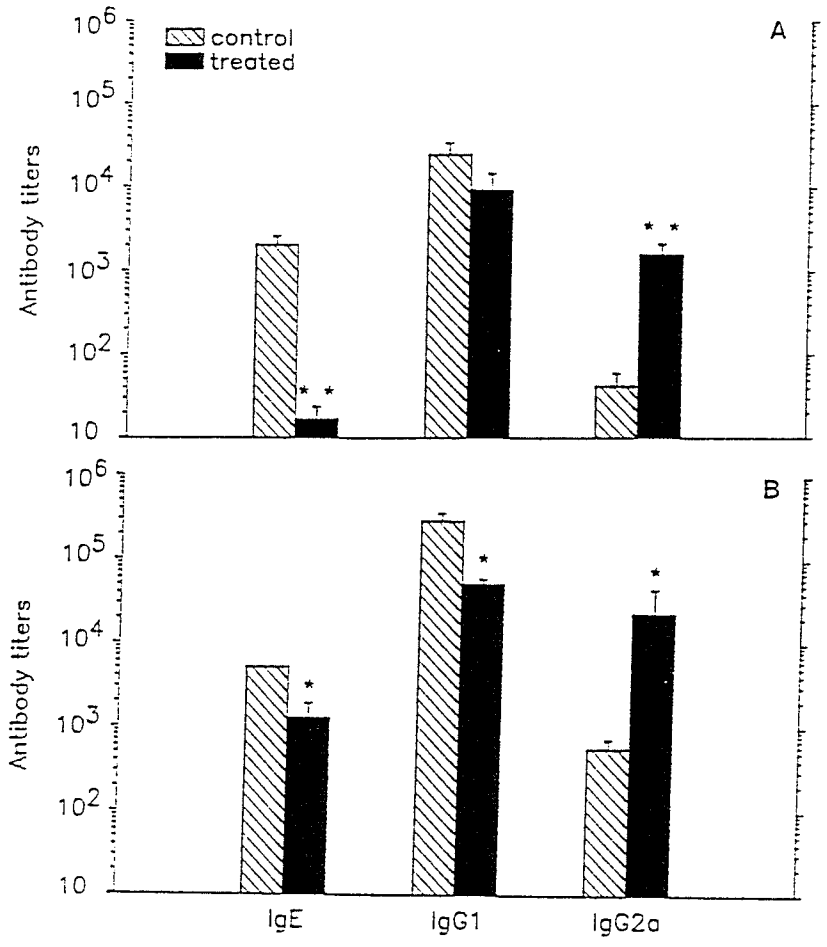

Figure 24. Reciprocal regulation of specific IgE and IgG2a Ab production by rAL treatment. B6D2F1 mice were injected with 250 μg per day of rKBG8.3 s.c. for two days, PBS injection as control, followed by immunization with rKBG8.3 plus alum 6 days later (A), 4 mice in each group. Secondary immunization was given around 40 days after primary immunization (B). On day 20 after primary immunization and on day 6 after secondary immunization, specific antibody titers were measured and expressed as geometric means ± SE.

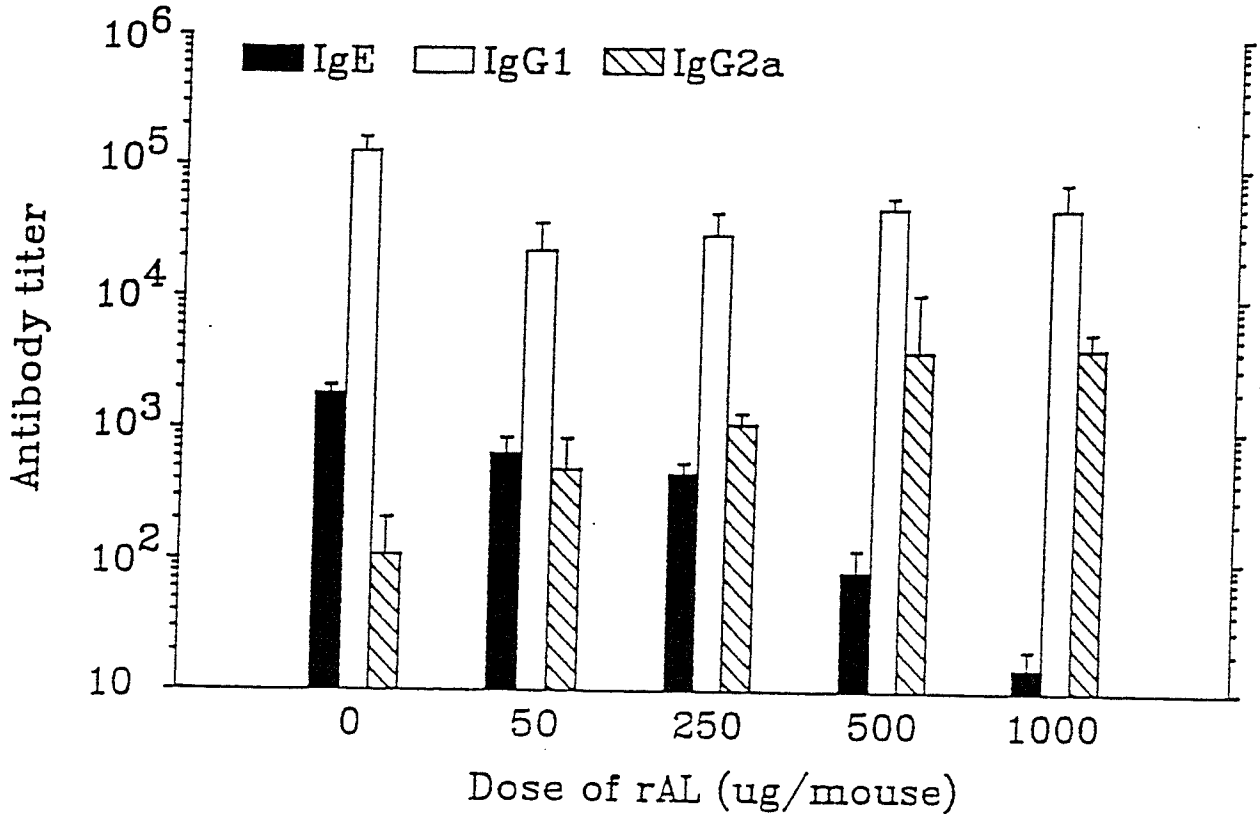

Figure 25. Dose dependent effect on the production of IgE and IgG2a Ab. Treatment was performed by s.c. injection with different doses of rKBG8.3, from 50 μg, 300 μg, 500 μg up to 1000 μg into each mouse, 4 mice in each group. Six days after treatment, the mice were immunized with 2 μg of rAL in alum. Specific antibody production was analysed from the mouse sera 22 days after immunization. Antibody titers were presented as geometric mean ± SE.

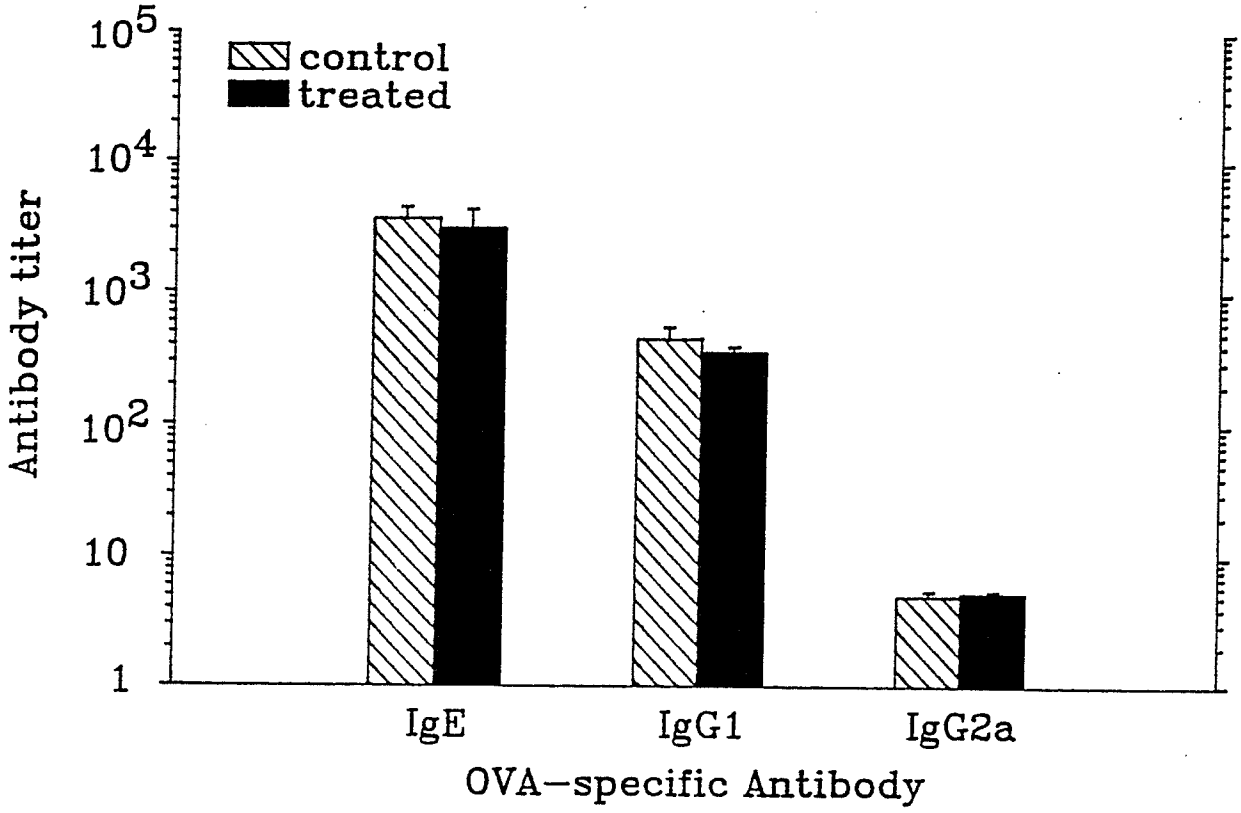

Figure 26. Antigenic specificity of rKBG8.3 treatment on antibody responses to unrelated antigen. Mice were injected (s.c.) with 250 µg of rKBG8.3 in PBS on day 1 and 2, control mice were injected with PBS, 4 mice in each group. The mice were then immunized with 2 µg of OVA in alum on day 7. Anti-OVA IgE, IgG1 and IgG2a antibodies were measured 14 days after OVA immunization. Antibody titers were presented as geometric mean ± SE.

with 200 μg/ml of rKBG8.3. In the absence of rKBG8.3 treatment in control mice, immunization with the rAL in alum elicited strong cytokine responses. In marked contrast, cultures established from the rAL treated mice under the same conditions exhibited marked reduction of IL-2 and IL-4 synthesis ($p<0.05$), production of IFN-γ was also reduced, but to a lesser degree ($p>0.05$; **Figure 27**). A similar cytokine secretion pattern was found in cultures of both spleen cells and lymph node cells from the mice treated with rAL.

Comparison of the ratio of IFN-γ:IL-4 in control and treated mice showed that mice treated with different doses (250 μg x 2, 500 μg x 2) resulted in an increase of the ratio. Furthermore, the ratio of IFN-γ:IL-4 was elevated in the mice treated with 250 μg of rAL more than those treated with 500 μg of rAL. Contrary to IFN-γ:IL-4 ratio, IL-2:IL-4 ratio decreased in the s.c. treated mice compared to the control (**Table 6**).

Taken together, these results lead to the inference that treatment with rAL results in deviation of cytokine response from IL-4- to IFN-γ-dominant.

2.4. Inhibition of Cytokine mRNA Expression by s.c. Treatment

To determine the effect of *in vivo* treatment on antigen-specific Th1- and Th2-like responses more directly, cytokine gene expression of IL-2, IFN-γ, and IL-4 in cultured spleen and lymph node cells from rKBG8.3 treated and untreated mice was examined by RT-PCR at the level of cytokine mRNA transcription. RT-PCR analysis for

Table 6. The ratio of IFN-γ:IL-4 and IL-2:IL-4 protein synthesis in s.c. treated mice

		IFN-γ:IL-4		IL-2:IL-4	
		SP[a]	LN[b]	SP	LN
Expt. 1	Control	114.3	80.9	57.96	77.3
	Treated (250μg)	1307	1029.5	11	6.5
Expt. 2	Control	119.7	960.6	27.35	50.5
	Treated (500μg)	667.5	1198.5	14	6

[a] spleen
[b] lymph node

cytokine messengers was performed with total RNA isolated from recovered cells cultured *in vitro* in the absence and presence of rKBG8.3 restimulation. As shown in **Figure 28**, mRNA expression of IL-2, IFN-γ, and IL-4 was induced in spleen and lymph node cells from control mice in response to *in vitro* restimulation with rAL. However, in spleen and lymph node cells from rAL treated mice, mRNA expression of IL-2, IFN-γ, and IL-4 was decreased, even totally abrogated in some mice. In this experiment the expression of cytokine mRNA was quantitated by comparison of relative OD of cytokine mRNA PCR product versus β-actin between treated and

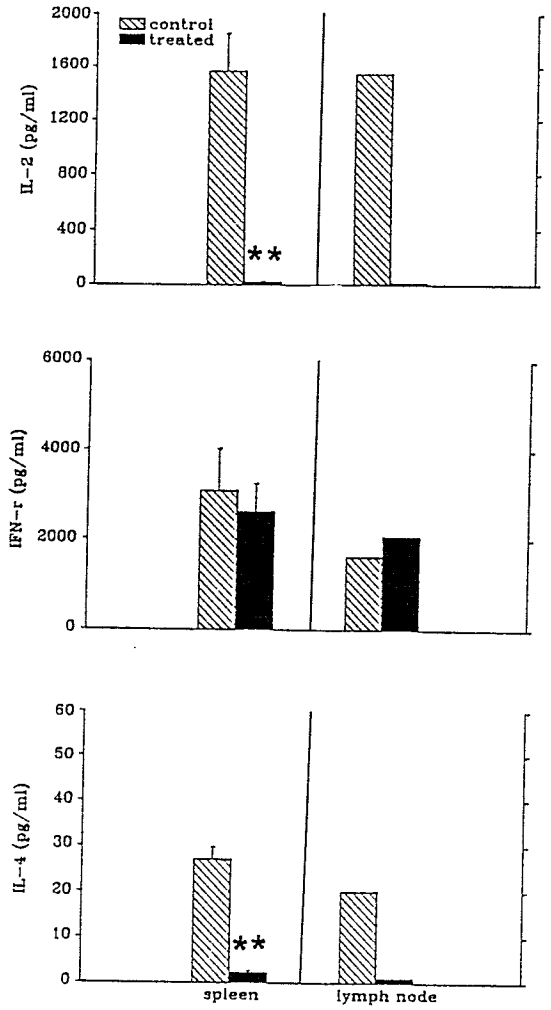

Figure 27. rAL treatment by s.c. route inhibits cytokine response to the following immunization. B6D2F1 mice were injected (s.c.) 250 µg per day of rKBG8.3 for two days, control mice were injected with PBS, 4 mice in each group. The mice were immunized with 2 µg of rKBG8.3 in alum on day 7 after treatment, sacrificed on day 5 after immunization for spleen and lymph node cell culture. Individual spleen cells and pooled lymph node cells were cultured in the presence of 200 µg/ml of rKBG8.3 for 24 h. Cytokine synthesis was analysed from the cultured supernatants and presented as arithmetic mean ± SE. This experiment was repeated once, similar results were reproduced.

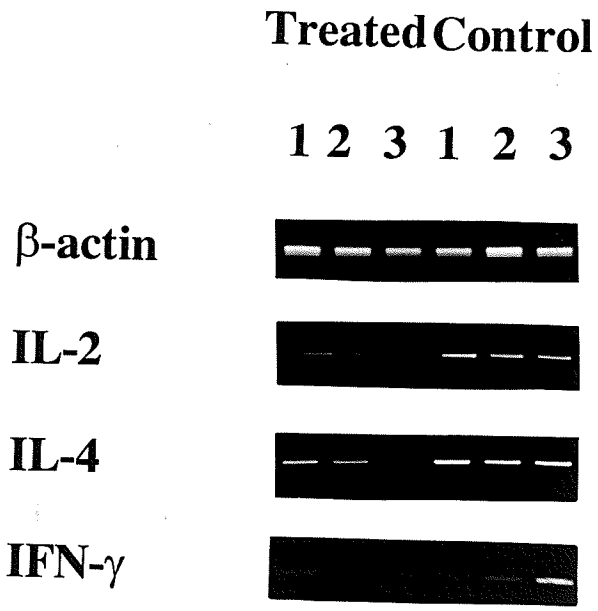

Figure 28. rAL pretreatment inhibits cytokine mRNA expression. The treatment, immunization, and cell culture were same as described in Figure 27. Total RNA was isolated from 24 h cultured individual spleen cells with rAL restimulation for RT-PCR assay. Treated, mice were treated with rAL and then immunized. Control, mice were injected with PBS and then immunized. 1, 2, 3, individual mouse.

control mice. These data indicated that specific antigen-driven cytokine mRNA expression was directly downregulated. Moreover, the ratio of IFN-γ:IL-4 mRNA expression was also elevated in spleen cells, as well as in lymph node cells (**Table 7**).

Table 7. rKBG8.3 treatment by s.c. (250 μg x 2) route prior to immunization inhibits Th1- and Th2-like cytokine mRNA expression

Gene	Control[c] (SP)[e]	Control (LN)[f]	Treated[d] (SP)	Treated (LN)
β-actin OD[a]	82.46	111.14	85.96	127.12
IL-2 ROD[b]	45.31	40.27	21.51	33.46
IFN-γ ROD	31.19	44.58	27.80	41.11
IL-4 ROD	64.90	52.71	29.02	31.41
IFN-γ/ IL-4	0.48	0.85	0.96	1.31

Mice were treated and immunized, spleen cells were cultured as described in Figure 27. Total RNA was extracted from the cells cultured for 24 h, reverse transcribed and amplified by PCR for 35 cycles. PCR products were analysed by electrophoresis on agarose gel containing 0.5 μg/ml ethidium bromide and further scanned by desitometry. Cytokine gene expression was normalised to OD values of β-actin PCR product.

[a] actual OD value x area.
[b] OD value of cytokine mRNA PCR products / OD value of β-actin mRNA PCR products.
[c] mice were injected with PBS and then immunized with rKBG8.3.
[d] mice were treated with rKBG8.3 and then immunized with rKBG8.3.
[e] individual spleen cells were cultured in the presence of rKBG8.3.
[f] pooled lymph node cells were cultured in the presence of rKBG8.3.

III. THERAPEUTIC MODULATION OF ANTIBODY AND CYTOKINE RESPONSES

1. Modulation of Established Immune Responses by rAL

The capacity to abrogate previously established immune responses is more difficult, but better reflects clinical situation in which an ongoing antigen-specific immune response is involved in a disease state (Hayglass, 1992; Briner et al., 1993). Therefore we examined whether *in vivo* treatment with rAL could induce immune deviation in antigen-primed animals. B6D2F1 mice were primed with 2 μg of rKBG8.3 in alum (i.p.). Around 148-170 days after priming, the mice were treated with 250 μg or 500 μg per day of rKBG8.3 in PBS for two days (s.c.). Five days after treatment these mice were challenged with 0.2 μg of rKBG8.3 in alum (i.p.). On day 6 after challenge, antibody and cytokine responses were analysed. Following administration of rKBG8.3, specific IgE response remained unchanged. The production of IgG1 and IgG2a isotypes increased in treated mice ($P<0.05$; **Figure 29**).

In cytokine assay, IL-2 production was inhibited in two repeated experiments, while IFN-γ synthesis was slightly inhibited in one experiment, elevated in the other experiment, but IL-4 synthesis was only decreased in one of the two repeated experiments (**Table 8**). However these changes were not as prominent as in naive mice pretreated *in vivo*.

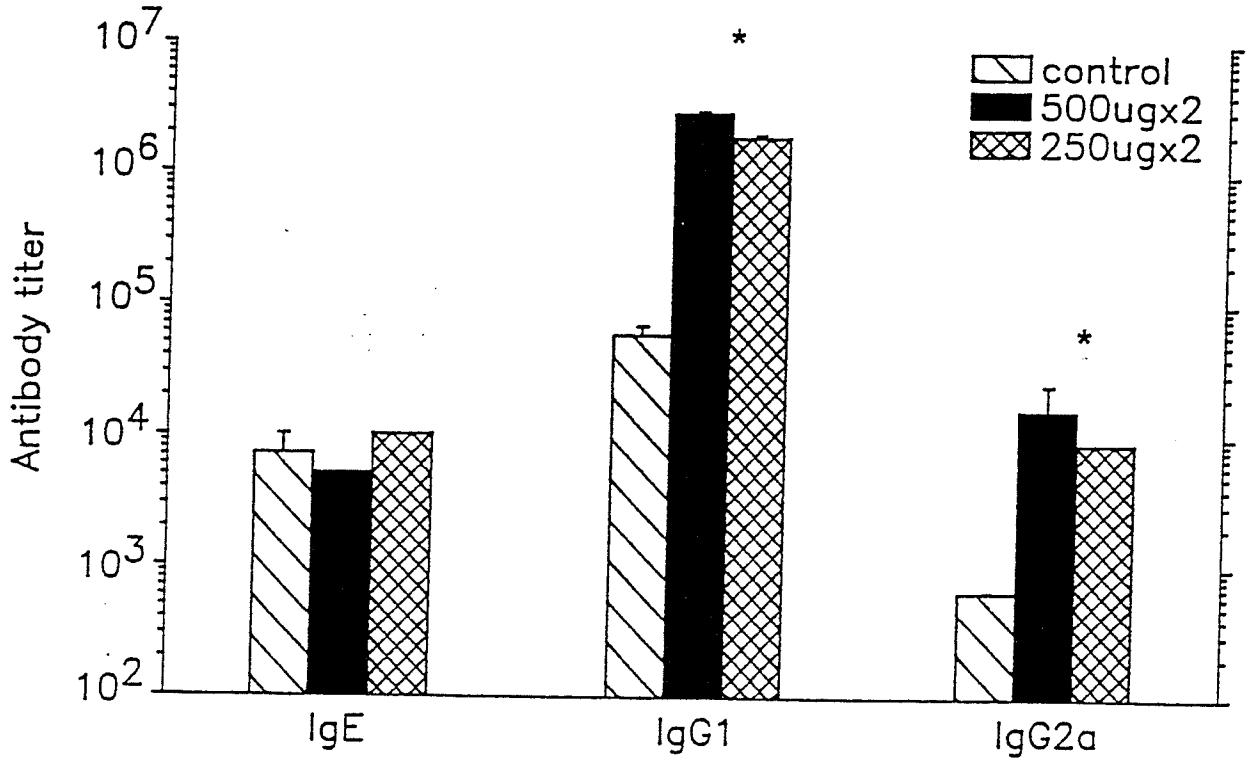

Figure 29. Modulation of antibody responses by rAL in primed mice. B6D2F1 mice were primed (i.p.) with 2 μg of rKBG8.3 in alum. Around 148 days after priming, these mice were treated (s.c.) with 500 μg or 250 μg per day of rKBG8.3 for two days, control mice were injected with PBS. Both of the treated and control mice were challenged (i.p.) with 0.2 μg of rKBG8.3 in alum. Five days later, specific antibody responses were analysed and presented as geometric mean ± SE.

Table 8. Effect of rAL treatment on cytokine synthesis in primed mice

Concentration (pg/ml)		IL-2		IFN-γ		IL-4	
		SP[a]	LN[b]	SP	LN	SP	LN
Expt. 1	Control	540	985	4400	9673	15	15
	Treated	218	91	3036	5421	11	3
Expt. 2	Control	106	79	2564	2642	9.5	2
	Treated	74	39	4348	3628	9	3

B6D2F1 mice were primed, treated and challenged as described in Figure 29, on the same day as the sera collected, individual spleen and pooled lymph node cells were cultured for cytokine assay.

[a] **spleen**

[b] **lymph node**

2. Modulation of kAL-Specific Immune Response by rAL

To examine whether recombinant allergen could modulate Ab responses to crude extract, we immunized mice with KBG extract in alum following rKBG8.3 treatment by s.c. and i.v. routes. We found that production of IgG2a and IgG1 Ab was dramatically elevated ($p < 0.001$), while antigen-specific IgE, total serum IgE (data not shown) did not change significantly ($p > 0.05$; **Figure 30**) compared to

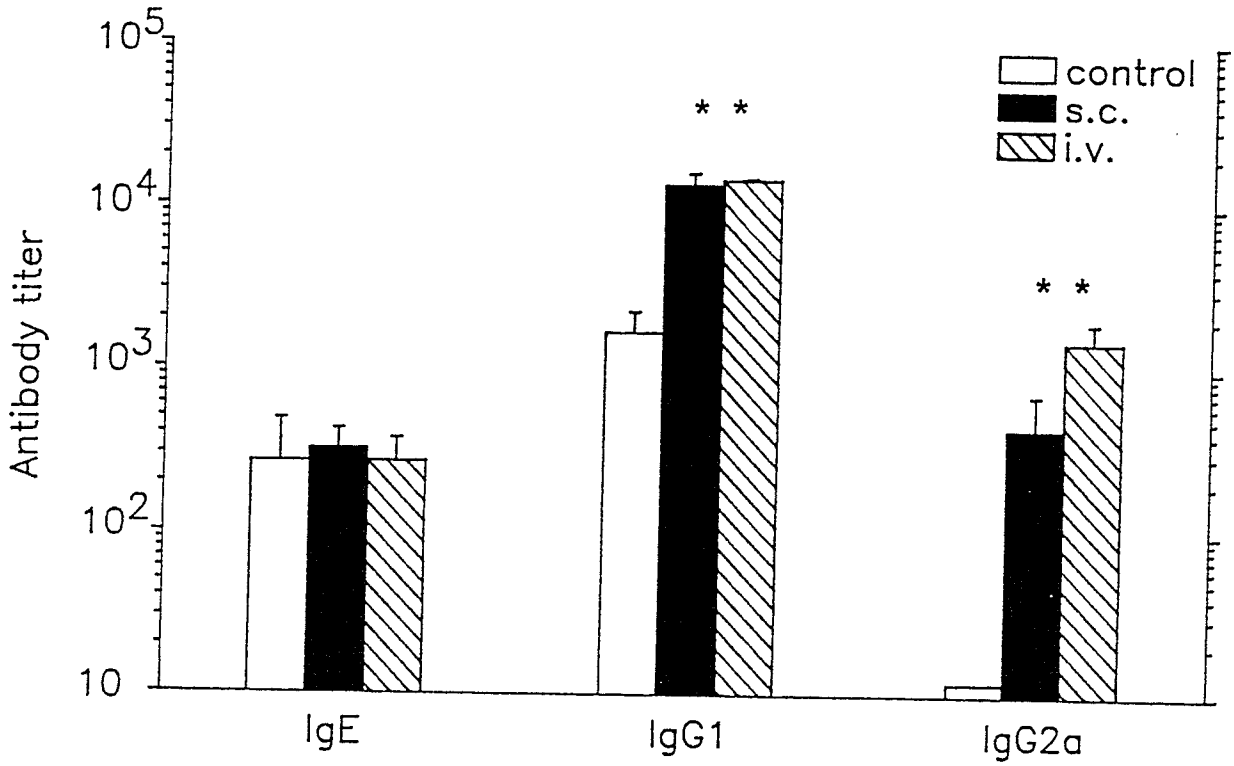

Figure 30. Enhancement of IgG2a and IgG1 Ab production to crude extract by rAL treatment. B6D2F1 mice were injected with 250 µg per day of rKBG8.3 for 2 days (s.c.), or 500 µg per day for 2 days (i.v.), or PBS, before immunization with 10 µg of KBG extract in alum. Anti-KBG extract antibodies were measured 14 days after immunization. Antibody titers were presented as geometric mean ± SE.

control mice.

IV. SOLUBLE rAL INDUCES HYPORESPONSIVENESS AND SKEWS IMMUNE RESPONSE

Preceding studies indicated that vaccination with rAL followed by immunization resulted in the increased IFN-γ:IL-4 ratio, however, the initial events which led to this condition remained unknown. To address this question, we set two parallel groups of mice, one immunized s.c. with 2 μg of rKBG8.3 in alum, the other one administered s.c. with 500 μg of rKBG8.3 in PBS, on the first day.

To analyse cytokine production, the mice were sacrificed on day 6. As shown in **Figure 31**, bulk culture of individual spleen cells from the mice immunized with rAL in alum exhibited strong cytokine response, whereas, spleen cells from the mice administered with rAL itself stimulated IFN-γ ($p=0.01$) and IL-4 ($p=0.16$) synthesis, although relatively lower than that from immunized mice. In contrast, IL-2 level was markedly reduced in the cultured spleen cells from treated mice compared to those from immunized mice ($P=0.005$).

To examine T cell proliferation, mice were sacrificed on day 11 and the individual spleens were collected for proliferation assay. Viable cells were recovered after stimulation with 50 μg/ml of rKBG8.3. The responsiveness of these cells to restimulation with rKBG8.3 is shown in **Figure 32**. Proliferation of spleen cells from the mice administered with rAL resulted in a 45% decrease compared

to that from the immunized mice. This state of hyporesponsiveness was reversed by addition of rIL-2 in the spleen cell culture.

Furthermore, antibody responses were examined on day 20. In rAL administered mice, rAL-specific IgE Ab was undetectable (<1:20; $P<0.001$), production of IgG1 Ab mounted certain level but less than that of immunized mice ($P<0.05$). In contrast, production of IgG2a Ab increased in rAL administered mice ($p<0.05$; **Figure 33**).

Therefore, it is conveivable that, once soluble rAL has initially triggered a IFN-γ- or IgG2a-dominant response via induction of hyporesponsiveness or anergy, rAL-specific T-cell repertoire will be further amplified following immunization with the same antigen, and more T cells with the same lymphokine profile will be recruited.

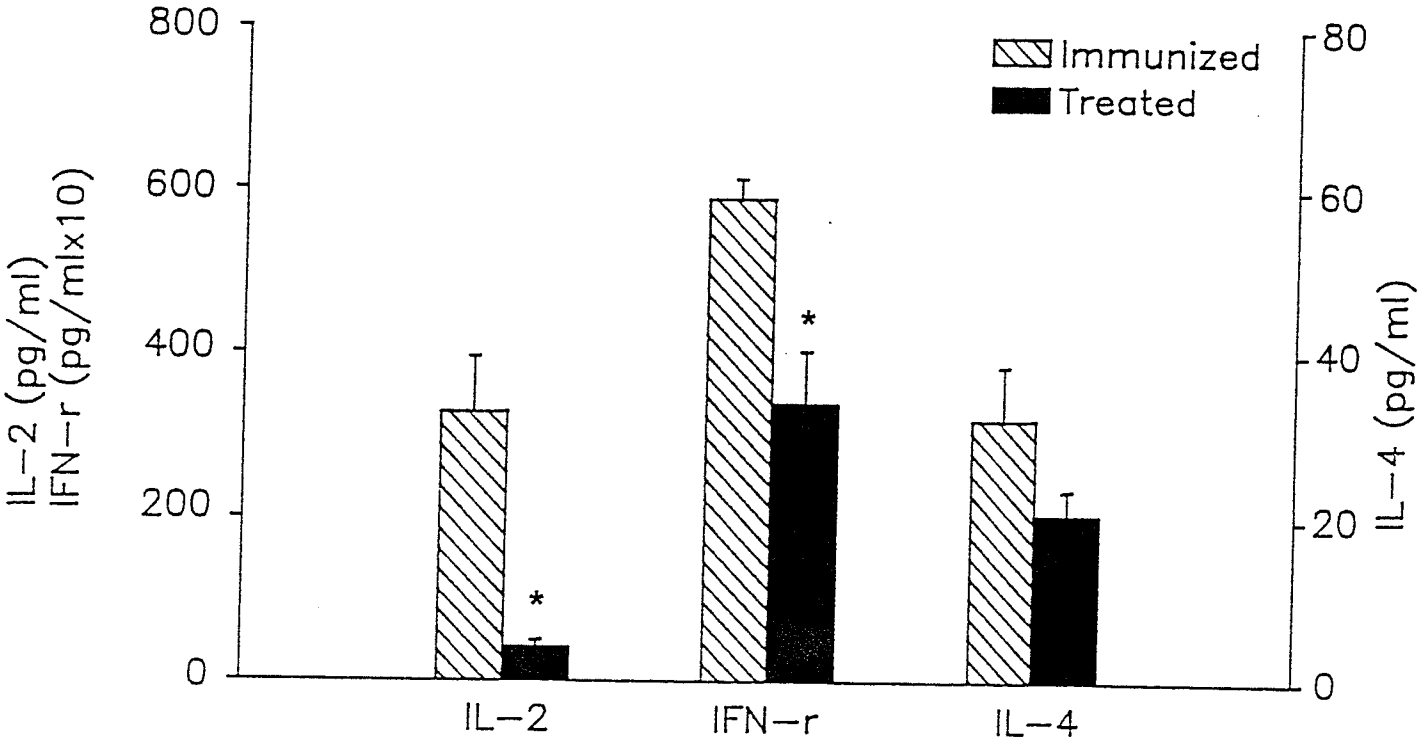

Figure 31. Administration of rAL could stimulate cytokine synthesis. Mice were treated with 500 μg of soluble rKBG8.3 in PBS, or immunized with 2 μg of rKBG8.3 absorbed in alum by s.c. route, 4 mice in each group. Five days later individual spleens were collected for cytokine assay. Cytokine synthesis was measured in the supernatants from spleen cells cultured in the presence of 200 μg of rKBG8.3 for 24 h. Cytokine concentration was presented as arithmetic mean ± SE. This experiment was repeated once, similar results were reproduced.

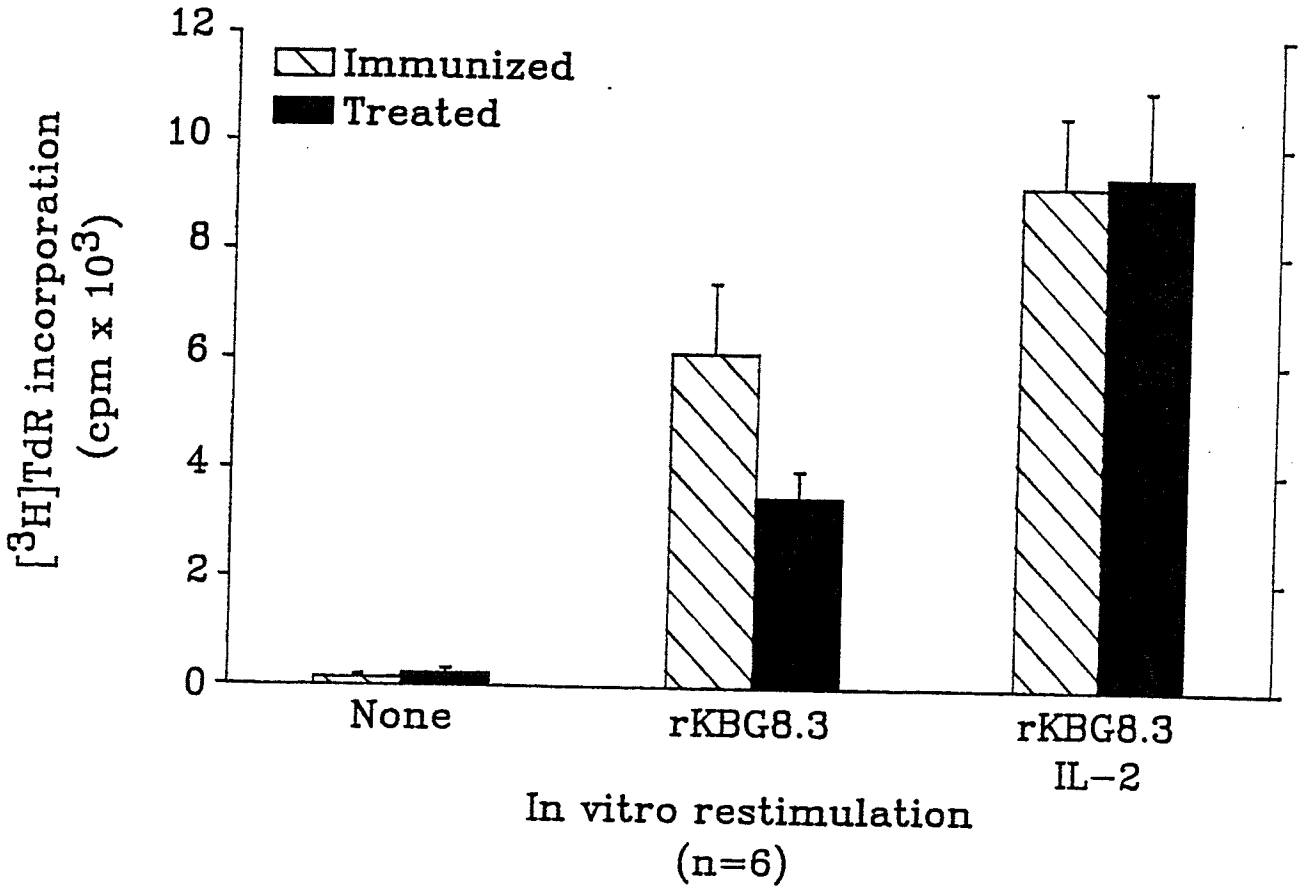

Figure 32. rIL-2 prevented the induction of hyporesponsiveness with rAL. Mice were administered with 500 μg of rAL or immunized with 2 μg of rAL in alum by s.c. route. Eleven days later, these mice were sacrificed for spleen cell culture. Individual spleen cells were cultured in triplicate in the presence of 50 μg/ml of rAL alone, or 50 μg/ml of rAL and 50 U/ml of rmIL-2 for 3 days. Proliferation was correlated with [^{3}H]thymidine incorporation which was expressed as arithmetic mean of cpm ± SE.

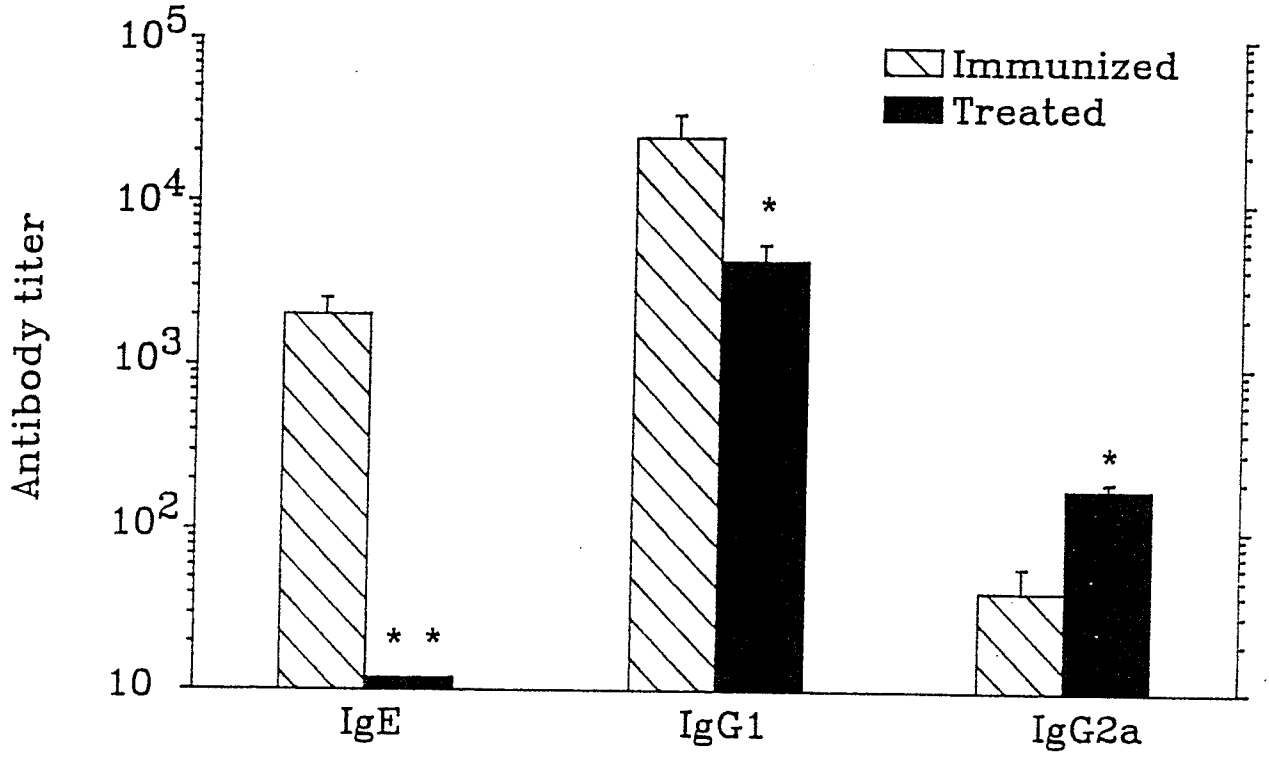

Figure 33. rAL administered in a soluble form increases production of specific IgG1 and IgG2a antibodies. Mice were treated or immunized same as in Figure 31. Specific antibody titers were measured on day 20 after and presented as geometric mean ± SE. This experiment repeated once, similar results were reproduced.

GENERAL DISCUSSION

One of the major challenges in allergy research is the development of therapies that safely induce suppression of allergen-specific IgE synthesis. Currently used allergen immunotherapy involves multiple injections of allergen extracts, which is effective in selected patients. However, the most crude extracts used in immunotherapy are complex mixtures, their compositions significantly vary in the preparations (Platts-Mills and Chapman, 1991), which may lead to unwanted side effects in allergen immunotherapy. It is expected that utilization of recombinant allergen(s) would standardize AIT and minimize the existing uncertainties.

It is known that T cell-derived cytokines play a pivotal role in determining the nature and intensity of immune responses, therefore, much effort has been focused on the mechanisms of cytokine regulation underlying successful allergen immunotherapy. Recent studies have demonstrated that in the successful immunotherapy, production of Th2-type of cytokines, such as IL-4, IL-5 is decreased, or Th1-type of cytokine IFN-γ is increased. (Jutel et al., 1995; Secrist et al., 1993; 1995). The results of these studies suggested that it is possible to modulate cytokine patterns, i.e., switch from Th2- to Th1-like dominant responses in allergic individuals.

Moreover, the studies by Holt and Hattevig's group suggested that early childhood is the period when specific T-cell responses to environmental antigens are initiated, and the ensuing T-cell selection process may take several years to complete (Holt, 1994;

Hattevig et al., 1987; 1993). It was inferred that it may be possible to stimulate the development of Th1 immunity in human by deliberate administration of allergens in childhood.

The present study, carried out with a multiepitopic recombinant grass allergen in a murine model of human allergy, examines (1) the difference between the rAL and kAL in eliciting immune responses, (2) modulation of antibody isotype patterns by vaccination with rAL, (3) mechanism underlying the prophylactic vaccination as evident by the change of cytokine patterns, and (4) therapeutic modulation of an established immune response to rAL and of *de novo* immune response to kAL.

I. IMMUNE RESPONSE TO rAL VERSUS kAL

In the present study, we have used a murine model and characterized *in vivo* immunological responses to rAL versus kAL. kAL induced similar pattern of specific IgE, IgG1 and IgG2a production to kAL and to rAL. Whereas, rKBG8.3 induced similar amount of specific IgE Ab to rAL as well as to kAL. These findings raised the possibility that this rAL may be used for *in vivo* diagnosis of KBG pollen allergy and for modulation of immune responses against kAL.

A major finding from our analysis of cytokine responses to kAL versus rAL, is that kAL and rAL significantly differ in eliciting IFN-γ production with *in vitro* restimulation. There was a complete lack of IFN-γ production in the culture supernatants of spleen cells after restimulation *in vitro* with kAL. This was irrespective

of the antigens used for immunization, whether the mice were immunized with kAL or rAL.

The reason for the differential IFN-γ production by kAL and rAL is not known. It may be due to the difference in dose of antigens used. As the rAL, rKBG8.3 constitutes approximately 20% of the total proteins in KBG extract (Zhang et al., 1992). In order to balance the dosage, mice were immunized with 2 μg rKBG8.3 and 10 μg KBG extract in alum respectively, moreover, the elevated IFN-γ production with rAL was observed in the culture supernatants of spleen cells with *in vitro* restimulation using different doses (4, 20, 200 μg/ml) of kAL and rAL. These results indicated that the elevated IFN-γ production was not due to dose difference of the antigens used for *in vivo* immunization and *in vitro* restimulation.

It is inferred from these results that spleen cells from rAL immunized mice comprise a mixture of Th1- and Th2-like cells which upon *in vitro* stimulation lead to increased IFN-γ production. This is in marked contrast to kAL, which induces predominantly Th2-like cells.

It is to be pointed out that the study of T cell clones (n=37) established from allergic patients specific for another recombinant *Poa p* IX allergen, rKBG7.2 (which constitutes a C-terminal fragment of rKBG8.3) showed that a majority (n=17) of these T cell clones produced large amounts of IL-4 and IL-5 but not IFN-γ. Another four clones of 37 were classified as Th1-like which secreted IFN-γ (Parronchi et al., 1996). The results in the present study are in agreement with the above study. Excess of IFN-γ production with

rKBG8.3 may be due to additional epitopes which may stimulate primary IFN-γ production (rKBG8.3, 201 amino acid; rKBG7.2, 140 amino acid). Moreover, as reported in our laboratory previously, T- and B-cell epitopes of human and murine systems are not completely overlapped, which may result in different responses (Mohapatra et al., 1995; Zhang et al., 1996).

II. VACCINATION WITH rAL RECIPROCALLY REGULATES IgE AND IgG2a Ab PRODUCTION

The goal of vaccination is induction of protective immunity. In mouse model of human allergic conditions, allergens induce Th2 cells which secrete IL-4, a switch factor for IgE Ab production. In contrast to Th2 cells, Th1 cells produce IFN-γ, which induces IgG2a Ab synthesis, together they represent protective immunity, as IFN-γ suppresses IL-4 synthesis, and IgG2a may act as blocking-antibody. In the present study, we demonstrated that vaccination of mice with rAL in different forms and by different routes induced suppression of IgE antibody production with a concomitant increase in IgG2a antibody production following immnization to these mice. Immune deviation induced by this approach has following characteristics, (i) dose-dependent inhibition of IgE Ab production; (ii) antigen-specific, since rAL has no effect on antibody production to unrelated antigen; (iii) isotype-selective, because both IgE and IgG2a responses were tightly controled by rAL vaccination, while IgG1 Ab showed variable reduction in treated mice opposed to

control mice which is presumably due to multifarious factors controling this isotype response *in vivo*. These results are consistent with a previous report using ovalbumin, although in the latter case the antigen was polymerized (Hayglass and Stefura, 1991). Our results obtained with rKBG8.3 differ from *Fel d* I study, where treatment with peptides suppressed antibody production irrespective of the isotype distribution (Briner et al., 1993). The the variations in experimental protocols, adjuvants and antigens used may account for the different outcomes.

Different forms of rAL affected the extent of IgE inhibition in this study. The insoluble form of rAL (rAL attached to beads, BFP) induced more profound IgE inhibition than soluble form of rAL (FP) at all doses used in primary response ($p<0.05$). However, subcutaneous administration in high doses of BFP and FP both suppressed IgE production, the difference between BFP and FP in local antigen maintenance and APC processing may affect their extent of antibody responses.

Administration of rAL by i.v. route induced a long-lasting (>87 days) suppression (>85-95%) of IgE Ab production although there were three immunizations following a single course of treatment. Administration of rAL by s.c. route induced profound IgE suppression (>99%, $p<0.001$) in primary response, less but still significant (75%; $p=0.04$) suppression in secondary response. Therefore, antigen-specific IgE tolerance could be achieved by administration of recombinant allergen in high doses subcutaneously and intravenously.

The finding that rAL could regulate *de novo* IgE responses in an antigen- and isotype-specific fashion suggests a potential applicability of this approach to allergen vaccination. However, it should be noted that the dosages used (250-500 μg/mouse), although consistent with similar animal studies (Briner et al., 1993; De Wit et al., 1992; Burstein and Abbas, 1993; Romball and Weigle, 1993; Vidard et al., 1995) are far more than could be used in human therapy. The antigen dose may also affect the cytokine profiles and antibody patterns, possibly by altering the type of APC or monokines involved (DeKruyff et al., 1992; Parish and Liew, 1972; Hayglass and Stefura, 1991; Manetti et al., 1993).

In the present time, the major objective in using rAL in murine model lies not only in its possible clinical application but more important in its development as an experimental model to examine role of cytokines in Ig isotype production *in vivo* and the regulation of immediate hypersensitivity. In murine system, IgE synthesis follows a Th2-like response to the antigen, whereas IgG2a antibody production results from elevated IFN-γ and a Th1-like response. It appears that an appropriate dose of an allergen can induce immune deviation as manifested by suppression of IgE antibody response and elevation of IgG2a response. The desirable antibody responses of reduced IgE and increased IgG2a synthesis by vaccination with rAL which reflect a shift from IL-4- to IFN-γ-dominant responses might facilitate symptomatic relief.

III. MODULATION OF CYTOKINE SYNTHESIS PATTERNS WITH rAL

In an effort to shed light on the mechanisms underlying the shift in immune response, we measured cytokine production. Vaccination with rAL resulted in decrease of expression of IL-2, IL-4 and IFN-γ proteins in both i.v. and s.c. routes for rAL administration; cytokine mRNA expression represented by RT-PCR products from transcription level confirmed ELISA results of cytokine protein synthesis, all of which revealed that the rAL treatment inhibited or even totally abrogated (in some cases) cytokine mRNA expression and ultimately protein synthesis. These results suggested that both Th1- and Th2-like cells were affected by the vaccination procedure.

In support of the results in the present study, it was previously demonstrated with deaggregated human γ-globulin as tolerogen, that $CD4^+$ cells from tolerized mice are tolerant for both IL-2 and IL-4 production and that both $CD45RB^{high}$ and $CD45RB^{low}$ cells are tolerized (Rombal and Weigle, 1993; Chu et al., 1995). Thus, under appropriate tolerizing conditions, it appears that both of the major subsets of $CD4^+$ cells can be readily tolerized.

Induction of T-cell unresponsiveness with peptides or rAL instead of crude extract is of particular interest for the treatment of allergies. Under the appropriate conditions of administration *in vivo* of proteins or peptides may be serve as effective tolerogens and may induce T-cell unresponsiveness, which has been attributed to T-cell anergy (O'Hehir et al., 1993; Briner

et al., 1993; Rogers et al., 1994). The extent of the decrease of antigen-specific IL-2, IFN-γ and IL-4 production which we have shown suggested that antigen-specific T cells of both Th1- and Th2-like may be anergized by our vaccination procedure. Reduction of proliferation in tolerized splenocytes and reversal of capacity to proliferate with exogenous rmIL-2 further suggest that the most likely explanation for our results is the induction of antigen-specific peripheral T-cell anergy. However, clonal deletion or apoptosis could not be excluded as a mechanism for induction of tolerance in the present study.

It is known that IL-2 and IFN-γ are proinflammatory cytokines (Katsikis et al., 1995). Therefore, reduction of IL-2 and IFN-γ synthesis by our vaccination protocol may attenuate inflammatory symptoms.

IV. IMMUNE DEVIATION FROM IL-4- TO IFN-γ-DOMINANT RESPONSE

As suggested and demonstrated by recent studies, the balance of cytokine responses rather than increased or decreased production of any single cytokine plays an essential role in determining the outcome of an immune response (Yang et al., 1993). The ratio of IFN-γ:IL-4 provides a picture of the balance of cytokine response at the population, rather than individual T cell level and does not indicate whether individual T cells secrete solely IL-4 or IFN-γ (Romagnani, 1994; Imada et al., 1995). In the present study, although all cytokine levels were reduced in vaccinated-immunized

mice, clearly there was an increase in the ratio of IFN-γ:IL-4 in comparison with the immunized mice. The parallel between the ratio of IFN-γ:IL-4 production and antibody isotype selection suggests that administration of rAL modulates *in vivo* antibody response via altered cytokine gene expression.

Our results of IFN-γ synthesis in vaccinated mice showed that IFN-γ production was least affected in anergic condition. This observation is consistent with other reports, which suggest that although IL-2 production and proliferation are dramatically reduced after induction of the anergic state, production of other lymphokines, such as, IFN-γ, is marginally affected, the anergized T cells could synthesize considerable amounts of IFN-γ (Schwartz, 1990; Schols et al., 1995). This is presumably due to the fact that IFN-γ relies on multiple signaling pathways for the expression, only some of which are inhibited in the anergic state (Gajewski et al., 1994).

An increase in IFN-γ:IL-4 ratio in this study suggested that the T cell response balanced in favour of developing a IFN-γ-dominant response in stead of the default IL-4-dominant response. Finally, the increased ratio led to enhanced IgG2a isotype, concomitantly reduction of IgE synthesis. The immune deviation from IL-4- to IFN-γ-dominant response by vaccinantion appears to result from antigen-specific T cell anergy.

It has been suggested that most IgE production in primary response is IL-4-dependent event. However, in our studies, although there was secretion of IL-4, IgE production was suppressed in the

treated mice compared to the immunized mice. We speculate that in the vaccinated mice low level of IL-2 (around 10 times less than immunized mice) could not provide paracrine growth factor to Th2-like cells for proliferation, the activity of IL-4 in eliciting IgE response could not be synergized by IL-2, and was antagonized by IFN-γ. Immunization at day 7 after treatment further expanded Th1-like cells, resulting in IgG2a-dominant responses. The ratio of IFN-γ:IL-4 only altered following immunization of vaccinated mice with rAL plus alum. These results demonstrated pivotal role of IL-2 during initiation of an immune response and in generation of a pool of Th1-like memory cells. Therefore, the effect of vaccination was further expanded by immunization with the same antigen, leading to an immune deviation from IL-4- to IFN-γ-dominant responses. It seems that IL-4 production and the Th2-like cell expansion in the mouse model vaccinated with rAL was largely IL-2-dependent in primary response.

V. THERAPEUTIC MODULATION OF ANTIBODY AND CYTOKINE RESPONSES

In preliminary investigation of the modulation on established immune response, treatment with rAL in primed mice showed consistent decrease of IL-2 synthesis, the levels of IFN-γ and IL-4 production varied. These mice showed elevated IgG1 and IgG2a production which suggest that immune deviation also occurred in these mice. However, ongoing IgE production remained unreduced in these mice following treatment with one course of rAL. These

results implied that T cell anergy may happen in this model presented by reduced IL-2 synthesis, the elevated IgG antibodies may function as blocking antibody competing with IgE antibody for binding to the immunodominant epitopes in the antigen for IgE response (Kolbe et al., 1995), both of which would lead to clinical improvement.

In our model of established immune response, 143 days after a priming with rAL, rAL-specific antibodies can still mount quite high levels. Generally, in mice the normal metabolic half-life of soluble immunoglobulin molecules varies between 12 hr and several days, depending on the immunoglobulin class. Thus a gradual disappearance of antibody can be expected, unless functionally active plasma cells are replenished continuously from an activated B-cell population (Vieira and Rajewsky, 1988; Dintzis HM and Dintzis RZ, 1992). Therefore, it is suggested that activated B cell population existed in the rAL primed mice for a period of time.

The protocol used in this study was not successful in downregulating IgE response, probably because of (1) the large dose of treatment is needed to overcome the high preexisting titers of specific serum antibodies in the primed mice, (2) relatively long duration and multiple times of treatment are required to persistently maintain antigen or tolerogen. Several other factors, such as adjuvant, age of mice, priming dose, route for treatment might affect the results.

As we have known that KBG extract are mixtures of allergens. To examine whether rKBG8.3 only could modulate immune response to

kAL, the mice were pretreated with rKBG8.3 and then immunized with KBG extract. We found that rKBG8.3 pretreatment by both s.c. and i.v. route could significantly enhance IgG2a production, without affecting IgE production. It is inferred that pretreatment with the major allergen may aid clinical improvement resulting from elevated IgG2a Ab production by IFN-γ-dominant response. However, with one of major allergens alone may not be sufficient to induce full protection in all patients. A combination of a few major recombinant allergens may be required to inhibit IgE production to the whole extract of KBG pollen allergens.

V. CONCLUSIONS

Taken together, it is concluded from the results in this study that (1) rKBG8.3 and KBG extract were similar in the ability to induce *in vivo* immune responses, except the major difference in the ability to induce IFN-γ production with *in vitro* restimulation. Therefore, rKBG8.3 is a potential candidate to substitute KBG extract for *in vivo* diagnosis. (2) Vaccination with rKBG8.3 in soluble form without adjuvant by i.v. and s.c. routes could tolerize antigen-specific T cells for subsequent challenge with the same allergen. The major mechanism of the tolerance involved in this study may be due to induction of antigen-specific T cell anergy, preferential Th2-like cell anergy, followed by immune deviation from IL-4- to IFN-γ-dominant response, resulting in a shift in antibody isotype distribution from IgE to IgG2a. (3)

Therapeutic treatment with rKBG8.3 in primed mice could also modulate established immune response although to a less extent than that of *de novo* immune response. (4) Treatment with rKBG8.3 could dramatically enhance IgG2a Ab production without change of IgE Ab production, in response to kAL.

These results suggest that recombinant allergens containing major multiple T- and B-cell epitopes used in vaccination may have potential for prevention of allergies.

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