

CpG oligodeoxynucleotide vaccination suppresses IgE induction but may fail to down-regulate ongoing IgE responses in mice

Zhikang Peng, Hongsheng Wang, Xiaojuan Mao, Kent T. HayGlass and F. Estelle R. Simons

Section of Allergy and Clinical Immunology, Department of Pediatrics and Child Health, and Department of Immunology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 3P5, Canada

Keywords: allergy, cytokines, T_H1/T_H2 , vaccination

Abstract

Antigen-specific IgE plays an important role in the pathogenesis of allergic disorders. Immunostimulatory CpG motifs (CpG) in bacterial DNA or synthesized oligodeoxynucleotides (ODN) are gaining recognition as potential immunomodulators for switching on protective T_H1 -mediated immunity and preventing or potentially inhibiting T_H2 -dependent allergic responses. To date, allergic models used in CpG ODN studies have been established by immunization of mice with allergen in the presence of adjuvant. This, in addition to failure to assess specific IgE production in most of the studies, has limited understanding of the role of CpG ODN vaccination in allergic responses. Here, we examine the effects of synthesized CpG ODN on both developing and ongoing IgE responses in mice sensitized using a recombinant mosquito salivary antigen (rAed a 2) without adjuvant. Pretreatment of mice with CpG ODN mixed with rAed a 2 successfully inhibited subsequent induction of serum rAed a 2-specific IgE (but not IgG1) and antigen-induced IL-4 and IL-5 production in spleen cells. This was associated with an increase of serum IgG2a and IL-12, and increased IFN- γ and IL-12 production by spleen cells. In this model, however, co-administration of CpG ODN with rAed a 2 to presensitized mice failed to down-regulate ongoing IgE responses despite significant up-regulation of serum IL-12 and specific IgG2a. Strikingly, a transient skin delayed-type hypersensitivity reaction occurred in CpG ODN-treated mice. These observations provide a new insight into the potential therapeutic application of CpG ODN to allergic disorders.

Introduction

Immediate hypersensitivity disorders are associated with increased IgE antibodies which cross-link the high-affinity Fc ϵ receptors on mast cells and basophils, leading to release of mediators that cause allergic reactions (1). It is well established that IgE production is regulated by a balance between two types of cytokines, secreted by T cell subsets T_H1 and T_H2 respectively (2,3). T_H1 -like lymphocytes produce IFN- γ , IL-2 and lymphotoxin, and promote immunity to intracellular pathogens as well as an Ig class switch to IgG2a. T_H2 -like cells secrete IL-4, IL-5, IL-10 and IL-13. IL-4 (and IL-13 in humans) is a strong promoter of switching on IgE production in B lymphocytes, while IL-5 plays an important role in activation and recruitment of eosinophils (4–6). Both

elevated IgE and eosinophils are important hallmarks of allergic disorders. IL-4 promotes differentiation of naive T cells into T_H2 -like cells and inhibits T_H1 activation, whereas IFN- γ suppresses IL-4-mediated T_H2 polarization both *in vitro* and *in vivo* (5,6). Fine tuning of the balance between production of T_H1 - and T_H2 -type cytokines is believed to be crucial for the down-regulation of IgE-mediated allergic responses and clinical sensitivity (7,8).

A general strategy proposed for down-regulation of T_H2 -dominant, IgE-mediated allergic disorders is to redirect pathogenic T_H2 responses toward less harmful T_H0/T_H1 responses by up-regulating the production of T_H1 -type cytokines (9). Unmethylated CpG motifs common in bacterial,

Correspondence to: Z. Peng University of Manitoba, 532 John Buhler Research Centre, 715 McDermot Avenue, Winnipeg, Manitoba R3E 3P5, Canada

Transmitting editor: Z. Ovary

Received 25 April 2000, *accepted* 8 August 2000

but not mammalian, DNA have been shown to induce strong T_H1 -biased immunity *in vivo* (10–14) and *in vitro* (15,16). Their use in infectious diseases, allergy and cancer in animal models may provide a broadly effective means of directing or redirecting immune responses (9,17,18). Synthetic CpG oligodeoxynucleotides (ODN) have recently been shown to be potential immunoregulators in prevention of airway inflammation and hyper-responsiveness in murine models (10–12,14). These studies suggest the therapeutic potential of bacterial DNA in prevention and perhaps in treatment of IgE-mediated allergic disorders.

CpG ODN can be produced in large quantities under controlled conditions and therefore have important potential as an immunotherapeutic agent. Many aspects remain to be clarified before their widespread application to immunoregulation in humans, however. Firstly, the animal models used extensively in the allergy and CpG ODN studies are sensitized either by addition of adjuvant (11,12,14,19) or by infrequent allergen injections via unnatural routes such as the peritoneum (10). Because adjuvants themselves induce non-specific T cell and inflammatory responses, and affect multiple cellular functions (20), these findings need to be translated into models which more closely approximate the triggering of IgE responses under natural conditions in humans, i.e. when the allergenic stimulus is not accompanied by an adjuvant, the portal entry of the allergen is natural and the exposure to the allergen is frequent. Secondly, since T_H1 immunity is enhanced following plasmid DNA or CpG ODN vaccination, the potential for harmful delayed-type hypersensitivity responses induced by CpG ODN vaccination needs to be determined. Finally, the production of antigen-specific IgE plays an important role in the mediation of allergic reactions, but the alterations of antigen-specific IgE production following CpG ODN administration are controversial and are not well investigated.

In this study, utilizing a mouse model sensitized with a recombinant mosquito saliva allergen (rAed a 2) via a natural route, without use of adjuvants, we examined the therapeutic effects of CpG ODN on both developing and ongoing IgE responses and characterized type 1/type 2 cytokine expression patterns. The occurrence of delayed-type hypersensitivity reactions, a potentially limiting side effect of CpG ODN vaccination, was also examined.

Methods

Mice

Female BALB/c and C57BL/6 mice (8–10 weeks old) were obtained from the Central Animal Care Services, University of Manitoba. All animals were kept under identical conditions in one room at the Service facility. The experiments were approved by the University of Manitoba and the Animal Care and Use Committee, and the investigators adhered to Canadian Council on Animal Care (CCAC) guidelines for humane treatment of animals.

CpG ODN and rAed a 2

The CpG ODN, consisting of 20 bases containing two CpG motifs (TCCATGACGTTCTGACGTT) (10,21), were produced

by Oligos Etc. (Wilsonville, OR) in a good manufacturing practice facility and had undetectable lipopolysaccharide contaminants.

Aed a 2 is a 37 kDa salivary protein of mosquito *Aedes aegypti* and is also found in many other mosquito species with worldwide distribution (22,23). This protein causes positive skin tests and binds to serum IgE in mosquito-allergic subjects (22,23). The cDNA coding for Aed a 2 was cloned (24) and the recombinant protein was expressed by a baculovirus/insect cell system and purified using DEAE-Sephacel as described elsewhere (25). Purified rAed a 2 induces a typical T_H2 -type response following repeated intradermal (i.d.) injections in mice (25).

Sensitization

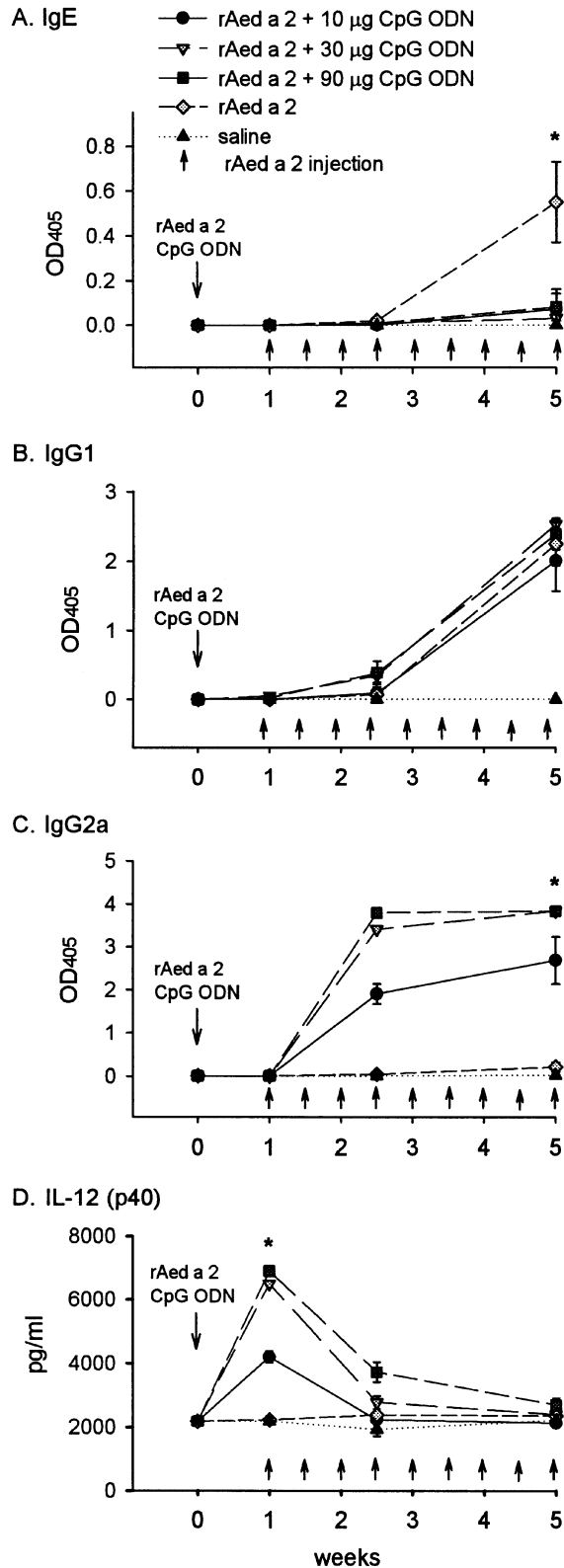
No adjuvants were used in these studies. ODN without immunostimulatory CpG motifs were not included in this study because previous studies clearly showed a CpG motif-dependent activity of synthetic ODN in immune regulation (10,12,14,19,21). The time-lines in the prevention study and in the ongoing IgE study are shown in Figs 1 and 3 respectively. Each immunization is represented by an arrow, CpG administration is shown by a CpG-labeled arrow and antibody measurements by symbols.

In the prevention study (Fig. 1), four BALB/c mice in each group were pretreated (i.d.) with a mixture of 10 μ g of rAed a 2 and 10, 30 or 90 μ g of CpG ODN in saline at day 0. On the same day, two other groups of mice were injected i.d. with 10 μ g of rAed a 2 as positive controls and saline as negative controls respectively. Seven days later, all mice were sensitized i.d. with 10 μ g of rAed a 2 in saline twice a week for 5 weeks, except for the negative control group which was treated with saline only. After confirming the induction of serum rAed a 2-specific IgE in the positive control group at week 5, all mice were sacrificed and spleen lymphocyte suspensions were prepared for antigen-induced cytokine assays. Blood samples were collected by tail bleeding at week 0, 2.5 and 5, and sera were stored at -70°C for measurement of antigen-specific IgE, IgG1 and IgG2a antibodies, and IL-12 (p40). To confirm the results, a similar experiment using ovalbumin as antigen was performed.

To determine the effect of CpG ODN vaccination on established IgE responses, CpG ODN vaccination was given twice after sensitization with rAed a 2 (Fig. 3). CpG ODN at 30 μ g was chosen as an optimal dose in these experiments. This dose is also compatible with the dose used in a previous study (10). In addition, two mouse strains, the T_H2 -biased BALB/c and T_H1 -biased C57BL/6, were utilized to evaluate the effect of genetic factors on the outcome of CpG ODN vaccination. BALB/c and C57BL/6 mice (four in each group) were first sensitized i.d. with 10 μ g of rAed a 2 twice weekly for 8 weeks. Serum rAed a 2-specific IgE was significantly increased in the positive control group at week 4. The mice were then challenged i.d. with 2 μ g of rAed a 2 at weeks 9.5 and 11.5. At weeks 4 and 8, one group of rAed a 2-sensitized mice was injected i.d. with a mixture of 30 μ g of CpG ODN and 10 μ g of rAed a 2. The other group of rAed a 2-sensitized mice was treated with 10 μ g of rAed a 2 alone. Control mice were injected with saline. Sera were collected biweekly.

Intradermal tests

Intradermal tests were performed as previously reported (25). Briefly, 1 μ g of rAed a 2 in 10 μ l of saline was injected i.d.



into the shaved back of each mouse; 10 μ l of saline was injected as a negative control 2 cm away from the antigen injection site. The immediate reaction was read 20 min after the injection by measuring the largest and orthogonal diameters of the wheal. The delayed reaction was read 24 and 48 h later by measuring the induration. The visibility of the edge of the wheal or induration was enhanced by wrinkling the skin surrounding the injection site. After subtraction of the saline-induced wheal or induration, the results were expressed using the average diameter of the wheal or the induration. A diameter ≥ 5 mm was considered to be positive.

Serum antigen-specific IgE, IgG1 and IgG2a

Serum samples were collected at weeks 0, 1, 2.5 and 5 in the prevention study, and every 2 weeks in the treatment study. rAed a 2-specific IgE was measured using a reverse-type, anti-IgE capture ELISA according to techniques previously developed in our laboratory (25). Briefly, 96-well microplates coated with monoclonal rat anti-mouse IgE (PharMingen, San Diego, CA) were incubated with test samples (1:10), followed by incubations with biotinylated rAed a 2 (0.5 μ g/ml) prepared in our laboratory according to an established procedure (26) and then streptavidin-alkaline phosphatase conjugate (1:1,000) (PharMingen).

rAed a 2-specific IgG1 and IgG2a were measured using an antigen capture ELISA as described previously (27,28). Briefly, the plates were coated with 0.25 and 1 μ g/ml of rAed a 2 for testing IgG1 and IgG2a respectively, and then incubated with serum samples or a standard reference serum (1:1000 for IgG1 and 1:40 for IgG2a) or PBS followed by incubation with alkaline phosphatase-conjugated monoclonal rat antibodies against mouse IgG1 (1:10,000) or IgG2a (1:1000). The standard reference serum was obtained from a pool of rAed a 2-immunized mice with high titer antibody levels.

Test samples were assayed in duplicate. The mean value of each samples was expressed by the optical absorbency at 405 nm (OD_{405}) after deduction of the PBS control, and then normalized using the standard reference serum (25).

Antigen-induced cytokine production

Mouse spleen cell suspensions were prepared as described previously (28) and cultured in triplicate to a final concentration of 2×10^6 cells/ml (total 200 μ l/well) in complete RPMI 1640 medium supplemented with 10% FCS. rAed a 2 was added to cultures at 50 μ g/ml. Cells cultured with the medium alone served as an unstimulated control. Culture supernatants were

Fig. 1. The effect of CpG ODN vaccination on prevention of subsequent rAed a 2-IgE responses. Four mice in each group were injected i.d. with rAed a 2 (10 μ g) or a mixture of rAed a 2 (10 μ g) and CpG ODN (10, 30 or 90 μ g) at the beginning of week 0. At week 1, the mice were sensitized with rAed a 2 (10 μ g in saline, i.d.) twice weekly for 5 weeks as indicated by arrows. Control mice were treated with saline. Serum rAed a 2-specific IgE, IgG1, IgG2a and IL-12 (p40) levels were measured by ELISA, and the results are expressed as mean $\pm$ SEM of four mice per group. * $P < 0.05$ comparing the CpG ODN-vaccinated groups versus positive control group.

harvested at day 3 and day 4, and stored at -70°C until cytokine analysis.

The levels of IL-4, IL-5 and IFN- γ in the culture supernatant and IL-12 (p40) in serum were determined by sandwich ELISA (28) with reagents purchased from PharMingen. Briefly, 96-well plates were coated with purified monoclonal rat anti-mouse IL-4 or IL-5, IL-12 (p40) and IFN- γ (1–2 $\mu\text{g/ml}$), and then incubated with test culture supernatants (undiluted and 1:2) or a recombinant standard (IL-4, 0.25–500 pg/ml; IL-5, 2.5–2500 pg/ml; IL-12, 4–4000 pg/ml; IFN- γ , 1–1000 pg/ml). This was followed by incubations with biotinylated monoclonal rat anti-mouse IL-4 or IL-5, IL-12 (p40) and IFN- γ (0.5–1 $\mu\text{g/ml}$), and finally streptavidin-alkaline phosphatase (1:1,000). The sensitivity of the assay was 2 pg/ml for IL-4, 10 pg/ml for IL-5, 8 pg/ml for IL-12 and 16 pg/ml for IFN- γ respectively.

Statistical analysis

The levels of specific antibodies and cytokines and the diameters of the skin reactions were expressed as mean $\pm$ SEM for each group. The statistical significance between groups was determined using Student's *t*-test.

Results

The effect of CpG ODN vaccination on prevention of IgE responses

The effect of CpG ODN vaccination on prevention of subsequent sensitization to rAed a 2 is shown in Fig. 1. After sensitization at week 5, positive control mice developed significantly higher levels of specific IgE and IgG1 but very low levels of IgG2a (Fig. 1A–C), consistent with our previous observations (25). CpG ODN vaccination suppressed specific IgE levels by 90% compared with positive control mice ($P < 0.05$) (Fig. 1A). Unlike IgE, rAed a 2-specific IgG1 production was not inhibited by CpG ODN treatment (Fig. 1B). As expected, a dose-dependent increase in rAed a 2-specific IgG2a was observed in CpG ODN-vaccinated mice ($P < 0.01$) (Fig. 1C). Moreover, all CpG ODN-treated mice appeared healthy, consistent with previous reports (21). The same results were obtained using ovalbumin; one dose of CpG ODN administration 1 week before sensitization induced a substantial suppression of IgE responses up to 7 weeks (29).

The effect of CpG ODN on skin reactions to rAed a 2 is shown in Table 1. Mice that were sham-vaccinated before i.d. sensitization developed positive immediate wheals, while CpG ODN-vaccinated mice generally produced smaller wheals. No delayed-type reactions were observed in any group. As IgG1 also plays a role in mast cell-mediated inflammation in mice, but not in humans (30), the unchanged IgG1 levels in CpG ODN-vaccinated mice may account for the incomplete suppression of the immediate skin reactions in this group.

The effect of CpG ODN on antigen-induced cytokine responses 5 weeks after CpG ODN vaccination is shown in Fig. 2. Compared to unimmunized mice, positive control mice produced significantly higher levels of IL-4 (>9 -fold, $P < 0.01$) and IL-5 (>40 -fold, $P < 0.0001$), and slightly more IFN- γ

(1.5-fold, $P < 0.01$) and IL-12 (1.8-fold, $P > 0.05$), confirming a T_H2 -like dominant response in this model. Consistent with their suppressed IgE production, CpG ODN-vaccinated mice showed significantly decreased production of IL-4 ($P < 0.01$) and IL-5 ($P < 0.001$), and increased secretion of IFN- γ and IL-12, indicating that a switch from a T_H2 - to a T_H1 -type immune response occurs following CpG ODN vaccination.

Analysis of the kinetics of serum IL-12 production revealed that 1 week after vaccination, an intense but transient increase in IL-12 production occurred in CpG ODN-vaccinated mice ($P < 0.01$) (Fig. 1D). The high IL-12 levels persisted for 1–2 weeks and then declined. This early production of IL-12 may be critical for the later establishment of T_H1 dominance in this model.

The effect of CpG ODN on down-regulation of ongoing IgE responses

Frequent immunizations with rAed a 2 in BALB/c mice resulted in a steady increase in serum rAed a 2-specific IgE and IgG1 levels, as shown in Fig. 3(A1). Surprisingly, compared with the positive controls, two CpG ODN vaccinations did not inhibit established IgE and IgG1 responses at all (Fig. 3A1 and B1), even although CpG ODN vaccinations resulted in an substantial increases in serum rAed a 2-specific IgG2a ($P < 0.05$ after week 6) (Fig. 3C1) and a transitory increase in serum IL-12, which reached a peak 2 weeks following CpG ODN administration and returned to baseline by 4 weeks ($P < 0.01$ at weeks 6 and 10 respectively) (Fig. 3D1).

To confirm this, two other experiments were performed using ovalbumin in which CpG ODN was administered at week 1 or weeks 2, 3 or 4 respectively after sensitization commenced. Similar results were found: ongoing IgE responses were not suppressed although serum IgG2a and IL-12 were up-regulated (29).

As shown in Fig. 3(right panel), in C57BL/6 mice, rAed a 2 sensitization induced an increase in IgE and IgG1, and a small increase in IgG2a. Compared with BALB/c mice, however, the overall levels of IgE, IgG1 and IgG2a were lower than those in BALB/c mice. Similar to the results in BALB/c mice, CpG ODN vaccination failed to down-regulate the ongoing IgE and IgG1 responses at all despite the fact that serum IL-12 levels were significantly up-regulated and IgG2a levels were slightly increased.

Immediate and delayed skin reactions were assessed at weeks 4, 8 and 12. There was no difference in the size of the immediate wheal reactions between the CpG ODN-vaccinated and positive control groups in both strains (Table 2). In the CpG ODN-vaccinated group, however, a delayed skin reaction was observed 24 h, not 48 h, after skin testing (Table 2). There was no delayed skin reaction observed in any other group during the study.

Discussion

In this study, pretreatment with a single dose of CpG ODN mixed with rAed a 2 consistently inhibited subsequent production of antigen-specific IgE and induced antigen-specific IgG2a for at least 5 weeks (Fig. 1). The inhibition of IgE responses was associated with increased production of IFN- γ and IL-12, and decreased production of IL-4 and IL-5 by

Table 1. Skin reactions to rAed a 2 in mice pretreated with CpG ODN vaccination before sensitization

Pretreatment	Sensitization (2/week for 4 weeks)	Wheal or induration diameter (mm) (mean $\pm$ SEM)		
		20 min	24 h	48 h
rAed a 2	rAed a 2	6.5 $\pm$ 0.1	0	0
rAed a 2 + CpG ODN 10 μ g	rAed a 2	6.3 $\pm$ 0.5 ($P < 0.67$)	0	0
rAed a 2 + CpG ODN 30 μ g	rAed a 2	5.2 $\pm$ 0.4 ($P < 0.02$)	0	0
rAed a 2 + CpG ODN 90 μ g	rAed a 2	5.4 $\pm$ 0.6 ($P < 0.14$)	0	0
Saline	saline	2.3 $\pm$ 0.60	0	0

Four mice in each group were pretreated i.d. with rAed a 2 + CpG ODN at week 0. One week later, the mice were sensitized i.d. with rAed a 2 from week 1 to 5 as described in Methods. Skin testing was performed at week 5. The immediate wheal sizes in rAed a 2-sensitized mice with or without CpG ODN treatment were significantly larger than those in saline control mice ($P < 0.001$). CpG ODN-vaccinated mice generally produced smaller wheals compared with untreated group (P values are shown in the parentheses).

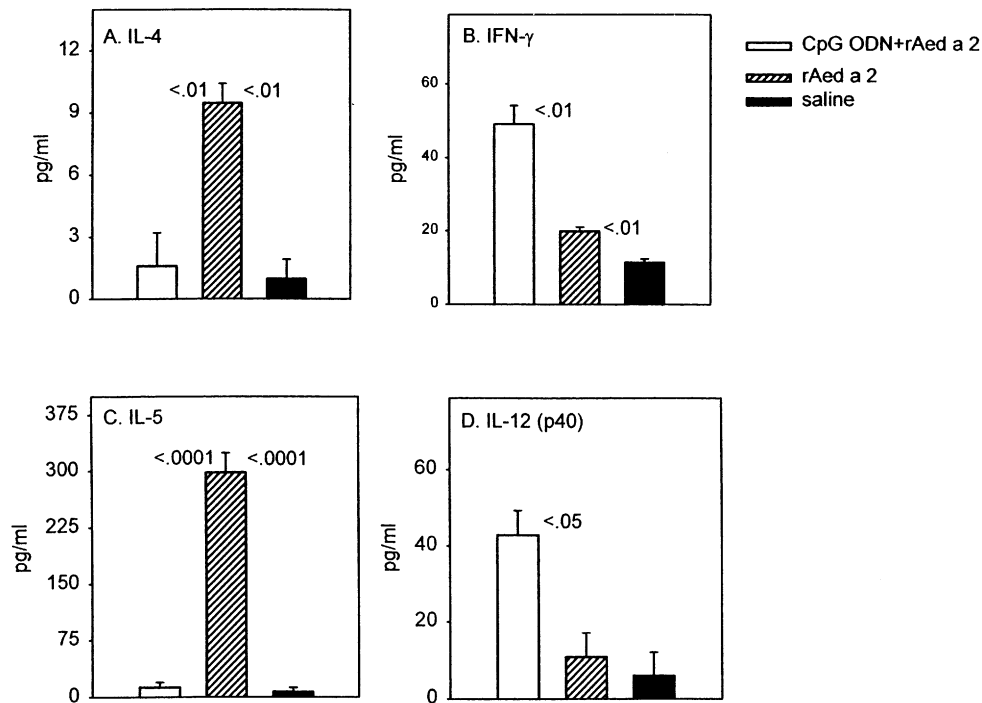


Fig. 2. The effect of CpG ODN vaccination on cytokine induction. Four mice in each group were treated as described in Fig. 1. Splenocytes from mice treated with 90 μ g of CpG ODN plus rAed a 2, or rAed a 2 alone, or saline were stimulated *in vitro* with 50 μ g/ml of rAed a 2 for 3 days. The levels of IL-4, IL-5, IL-12 (p40) and IFN- γ (mean $\pm$ SEM) were measured by ELISA. Cytokine production in the absence of rAed a 2 in culture was undetectable. The cytokine levels from day 4 culture exhibited similar patterns (data not shown).

rAed a 2-re-stimulated spleen cells. Our data support a role of CpG ODN in switching on T_H1 immunity to a protein antigen through up-regulation of T_H1 -type cytokines and down-regulation of T_H2 -type cytokines, indicating the ability of CpG ODN to hinder allergic sensitization (10,11,13) in mice sensitized without adjuvant. Although both IFN- γ and IL-12 are involved in the protection against the development of T_H2 -mediated allergic responses, other mechanisms may also be important in inducing this protection (31).

The successful inhibition of the production of IgE, but not IgG1, by CpG ODN in primary immune responses to rAed a 2 indicates that the production of IgG1 and IgE antibodies to rAed a 2 is differentially regulated. This is not surprising because the dissociation in regulation of IgE and IgG1

production has already been demonstrated *in vitro* (32) and *in vivo* (33,34). Similar findings, a T_H0 -type response, i.e. an increase in both IgG1 and IgG2a, have been observed following intranasal co-administration of hepatitis B surface antigen with CpG ODN (35).

Strikingly, treatment with CpG ODN in rAed a 2-sensitized mice failed to inhibit established IgE responses in both BALB/c and C57BL/6 mice (Fig. 3). Presumably, when CpG ODN are administered 4 weeks after antigen sensitization, IgE-secreting plasma cells would already be present in the bone marrow and would likely be refractory to further T_H1 stimuli. This is supported by the evidence that IgE-committed B lymphocytes may be resistant to exogenous stimuli such as IL-4 and anti-CD40 antibodies (36). Recently, it has been

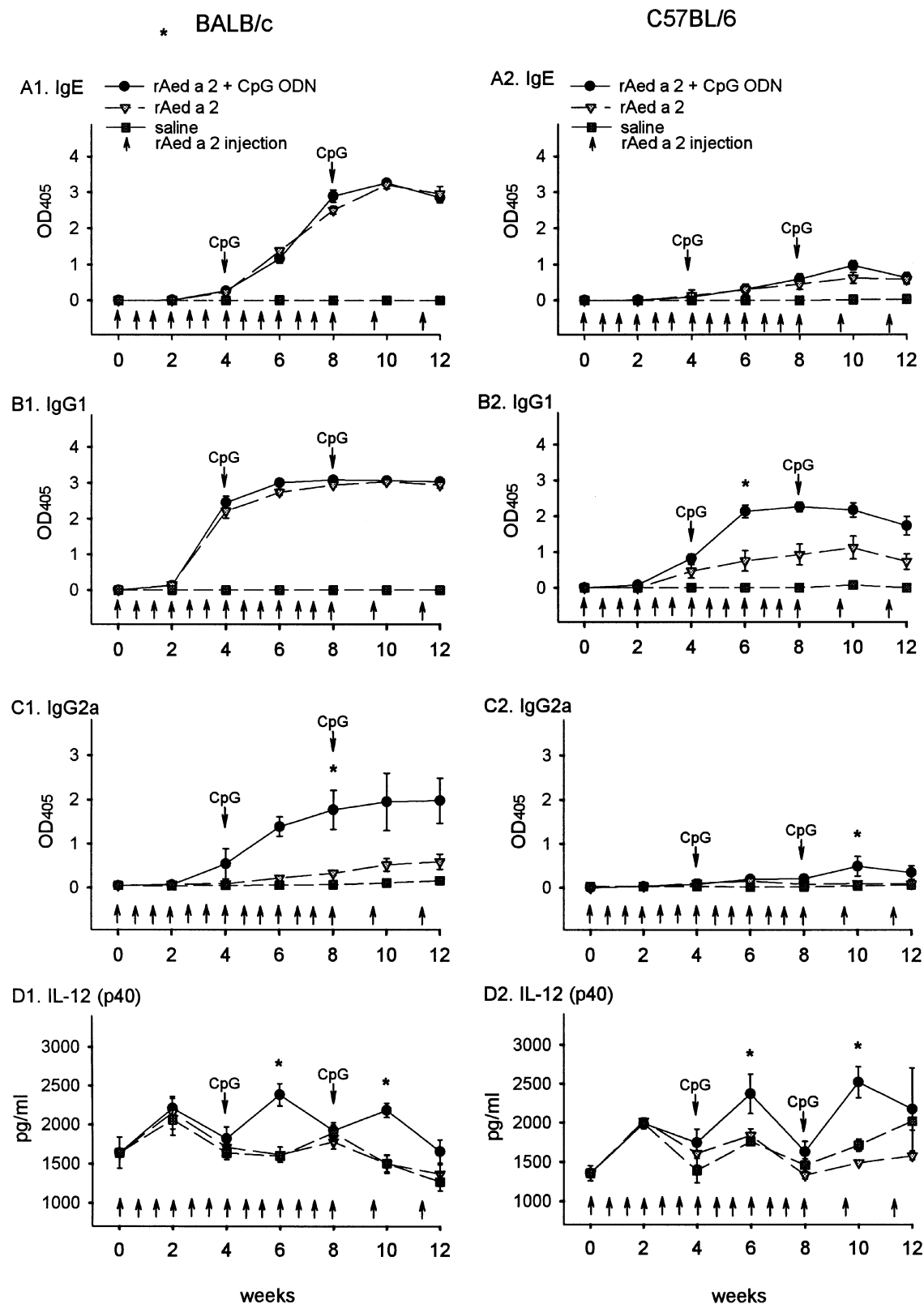


Fig. 3. The effect of CpG ODN vaccination on established IgE responses. BALB/c (left panel) and C57BL/6 (right panel) mice were immunized i.d. with rAed a 2 (10 μ g) twice weekly for 8 weeks. The mice were then given injections with 2 μ g of rAed a 2 at weeks 9.5 and 11.5. A mixture of rAed a 2 (10 μ g) and CpG ODN (30 μ g) was administered i.d. at weeks 4 and 8. Serum rAed a 2-specific IgE (A), IgG1 (B), IgG2a (C) and IL-12 (p40) (D) were measured by ELISA. Data shown are mean $\pm$ SEM of four mice per group. * P < 0.05 comparing the CpG ODN-vaccinated group versus positive control group.

Table 2. Skin reactions to rAed a 2 in mice receiving CpG ODN vaccination after sensitization

Strain	Treatment	Wheal or induration diameter (mm) (mean $\pm$ SEM)								
		Week 4			Week 8			Week 12		
		20 min	24 h	48 h	20 min	24 h	48 h	20 min	24 h	48 h
BALB/cr	Aed a 2	8.8 $\pm$ 0.4	0	0	9.3 $\pm$ 0.7	0		9.5 $\pm$ 0.4	0	0
	rAed a 2 + CpG	8.9 $\pm$ 0.6	0	0	9.4 $\pm$ 0.8	5.7 $\pm$ 0.4 ^a	0	9.7 $\pm$ 0.6	0	0
	Saline	<3.0	0	0	<3.0	0	0	<3.0	0	0
C57BL/6	rAed a 2	8.9 $\pm$ 0.6	0	0	6.3 $\pm$ 0.3	0	0	7.7 $\pm$ 0.6	0	0
	rAed a 2 + CpG	8.2 $\pm$ 0.6	0	0	6.7 $\pm$ 1.0	0	0	6.4 $\pm$ 0.4	0	0
	Saline	<3.0	0	0	<3.0	0	0	<3.0	0	0

^a $P < 0.02$.

Four mice in each group were sensitized from weeks 0 to 12. CpG ODN vaccination (30 μ g/mouse) was administered at weeks 4 and 8. Skin testing was performed at weeks 4, 8 and 12. There was no difference in the sizes of the immediate wheal reactions between the CpG ODN-vaccinated and positive control groups in both strains, although all of them developed significantly larger immediate wheal sizes than those in saline controls ($P < 0.001$). In the CpG ODN vaccinated group, however, a delayed skin reaction was observed in three out of four mice ($P < 0.02$).

found that incubation of CpG ODN with human peripheral blood mononuclear cells from allergic donors results in suppression of total IgE production, whereas the antigen-specific IgE secretion remains unaltered (16). This is accompanied by elevated expression of IL-12 and IL-18 mRNA (16). Our analysis of serum IL-12 and total IgE levels revealed an increased IL-12 (Fig. 3D) but an unchanged total IgE or rAed a 2-specific IgE production following CpG ODN administration (Fig. 3A; total IgE responses similar to rAed a 2-specific IgE, data not shown). As shown in Fig. 3(D), even though serum IL-12 levels are maintained at a high level after week 4 by repeated administration of CpG ODN, the ongoing IgE responses are not affected. Our data obtained in mice frequently sensitized without adjuvant suggests that increased T_H1 immunity induced by CpG ODN does not inhibit established IgE responses. This is supported by recent studies in which passively transferred T_H1 cells did not inhibit T_H2 responses efficiently *in vivo* (37) and CpG ODN was unable to redirect a neonatally established T_H2 response to tetanus toxoid antigen (19). Interestingly, we have recently found that although CpG ODN has little effect on down-regulation of ongoing IgE production, administration of this vaccine intranasally is highly effective in suppression of airway eosinophilia in mice with elevated serum IgE (29).

Other studies in murine asthma models have shown that CpG ODN efficiently suppresses airway eosinophilia and hyper-responsiveness. In these studies, the mice were sensitized infrequently (2–4 times) with allergen either through an unnatural route such as the peritoneum (10) or using alum as an adjuvant (11,12,14). It has long been known that adjuvants can influence the nature and cellular functions of the immune response (20). Systemic sensitization with allergen plus adjuvant $Al(OH)_3$ induces the highest levels of IgE and eosinophil infiltration compared with protocols without adjuvants (38,39). Thus, the non-specific effect of the adjuvant may increase the complexity of the responses to CpG ODN. In the study by Sur *et al.*, antigen-specific IgE induced by immunization with ragweed and alum was found to be significantly reduced by two or three doses of CpG ODN (12). This contrasts with our observations. One explanation

for this discrepancy may be the alum adjuvant used and/or the route of administration delivering CpG ODN through mucus membrane or skin, and the frequency of sensitization, which may influence the IgE production differently in the models. Another explanation may be that the effect of CpG ODN is antigen dependent. This is not likely because we repeated the experiment with ovalbumin and obtained similar results that administration of CpG ODN even 1 week after sensitization started did not down-regulate IgE responses (29).

Consistent with the unaltered specific IgE levels in CpG ODN-vaccinated, pre-sensitized mice, immediate skin reactions were also unchanged. Surprisingly, CpG ODN vaccination induced transient delayed skin reactions at week 8 in BALB/c mice after the second injection of CpG ODN (Table 2). To our knowledge, this is the first time a potential visible side effect of CpG ODN on the treatment of IgE responses to an allergen has been reported. Delayed skin reactions were not found in mice receiving one CpG ODN injection before sensitization, suggesting that delayed skin reactions may be correlated to the number of injections of CpG ODN. Genetic background may also influence the severity of this side effect because C57BL/6 mice, a T_H1 -biased strain (40), produced rather lower levels of IgE, IgG1 and IgG2a, and did not develop delayed hypersensitivity skin reactions following CpG ODN vaccination.

IgE-mediated disorders are a rapidly increasing problem, especially in developed countries (41,42). Our study performed in a mouse model in which mice were frequently sensitized without adjuvant demonstrated that CpG ODN vaccination may provide an effective and economic approach for preventing IgE production, and reducing the risk of developing allergic disorders. The therapeutic potential of CpG ODN vaccination for established allergic disease clearly needs to be investigated further using animal models that relate more closely to humans.

Acknowledgements

This study was supported by a grant and a personal award (K. T. H.) from the Medical Research Council of Canada, and by a

grant and personnel awards (Z.P. and F. E. R. S.) from the Children's Hospital Foundation, Winnipeg, Manitoba, Canada

Abbreviations

i.d.	intradermal
ODN	oligodeoxynucleotide
rAed a 1	recombinant Aed a 1

References

- Janeway, C. and Travers, P. 1997. *Immunobiology: The Immune System in Health and Disease*, 3rd edn, p. 11:1. Garland, London.
- Murphy, K. M. 1998. T lymphocyte differentiation in the periphery. *Curr. Opin. Immunol.* 10:226.
- O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 8:275.
- Mosmann, T. R. and Sad, S. 1996. The expanding universe of T-cell subsets: T_H1, T_H2 and more. *Immunol. Today* 17:138.
- Borish, L. and Rosenwasser, L.J. 1998. Cytokines in allergy inflammation. In Middleton, E. J., Reed, C. E., Ellis, E. F., Adkinson, N. F., Jr, Yunginger, J. W. Jr and Busse, W. W., eds, *Allergy Principles and Practice*, 5th edn, p. 108. Mosby, St Louis, MO.
- Coffman, R. L., Seymour, B. W., Lebman, D. A., Hiraki, D. D., Christiansen, J. A., Shrader, B., Cherwinski, H. M., Savelkoul, H. F., Finkelman, F. D., Bond, M. W., et al. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 102:5.
- McHugh, S. M., Deighton, J., Stewart, A. G., Lachmann, P. J. and Ewan, P. W. 1995. Bee venom immunotherapy induces a shift in cytokine responses from a TH-2 to a TH-1 dominant pattern: comparison of rush and conventional immunotherapy. *Clin. Exp. Allergy* 25:828.
- Jutel, M., Pichler, W. J., Skrbic, D., Urwyler, A., Dahinden, C. and Muller, U. R. 1995. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J. Immunol.* 154:4187.
- Van Uden, J. and Raz, E. 1999. Immunostimulatory DNA and applications to allergic disease. *J. Allergy Clin. Immunol.* 104:902.
- Kline, J. N., Waldschmidt, T. J., Businga, T. R., Lemish, J. E., Weinstock, J. V., Thorne, P. S. and Krieg, A. M. 1998. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J. Immunol.* 160:2555.
- Broide, D., Schwarze, J., Tighe, H., Gifford, T., Nguyen, M. D., Malek, S., Van Uden, J., Martin-Orozco, E., Gelfand, E. W. and Raz, E. 1998. Immunostimulatory DNA sequences inhibit IL-5, eosinophilic inflammation, and airway hyperresponsiveness in mice. *J. Immunol.* 161:7054.
- Sur, S., Wild, J. S., Choudhury, B. K., Sur, N., Alam, R. and Klinman, D. M. 1999. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J. Immunol.* 162:6284.
- Hartl, A., Kiesslich, J., Weiss, R., Bernhaupt, A., Mostbock, S., Scheibhofer, S., Ebner, C., Ferreira, F. and Thalhamer, J. 1999. Immune responses after immunization with plasmid DNA encoding Bet v 1, the major allergen of birch pollen. *J. Allergy Clin. Immunol.* 103:107.
- Jahn-Schmid, B., Wiedermann, U., Bohle, B., Repa, A., Kraft, D. and Ebner, C. 1999. Oligodeoxynucleotides containing CpG motifs modulate the allergic TH2 response of BALB/c mice to Bet v 1, the major birch pollen allergen. *J. Allergy Clin. Immunol.* 104:1015.
- Parronchi, P., Brugnolo, F., Annunziato, F., Manuelli, C., Sampognaro, S., Mavilia, C., Romagnani, S. and Maggi, E. 1999. Phosphorothioate oligodeoxynucleotides promote the *in vitro* development of human allergen-specific CD4⁺ T cells into T_H1 effectors. *J. Immunol.* 163:5946.
- Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D. and Ebner, C. 1999. Oligodeoxynucleotides containing CpG motifs induce IL-12, IL-18 and IFN-gamma production in cells from allergic individuals and inhibit IgE synthesis *in vitro*. *Eur. J. Immunol.* 29:2344.
- Tighe, H., Corr, M., Roman, M. and Raz, E. 1998. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today* 19:89.
- Spiegelberg, H. L., Tighe, H., Roman, M., Broide, D. and Raz, E. 1998. Inhibition of IgE formation and allergic inflammation by allergen gene immunization and by CpG motif immunostimulatory oligodeoxynucleotides. *Allergy* 53:93.
- Kovarik, J., Bozzotti, P., Love-Homan, L., Pihlgren, M., Davis, H. L., Lambert, P. H., Krieg, A. M. and Siegrist, C. A. 1999. CpG oligodeoxynucleotides can circumvent the T_H2 polarization of neonatal responses to vaccines but may fail to fully redirect T_H2 responses established by neonatal priming. *J. Immunol.* 162:1611.
- Bomford, R. 1980. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. I. Effect on the antibody response to bovine serum albumin and sheep red blood cells of Freund's incomplete and complete adjuvants, alhydrogel, *Corynebacterium parvum*, *Bordetella pertussis*, muramyl dipeptide and saponin. *Clin. Exp. Immunol.* 39:426.
- Chu, R. S., Targoni, O. S., Krieg, A. M., Lehmann, P. V. and Harding, C. V. 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (T_H1) immunity. *J. Exp. Med.* 186:1623.
- Peng, Z., Lam, H., Xu, W., Cheng, L., Chen, Y. L. and Simons, F. E. R. 1998. Characterization and clinical relevance of two recombinant mosquito *Aedes aegypti* salivary allergens, rAed a 1 and rAed a 2. *J. Allergy Clin. Immunol.* 101:S32.
- Peng, Z., Li, H. and Simons, F. E. R. 1998. Immunoblot analyses of salivary allergens in 10 mosquito species with world-wide distributions, and the IgE responses to these allergens. *J. Allergy Clin. Immunol.* 101:498.
- James, A. A., Blackmer, K., Marinotti, O., Ghosn, C. R. and Racioppi, J. V. 1991. Isolation and characterization of the gene expressing the major salivary gland protein of the female mosquito, *Aedes aegypti*. *Mol. Biochem. Parasitol.* 44:245.
- Wang, H., Mao, X., Simons, F. E. R. and Peng, Z. 1999. Induction of IgE responses using a recombinant mosquito salivary allergen rAed a 2 without adjuvant in mice. *Int. Arch. Allergy Immunol.* 120:135.
- Jansson, B., Brodin, T. and Sjogren, H. O. 1988. A biotin-labelled antigen radioimmunoassay (BILA) for antibodies to membrane antigens useful for monoclonal antibody screening. *J. Immunol. Methods* 115:219.
- Peng, Z., Yang, M. and Simons, F. E. R. 1995. Measurement of mosquito *Aedes vexans* salivary gland-specific IgE and IgG antibodies and the distribution of these antibodies in human sera. *Ann. Allergy Asthma Immunol.* 74:259.
- Chen, Y. L., Simons, F. E. R. and Peng, Z. 1998. A mouse model of mosquito allergy for study of antigen-specific IgE and IgG subclass responses, lymphocyte proliferation, and IL-4 and IFN-gamma production. *Int. Arch. Allergy Immunol.* 116:269.
- Peng, Z., Mao, X. and Simons, F. E. R. 2000. Prevention of airways eosinophilia in mice receiving subsequent sensitization and in mice with ongoing IgE responses, using CpG Oligodeoxynucleotides. *J. Allergy Clin. Immunol.* 105:576.
- Ishizaka, T. 1988. Mechanisms of IgE-mediated hypersensitivity. In Middleton, E. J., Reed, C. E., Ellis, E. F., Adkinson, N. F. J. and Yunginger, J. W. J., eds, *Allergy: Principles and Practice*, 3rd edn, p. 71. Mosby, St Louis, MO.
- Kline, J. N., Krieg, A. M., Waldschmidt, T. J., Ballas, Z. K., Jain, V. and Businga, T. R. 1999. CpG oligodeoxynucleotides do not require TH1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J. Allergy Clin. Immunol.* 104:1258.
- Snapper, C. M., Finkelman, F. D., Stefany, D., Conrad, D. H. and Paul, W. E. 1988. IL-4 induces co-expression of intrinsic membrane IgG1 and IgE by murine B cells stimulated with lipo polysaccharide. *J. Immunol.* 141:489.
- HayGlass, K. T. and Stefura, B. P. 1991. Anti-interferon gamma treatment blocks the ability of glutaraldehyde-polymerized allergens to inhibit specific IgE responses. *J. Exp. Med.* 173:279.
- Van Halteren, A. G., van der Cammen, M. J., Cooper, D., Savelkoul, H. F., Kraal, G. and Holt, P. G. 1997. Regulation of antigen-specific IgE, IgG1, and mast cell responses to ingested allergen by mucosal tolerance induction. *J. Immunol.* 159:3009.

- 35 McCluskie, M. J. and Davis, H. L. 1998. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J. Immunol.* 161:4463.
- 36 Steinberger, P., Bohle, B., di Padova, F., Wrann, M., Liehl, E., Scheiner, O., Kraft, D. and Valenta, R. 1995. Allergen-specific IgE production of committed B cells from allergic patients *in vitro*. *J. Allergy Clin. Immunol.* 96:209.
- 37 Randolph, D. A., Carruthers, C. J., Szabo, S. J., Murphy, K. M. and Chaplin, D. D. 1999. Modulation of airway inflammation by passive transfer of allergen-specific T_H1 and T_H2 cells in a mouse model of asthma. *J. Immunol.* 162:2375.
- 38 Hamelmann, E., Tieda, K., Oshiba, A. and Gelfand, E. W. 1999. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness—a murine model. *Allergy* 54:297.
- 39 Taylor, W. A., Sheldon, D. and Francis, D. H. 1980. IgE antibody formation in BALB/c mice without adjuvant: induction of responses to grass pollen extract and to a hapten-carrier conjugate. *Immunology* 39:583.
- 40 Yang, X., HayGlass, K. T. and Brunham, R. C. 1996. Genetically determined differences in IL-10 and IFN-gamma responses correlate with clearance of *Chlamydia trachomatis* mouse pneumonitis infection. *J. Immunol.* 156:4338.
- 41 Beasley, R., Keil, U., von Mutius, E. and Pearce, N. 1998. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee [see Comments]. *Lancet* 351:1225.
- 42 Holgate, S. T. 1999. The epidemic of allergy and asthma. *Nature* 402:B2-4.