

Oligodeoxynucleotides containing CpG motifs modulate the allergic T_{H2} response of BALB/c mice to Bet v 1, the major birch pollen allergen

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Background: The use of adequate adjuvants to modulate the allergic T_{H2} -type immune response is a promising concept for future immunotherapy of type I allergy. Bacterial DNA or oligodeoxynucleotides containing CpG motifs (CpG-ODNs) have been demonstrated to foster T_{H1} -type immune responses. **Objective:** We investigated the adjuvanticity of CpG-ODNs and their capability to modulate the allergic T_{H2} response in a mouse model.

Methods: BALB/c mice were treated with CpG-ODNs and Bet v 1, the major birch pollen allergen, in different experimental setups. Allergen-specific antibody responses, T_H cytokines, and eosinophilic infiltration of the airways were investigated.

Results: Intraperitoneal administration of Bet v 1 together with aluminium hydroxide led to a typical T_{H2} response. In contrast, coadministration of CpG-ODNs with Bet v 1 in aluminium hydroxide resulted in markedly increased T_{H1} activities (high IgG2a levels) and subsequently to reduced airway inflammation. The T_{H1} -like immune response indicated by these humoral findings was also reflected by decreased IL-5 and increased IFN- γ levels in cell cultures. CpG-ODNs as sole adjuvants with Bet v 1 did not lead to measurable Ig responses after subcutaneous or intraperitoneal immunizations; after intranasal application, 3 of 10 mice reacted. Nevertheless, a prophylactic effect was obtained with all routes tested; that is, mice treated subsequently with an established aerosol sensitization protocol displayed altered immune responses characterized by drastically elevated levels of Bet v 1-specific IgG2a, indicating a T_{H1}/T_{H0} -like immunity. Application of CpG-ODNs after aerosol sensitization also induced IgG2a.

Conclusion: By inducing T_{H1}/T_{H0} -biased immune responses to allergens, the use of CpG-ODNs as adjuvants may have important impacts for new forms of specific immunotherapy in type I hypersensitivity. (*J Allergy Clin Immunol* 1999;1015-23.)

Key words: CpG oligodeoxynucleotides, immunomodulation, type I allergy, T_{H1}/T_{H2} , Bet v 1, BALB/c mice

Abbreviations used

AS:	Aerosol sensitization
BAL:	Bronchoalveolar lavage
CpG-ODN:	Oligodeoxynucleotide containing CpG motifs
ISS:	Immunostimulatory sequence
SIT:	Specific immunotherapy

The prevalence of type I allergy has tremendously increased in recent years, with approximately 20% of the population having allergic rhinitis, conjunctivitis, or bronchial asthma mostly caused by airborne inhalant allergens.¹ Among these, birch pollen represents an important allergen source, containing Bet v 1 as the major allergen.² In allergic individuals the dominance of T_{H2} -type cytokines (IL-4, IL-5, and IL-13) leads to the production of IgE antibodies against allergenic molecules.³ Specific immunotherapy (SIT) has been the therapy of choice for more than 80 years in the treatment of type I hypersensitivity to airborne allergens.⁴ Successful SIT (ie, injection of increasing doses of specific allergen to induce tolerance) has been associated with a shift from T_{H2} - to T_{H1}/T_{H0} -like cytokine responses indicated by decreased IL-4 and/or increased IFN- γ production of allergen-specific T cells.⁴ In addition, a strong inverse association between delayed-type hypersensitivity responses (T_{H1}) to *Mycobacterium tuberculosis* and atopic disorders was found recently.⁵ Thus immune deviation of T cells toward T_{H0}/T_{H1} responses may represent a promising approach in therapy and possibly even in prophylaxis of type I allergy.

In this respect the use of T_{H1} -inducing adjuvants in SIT should be considered in the development of allergy vaccines.^{6,7} Besides well-known surface components of bacterial origin, lately bacterial DNA and synthetic oligodeoxynucleotides (ODNs) containing immunostimulatory DNA sequences have been shown to exhibit adjuvant activity and to induce T_{H1} -like immune responses.⁸⁻¹² These effects are mainly due to unmethylated CpG dinucleotides in certain sequence contexts (CpG motifs), which are present at the expected frequency in bacterial DNA but are underrepresented (CpG suppression) in vertebrate DNA.¹³ ODNs containing CpG motifs (CpG-ODNs) elicit an innate immune response characterized by polyclonal B-cell activation and T_{H1} -type cytokine production dominated by IL-12 and IFNs, leading to natural killer-cell activation and induction of cytotoxic T lymphocytes.^{8-10,14,15} The potent immunologic response

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to unmethylated CpG motifs suggests that bacterial DNA serves as an evolutionary conserved ligand for the vertebrate immune system, which recognizes this structural pattern as a danger signal.¹⁶ The mechanisms through which immunostimulatory DNA exerts its drastic immune activation are still unclear. Cells directly responsive to CpG-ODNs include macrophages, dendritic cells, B cells, and natural killer cells.¹⁷ Uptake of DNA is sequence independent and temperature and energy dependent. Once internalized, nuclear factor κ B, activator protein, or both are activated, which leads to expression of early-response genes and subsequent cytokine production.¹⁸

Recent studies in mice with protein antigens, such as ovalbumin, hen egg lysozyme, fowl γ -globulin, or hepatitis B surface antigen, demonstrated an adjuvant effect of systemically administered CpG-ODNs, especially when applied together with liposomes, incomplete Freund's adjuvant, or aluminium hydroxide (Al(OH)₃).^{10,12,19,20} With respect to allergic immune responses, it has been reported that genetic immunization with plasmids containing immunostimulatory sequences (ISS) resulted in a preferential induction of T_{H1} responses,^{21,22} such as immunoprophylaxis.²³ Thus far CpG-ODNs have only been reported as immunomodulating adjuvants for model allergens, such as β -galactosidase or ovalbumin.^{11,24}

The objective of this study was to investigate whether CpG-ODNs may serve as adjuvants for allergens to possibly improve the efficacy of conventional SIT. Recombinant Bet v 1,²⁵ a relevant inhalant allergen, was used in BALB/c mice.²⁶ CpG-ODNs were examined for (1) their modulatory effect on a T_{H2} response induced by Al(OH)₃, which is currently used in SIT; (2) their adjuvant effect per se, comparing mucosal and parenteral routes for immunization; (3) their effect in a presensitization (prophylaxis) experimental set-up; and (4) their effect in a postsensitization (therapy) experimental set-up. To evaluate immune responses induced by CpG-ODNs, Ig subclass distribution and cytokine expression were assessed, as well as their effect on airway inflammation. Our results, showing T_{H0}/T_{H1}-biased allergen-specific immune responses induced by CpG-ODNs, support the potential use of such immunomodulators in the development of new prophylactic and therapeutic strategies in the treatment of type I allergy. The mucosal (eg, nasal) application of CpG-ODNs appears to be especially promising.

METHODS

ODNs and allergens

ODNs with phosphorothioate modification of the backbone were synthesized and HPLC purified by MWG Biotech (Munich, Germany). The 20-mer used contained 3 CpG motifs (ie, ATCGACTCTCGAGCGTTCTC).⁹ In some experiments a control ODN lacking CpG motifs was used (ie, ATGCACTCTGCAGGCTTCTC). The endotoxin content of the ODN was undetectable (<0.1 EU/6 μ g of DNA) by means of Limulus assay (BioWhittaker Bioproducts, Walkersville, Md). Birch pollen (Allergon AB, Engelholm, Sweden) was extracted in PBS, as previously described,⁷ containing approximately 25% natural Bet v 1. Recombinant (r)Bet v 1 was obtained from Biomay GmbH (Linz, Austria).

Animals

Female BALB/c mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) and used at an age of 7 to 10 weeks. All experiments were approved by the Animal Experimentation Ethics Committee of the University of Vienna and the Ministry of Science and Research.

Experimental design

Four approaches were made to test the adjuvanticity of CpG-ODNs in the context of Bet v 1. First, for CpG-ODNs as immunomodulators of the Al(OH)₃-induced T_{H2} response, CpG-ODNs or control ODNs were coinjected 3 times, or CpG-ODNs were preinjected (3 days in advance) intraperitoneally with Bet v 1 and Al(OH)₃ (group 1). Second, for CpG-ODNs as sole adjuvants, mice were immunized 3 times intranasally, subcutaneously, or intraperitoneally with CpG-ODNs and Bet v 1 (group 2). Third, to trace possible priming or prophylactic effects, parallel immunized groups (as in group 2) were subsequently subjected to the aerosol sensitization protocol, as described below (group 3). Fourth, for CpG-ODNs in postsensitization treatment, sensitized mice were treated simultaneously 3 times both intranasally and intraperitoneally with ISS-ODN plus Bet v 1, whereas the respective sensitized control group received PBS plus allergen (group 4). For groups 3 and 4, see Fig 1. Groups consisted of 5 mice each.

Immunization-aerosol sensitization

For intraperitoneal coadministration experiments (group 1), 10 μ g of Bet v 1 and 10 nmol of CpG-ODNs (or control ODNs) were mixed with 100 μ L (2 mg) of Al(OH)₃ (Serva, Heidelberg, Germany). Control groups or mice that had been pretreated with CpG-ODNs intraperitoneally before received Bet v 1 and Al(OH)₃ alone. For mucosal immunizations (groups 2 and 3), 10 μ g of Bet v 1 and 10 nmol of CpG-ODN in 40 μ L of PBS were applied intranasally after mice had been anesthetized with metoxyfluorane (Metofane; Mallinckrodt Veterinary Inc, Mundelein, Ill). For systemic immunizations (groups 2 and 3), 10 μ g of Bet v 1 and 10 nmol of CpG-ODN in 150 μ L of PBS were injected subcutaneously at 3 different sites on the back or intraperitoneally, respectively. Aerosol sensitization (AS; groups 3 and 4) was performed by means of one intraperitoneal injection of 1 μ g of Bet v 1 in 50 μ L of PBS adsorbed to 100 μ L of Al(OH)₃. Fourteen days later, mice were exposed 10 times to aerosolized birch pollen extract, as previously described in detail.⁷ To assess postsensitization effectiveness (group 4), 10 μ g of Bet v 1 plus 10 nmol of CpG-ODN in PBS were applied both intranasally and intraperitoneally 14 days after sensitization. Control mice received Bet v 1 in PBS only.

Sampling

Blood samples were taken by means of tail bleeding before each immunization-sensitization or before mice were killed 7 days after the last immunization. Sera were prepared and stored at -20°C until analysis. Bronchoalveolar lavage (BAL) fluid from killed mice was obtained as described previously.⁷ Lungs were washed twice with 0.8 mL of ice-cold PBS and with approximately 1.4-mL recovered volume of BAL volume.

Airway eosinophilia

To evaluate the effect of CpG-ODNs on airway inflammation, mice were immunized 3 times intraperitoneally at 14-day intervals with 10 nmol of CpG-ODNs with Al(OH)₃ and Bet v 1 or with Al(OH)₃ and Bet v 1 without CpG-ODNs as a control. Thereafter, mice were challenged twice 7 and 8 days after the last immunization with 40 mg/4 mL aerosolized birch pollen extract. Three days later, BAL was collected, and samples were spun onto microscope

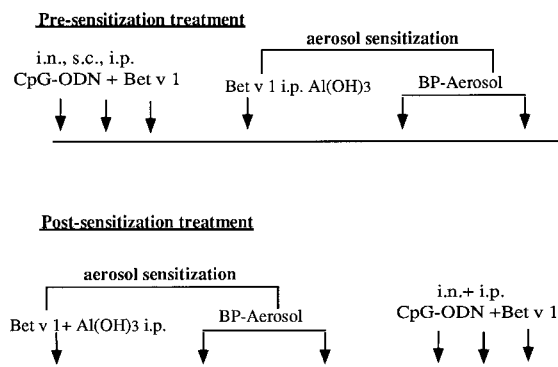


FIG 1. Experimental approach: presensitization and postsensitization treatment with CpG-ODNs plus Bet v 1. Mice were immunized at 14-day intervals and killed 7 days after the last treatment. *i.n.*, Intranasal; *s.c.*, subcutaneous; *i.p.*, intraperitoneal; *BP*, Birch pollen.

slides, air-dried, and stained with hematoxylin and eosin (Hemacolor; Merck, Darmstadt, Germany). At least 200 cells were counted and differentiated by light microscopy according to standard morphologic criteria, and the percentage of eosin-positive cells was determined. At day 6 after challenge, lung-associated lymphocytes were isolated from identically treated mice and analyzed as described below.

Determination of allergen-specific antibodies

Bet v 1-specific antibodies were measured in an ELISA, as previously described.²⁶ For coating, 2 µg of Bet v 1 were used. Serum samples were diluted 1:2000 for IgG1, 1:500 for IgG2a, and 1:10 for IgE, and BAL fluid was used undiluted to determine IgA levels. Subclass-specific rat anti-mouse antibodies to IgG1, IgG2a, IgE, and IgA were diluted 1:500 (Pharmingen, San Diego, Calif), and the peroxidase-conjugated mouse anti-rat IgG was diluted 1:2000 (Jackson ImmunoLabs Inc, Westgrove, Pa). Antibody levels were indicated in OD units.

Determination of proliferation and cytokines

Allergen-specific proliferation of spleen cells or lung-associated lymphocytes was assessed in RPMI medium containing 10% FCS, as described previously.²⁶ Here, 2 × 10⁵ cells/200 µL were stimulated with or without birch pollen extract (25 µg/mL) or Bet v 1 (10 µg/mL) for 5 days. Tritiated thymidine was added for the last 16 hours of incubation, and proliferation was determined by scintillation counting. To determine cytokine production, 5 × 10⁶ spleen cells/500 µL were stimulated with and without birch pollen extract (20 µg/mL). Supernatants were collected after 24 hours (IL-5) and 48 hours (IFN-γ). Samples within the same group were pooled and stored at -20°C. The cytokine concentrations were determined by using commercially available ELISA kits (Endogene, Cambridge, Mass; sensitivity limits: 15 pg/mL for IFN-γ and 5 pg/mL for IL-5). Allergen-induced cytokine production was obtained by subtracting the values found in control cultures without stimulant.

Statistical analysis

The Mann-Whitney *U* test was used for statistical analysis.

RESULTS

Humoral immune responses

Modulation of the Al(OH)₃-induced response to Bet v 1 (group 1). To induce T_{H2} responses to Bet v 1, mice

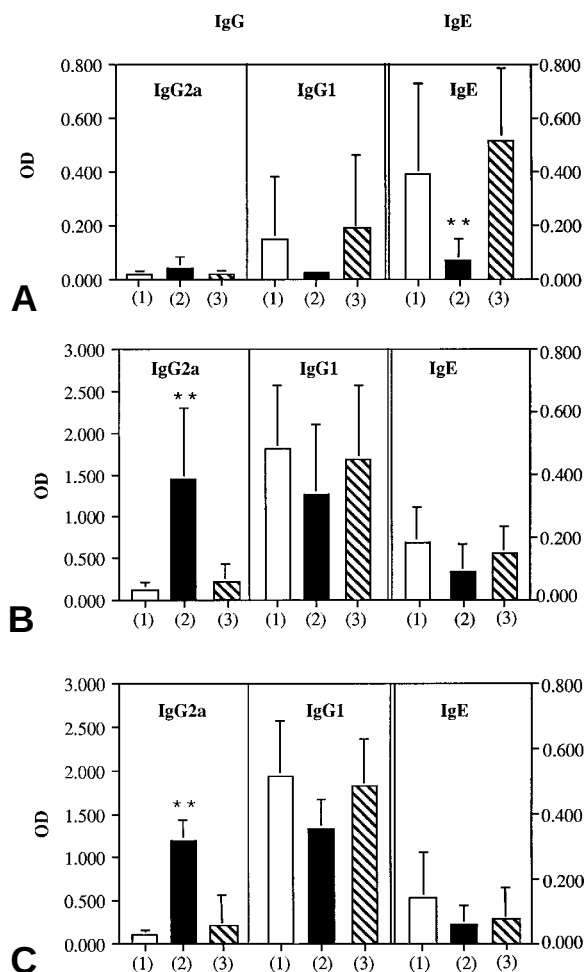


FIG 2. Coadministration of CpG-ODNs modulates the Al(OH)₃-induced immune response to Bet v 1. Ig isotype distribution of Bet v 1-specific antibodies in sera after 1 (**A**), 2 (**B**), or 3 (**C**) intraperitoneal immunizations. Comparison of mice that had received Bet v 1 in Al(OH)₃ alone (1), in Al(OH)₃ plus CpG-ODNs (2), or in Al(OH)₃ plus control ODNs (3) is shown. Sera were diluted 1:2000 for IgG1, 1:500 for IgG2a, and 1:10 for IgE. ELISA OD units are shown (means ± SD; ***P* < .01).

were immunized 3 times intraperitoneally with Bet v 1 in Al(OH)₃ at 14-day intervals and killed 7 days after the last treatment. Bet v 1-specific antibodies were investigated in the respective sera by means of ELISA. The humoral immune response was dominated by IgG1 and IgE production (Fig 2, A-C, 1). To assess whether CpG-ODNs can modulate the Al(OH)₃-induced T_{H2} response, CpG-ODNs were coadministered with Bet v 1 and Al(OH)₃, and results were compared with those from mice immunized with allergen in Al(OH)₃ alone. A 20-mer ODN with a nuclease-resistant phosphorothioate backbone containing 3 CpG motifs was used (Fig 2, A-C, 2). A significant increase of IgG2a antibodies (*P* = .002) was observed after 2 and 3 immunizations when compared with the control group, which had received Bet v 1 in Al(OH)₃ only. IgE antibodies were significantly suppressed after the first immunization (*P* = .011), whereas

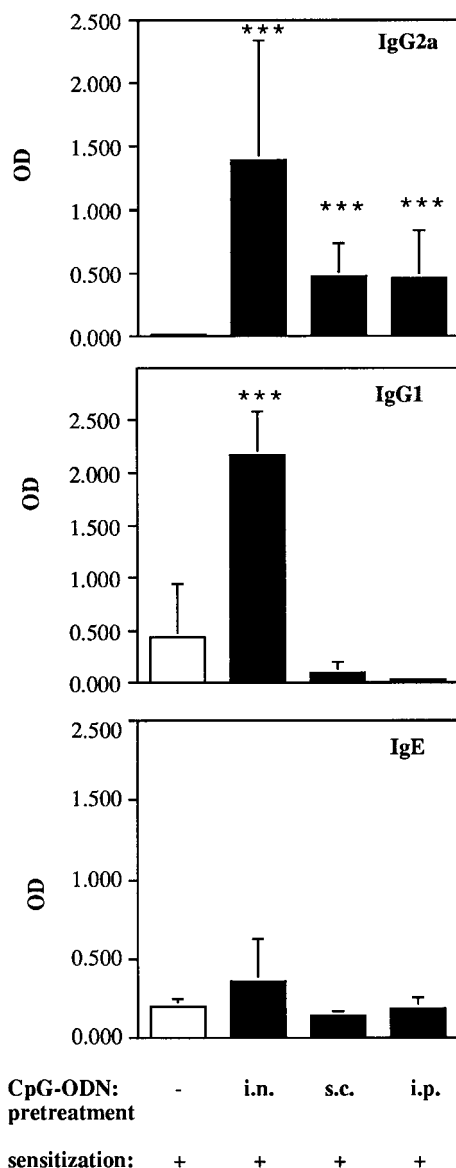


FIG 3. Treatment with CpG-ODNs before aerosol sensitization. Ig isotype distribution of Bet v 1-specific antibodies in sera of BALB/c mice that had been subjected to an AS protocol (see the "Methods" section) or that had been treated 3 times with CpG-ODNs intranasally (*i.n.*), subcutaneously (*s.c.*), or intraperitoneally (*i.p.*) before AS. ELISA OD units are shown (means $\pm$ SD; *** P < .001).

similar levels were found in both groups after the following injections. No significant differences were obtained concerning Bet v 1-specific IgG1 levels. In parallel coadministration experiments mice were immunized with control ODNs lacking CpG motifs in addition to $\text{Al}(\text{OH})_3$ plus Bet v 1. When compared with $\text{Al}(\text{OH})_3$ plus Bet v 1 alone, no effect on the Ig isotype distribution was observed (Fig 2, A-C, 3).

We presumed that a preceding injection of CpG-ODNs might increase their modulatory capacity by activating the innate immune system and creating a favorable

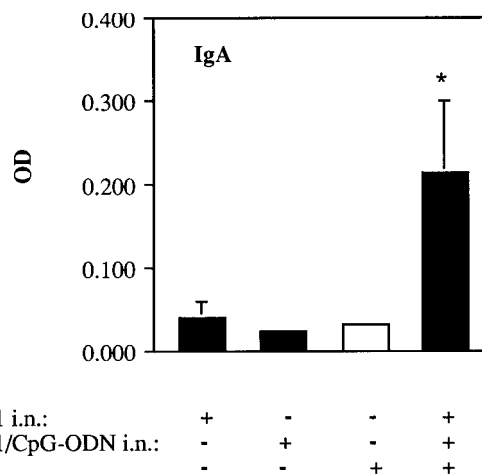


FIG 4. Priming for Bet v 1-specific IgA by intranasal (*i.n.*) treatment with Bet v 1 and CpG-ODNs. IgA levels of BALB/c mice after 3x Bet v 1 administered intranasally (*i.n.*), 3x Bet v 1 plus CpG-ODNs administered intranasally, AS (see the "Methods" section), or 3x Bet v 1 plus CpG-ODNs administered intranasally and subsequent AS. ELISA OD units are shown (means $\pm$ SD; * P < .05).

cytokine environment in the mice before they were immunized specifically with allergen in $\text{Al}(\text{OH})_3$. However, preadministration of CpG-ODNs 3 days before immunization with Bet v 1 in $\text{Al}(\text{OH})_3$ did not lead to markedly different results compared with those achieved by coadministration (data not shown).

CpG-ODNs as sole adjuvant for Bet v 1 (group 2). Mucosal and systemic application of CpG-ODNs as an adjuvant for Bet v 1 were examined. Systemic subcutaneous or intraperitoneal immunizations of 10 mice each did not lead to detectable Bet v 1-specific antibody production. After 3 intranasal applications, Bet v 1-specific antibody responses with IgG2a as the dominating Ig isotype developed in 3 of 10 mice (data not shown).

CpG-ODN treatment before aerosol sensitization (group 3). In contrast, a distinct priming effect was obtained by all 3 routes of applying CpG-ODNs plus Bet v 1, when mice were subsequently subjected to an established aerosol-sensitization protocol (Fig 3). Sensitized control animals showed a typical T_H2 response to allergen, namely very low levels of IgG2a and high concentrations of IgG1 and IgE. Compared with these control mice, intranasal, subcutaneous, and intraperitoneal preimmunizations with Bet v 1 and CpG-ODNs caused a prominent induction of IgG2a (P = .001). IgG1 was significantly higher only in intranasally pretreated animals (P < .001). At the same time, IgE levels were increased (intranasal) or not influenced (subcutaneous and intraperitoneal). Moreover, mucosal priming, as indicated by allergen-specific IgA levels in BAL fluid, was observed in mice pretreated intranasally with CpG-ODNs plus Bet v 1 (Fig 4).

CpG-ODN treatment after aerosol sensitization (group 4). To investigate whether an ongoing T_H2 immune response can be influenced by vaccination

with CpG-ODNs, sensitized mice were subsequently treated 3 times intranasally and intraperitoneally with Bet v 1 and CpG-ODNs or with Bet v 1 in PBS as control (Fig 1, B). The results are shown in Fig 5. AS led to a T_{H2} -like Bet v 1-specific response with a predominant expression of IgG1 and IgE. At later time points, the antibody levels of the control group decreased slightly. In contrast, a drastic and gradual increase of Bet v 1-specific IgG2a was observed within the group treated with CpG-ODNs plus Bet v 1. Consequently, the CpG-ODN-treated mice displayed significantly higher IgG2a levels compared with those found in the control group ($P = .044$, $P = .007$, and $P = .003$, respectively). IgG1 was not significantly ($P > .05$) enhanced by the treatment with Bet v 1 and CpG-ODNs. However, compared with control mice, IgG1 antibodies were significantly increased ($P = .004$, $P = .001$, $P = .004$, Fig 5). This difference is due in part to the time-dependent decrease of IgG1 in the control mice after sensitization. Allergen-specific IgE was not boosted by the application of CpG-ODNs with Bet v 1 and was not significantly increased compared with the control group. No differences were obtained in IgA levels from BAL fluid (OD, 0.119 ± 0.027 vs 0.130 ± 0.04 , respectively; data not shown).

Cellular responses

Antigen-specific stimulation of spleen cell cultures with Bet v 1 or birch pollen extract generally resulted in similar proliferations in all groups immunized with allergen (data not shown). To characterize the T_H cytokine profile in supernatants of spleen cell cultures, IFN- γ and IL-5 were determined as representative T_{H1} versus T_{H2} cytokines. Table I summarizes the cytokine production of splenocytes stimulated with birch pollen extract. Compared with the respective control groups, CpG-ODNs led to markedly increased IFN- γ production in groups 2 (after intranasal application of CpG-ODNs plus Bet v 1) and 3 (treated intranasally or subcutaneously with Bet v 1 plus CpG-ODNs before sensitization). Addition of CpG-ODNs to intraperitoneal immunizations with Bet v 1 in $Al(OH)_3$ (group 1) did not alter the IFN- γ production in our *in vitro* system. The application of CpG-ODNs after sensitization (group 4) caused no marked increase in IFN- γ levels.

In coadministration experiments (group 1) IL-5 was drastically reduced compared with levels found in the respective control mice. A distinct reduction of IL-5 compared with immunization with $Al(OH)_3$ was observed in mice immunized with CpG-ODNs intranasally, subcutaneously, or intraperitoneally as the sole adjuvant (group 2). In parallel groups with subsequent sensitization, IL-5 was diminished in intranasally pretreated mice but was even more drastically reduced in the systemically pretreated mice when compared with the sensitized control group (group 3). Application of CpG-ODNs to already sensitized mice (ie, after the allergic $Th2$ response was already established; group 4) did not lead to a marked reduction of IL-5.

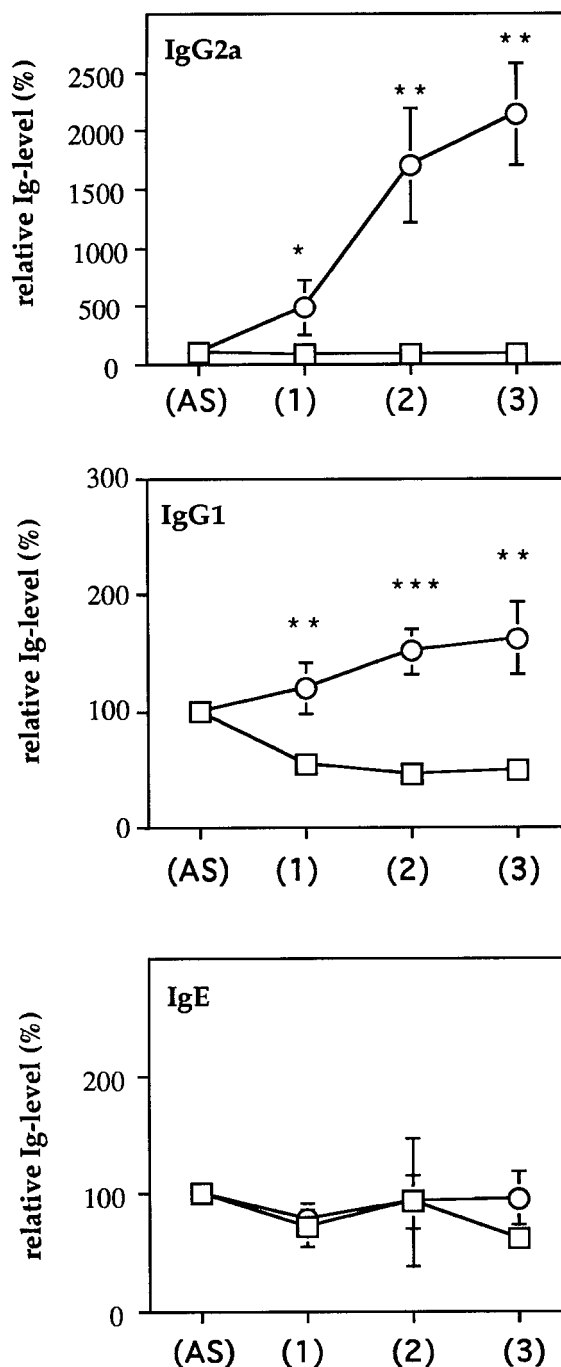


FIG 5. Treatment after aerosol sensitization. Aerosol-sensitized mice were treated with Bet v 1 plus CpG-ODNs simultaneously intranasally and intraperitoneally (circles) compared with Bet v 1 in PBS (squares). Ig isotype distribution of Bet v 1-specific antibodies at different time points: after sensitization (AS), and the first (1), second (2), and third application (3). Because of differences between the 2 groups at the time of sensitization, Ig levels were calculated as the percentage of the OD measured after sensitization (* $P < .05$, ** $P < .01$, and *** $P < .001$).

Airway inflammation

As shown in Table II, eosinophilic infiltration in the lung can be induced in mice immunized intraperitoneal-

TABLE I. Allergen-induced cytokine production of spleen cell cultures

Treatment	IFN- γ (pg/mL)	IL-5 (pg/mL)
Coadministration		
3 $\times$ Al(OH) ₃ + Bet v 1 ip	388*	8.9
3 $\times$ Al(OH) ₃ + Bet v 1 + CpG-ODN ip	401	0.4
ISS-ODN as adjuvant		
3 $\times$ Al(OH) ₃ + Bet v 1 ip	310	19.2
3 $\times$ CpG-ODN + Bet v 1 in	1913	0.8
3 $\times$ CpG-ODN + Bet v 1 sc	372	0.8
3 $\times$ CpG-ODN + Bet v 1 ip	571	0.5
ISS-ODN treatment before AS†		
AS	184	10.5
AS/3 $\times$ CpG-ODN + Bet v 1 in	1078	7.2
AS/3 $\times$ CpG-ODN + Bet v 1 sc	947	1.0
AS/3 $\times$ CpG-ODN + Bet v 1 ip	254	0.0
ISS-ODN treatment after AS		
AS/3 $\times$ PBS + Bet v 1 ip + in	0	7.5
AS/3 $\times$ CpG-ODN + Bet v 1 ip + in	25	6.3

ip, Intraperitoneal administration; in, intranasal administration; sc, subcutaneous administration.

*Concentration of cytokines in pooled supernatants of spleen cell cultures after 24 hours (IL-5) or 48 hours (IFN- γ) are shown. Values indicated represent change in picograms per milliliters (cytokine concentration after stimulation with allergen – medium control).

†For AS protocol, see the “Methods” section.

ly with Al(OH)₃ and Bet v 1 after subsequent exposure to aerosolized allergen. However, addition of CpG-ODNs to intraperitoneal immunizations with Bet v 1 in Al(OH)₃ resulted in significantly reduced eosinophilic inflammation and concomitantly decreased IL-5 expression in cell cultures of lung-associated lymphoid tissue (Table II).

DISCUSSION

SIT is the treatment of choice in type 1 allergy and has been successfully used in venom hypersensitivity and in patients with pollinosis.²⁷ A possible approach to further improve the effectiveness of SIT is to develop adjuvants that may be better suited than Al(OH)₃, which is conventionally injected subcutaneously together with allergen during SIT and has been shown to induce T_{H2} responses.^{7,28} In addition, other routes of application, especially mucosal application (oral, sublingual, and intranasal) could ease the tedious long-term SIT and favorably influence patient compliance.^{29,30} In the present study we investigated the adjuvanticity of CpG-ODNs, which are known to induce strong innate and antigen-specific T_{H1}-like immune responses. We show that CpG-ODNs, given together with allergens, can modulate the characteristic allergen-specific T_{H2} response in mice. A relevant inhalent allergen, recombinant Bet v 1, was used as the model allergen. Pilot experiments with phosphodiesterase CpG-ODNs together with Al(OH)₃ resulted in significantly increased allergen-specific IgG2a levels and no significant differences for allergen-specific IgG1 or IgE (data not shown). In our present study, CpG-ODNs with a more nuclease-resistant phosphorothioate modifi-

TABLE II. Reduced eosinophilic infiltration and IL-5 production in lungs of mice induced by means of CpG-ODNs

Treatment*	Eosinophils (%)	IL-5 (pg/mL)†
3 $\times$ Al(OH) ₃ + Bet v 1 ip	7.6 $\pm$ 2.8	599 $\pm$ 398
3 $\times$ Al(OH) ₃ + Bet v 1 + CpG-ODN ip	3.4 $\pm$ 2.9‡	210 $\pm$ 145

*After intraperitoneal immunization, mice were challenged with 1% aerosolized birch pollen extract; eosinophils were determined in BAL fluid at day 3 (n = 5) after challenge, and allergen-induced IL-5 production was determined at day 6 (n = 6) after challenge.

†IL-5 in 24-hour supernatants of lung-associated lymphoid cells. Values indicated represent the change in picograms per milliliters (cytokine concentration after stimulation with allergen – medium control).

‡P < .05.

cation of the backbone were used to increase the half-life of the oligonucleotides. It was demonstrated that first intraperitoneal coadministration (or pretreatment) of phosphorothioate CpG-ODNs induced a drastic increase of allergen-specific IgG2a and an initial suppression of Bet v 1-specific IgG1 and IgE. Second, CpG-ODNs proved to be weak immune-enhancing agents when applied as the sole adjuvant for Bet v 1. Only the intranasal route, but not systemic injections, led to detectable Ig responses; however, all routes of CpG-ODN pretreatment led to IFN- γ production in spleen cells, thereby creating a milieu for the T_{H1}-like priming effect, which was observed in mucosally and systemically preimmunized mice when they were subsequently subjected to an aerosol sensitization protocol. Third, treating sensitized animals with CpG-ODNs plus allergen led to strongly increased IgG2a levels. In general, the Ig-isotype distribution was consistent with the T_{H1}-biased cytokine profile of allergen-stimulated spleen cells.

Within our system, we confirm findings from other groups who characterized systemically applied CpG-ODNs as weak immune enhancers when used as the sole adjuvant for fowl γ -globulin immunization¹⁹ but as strong immunomodulators when applied together with other adjuvants.^{10,19,20,24} In our investigations the preferential induction of allergen-specific IgG2a in every immunization protocol tested demonstrates a strongly T_{H1}-biased immune response. The presence of IgG2a indicates the induction of IFN- γ , which is necessary as a switch factor for this isotype.³¹ Increased allergen-induced IFN- γ levels were especially found in spleen cell cultures of mice that had obtained CpG-ODNs by intranasal application. These findings are remarkable considering BALB/c mice as a strain exceptionally disposed to T_{H2}-like immune responses in general and in response to Bet v 1.^{26,32} IL-5 production, which is indicative for T_{H2}-type immune responses, was reduced in all groups that had received CpG-ODNs. When CpG-ODNs were used in a postsensitization regimen, only little effect was observed on IgE serum levels. However we obtained elevated IgG levels, especially IgG2a. This phenomenon is also noticed in the human disease. Even after successful immunotherapy, persistent IgE levels

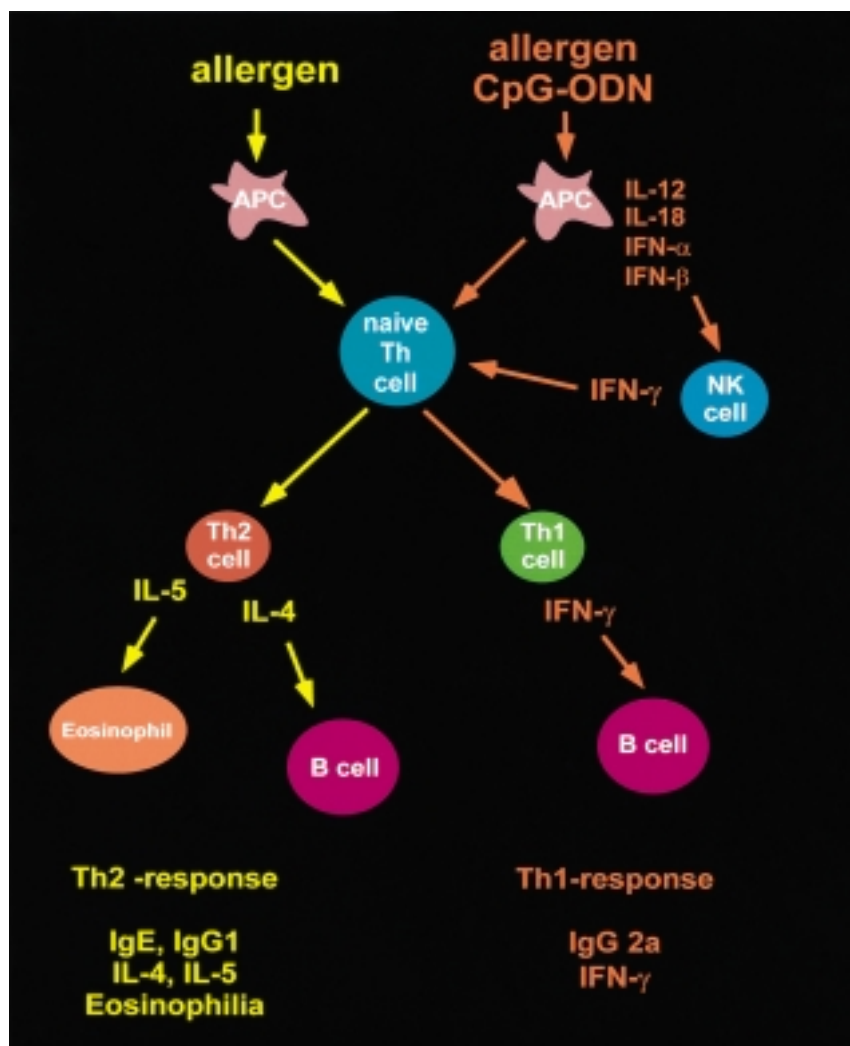


FIG 6. Proposed mechanism of the immunomodulating effects of CpG-ODNs on allergic T_H2 immune responses.

have been observed opposed to increased and possibly *blocking* IgG antibodies.⁴ It has been reported that extreme priming effects leading to established immune responses of either T_{H1} or T_{H2} type are difficult to redirect. Long-term effects of priming rather than boosting determine the type of Ig and cytokine responses.³³ However, in other experimental systems (eg, murine model of asthma with *Schistosoma* eggs as antigen or ovalbumin sensitization), CpG-ODNs have been shown to reverse ongoing allergic reactions, such as reduction of IgE production or protection from eosinophilia.^{21,23,24,34} Our opposing findings may be due to intrinsic properties of the allergen used.³⁵

The nasal-associated lymphoid tissue has been found to be a convenient site of vaccine application.³⁶ Recently, it was shown that in mice CpG-ODNs are also potent mucosal adjuvants for vaccination with influenza virus or hepatitis B surface antigen. CpG-ODNs applied intranasally together with inactivated virus³⁷ or viral pro-

tein³⁸ induced systemic IgG and mucosal sIgA production. Also in our system, allergen-specific sIgA in BAL was inducible by intranasal pretreatment with CpG-ODNs plus Bet v 1 (Fig 4). The importance of specific IgA in allergy is a matter of debate.³⁹ However, IgA induction by CpG-ODNs has important implications in vaccine development for infectious diseases transmitted through mucosal surfaces, such as influenza.³⁷ Other possible applications for CpG-ODNs in future vaccines have been proposed (eg, against intracellular parasites^{40,41} or in tumor therapy⁴²).

The doses of CpG-ODNs used in our study did not lead to any obvious vital complications compared with those found in control mice. However, for the use of CpG-ODNs as the adjuvant in human individuals, the potential toxicity is of concern. Perhaps because of sequence properties, CpG-ODNs have been described to induce high levels of TNF- α and thus lead to shock symptoms in mice.⁴³ Other side effects may include

excessive inflammatory reactions⁴⁴ or activation of autoreactive T cells.⁴⁵ Because immunogenicity is improved when CpG-ODNs are physically linked to the relevant antigen,⁴⁶ allergen-CpG-ODN conjugates may permit the reduction of CpG-ODN doses in future SIT vaccines. The application of CpG-ODNs together with hypoallergenic variants of allergens^{47,48} could provide us with highly efficient and safe new forms of SIT (Fig 6).

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