

Vaccination with the Recombinant Allergen ProDer p 1 Complexed with the Cationic Lipid diC14-amidine Prevents Allergic Responses to House Dust Mite

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Available online 9 March 2005

The present study evaluated the prophylactic potential of ProDer p 1, the recombinant precursor form of the major mite allergen Der p 1, combined with the cationic lipid diC14-amidine in a murine model of house dust mite allergy. Naïve mice vaccinated with the amidine/allergen complex developed a Th1-biased immune response characterized by the absence of specific IgE, the production of specific IgG2a, and the presence of IFN- γ in splenocyte cultures. In contrast, ProDer p 1 adjuvanted with alum induced typical strictly Th2-biased allergic responses with strong IgG1 and IgE titers and IL-5 secretion. Removal of negatively charged sialic acids in ProDer p 1 or increasing the ionic strength reduced the binding of ProDer p 1 to the cationic liposomes and resulted in a decrease of the allergen immunogenicity, suggesting that complexation is required for triggering an optimal immune response. Finally, prophylactic vaccination with ProDer p 1–diC14-amidine reduced drastically the production of specific IgE and airway eosinophilia following subsequent immunization with Der p 1–alum and challenge with aerosolized house dust mite extracts. In conclusion, recombinant ProDer p 1 complexed with diC14-amidine could represent an efficient prophylactic vaccine against house dust mite allergy.

Key Words: allergy, ProDer p 1, diC14-amidine, IgE, eosinophilia, cationic liposome, challenge

INTRODUCTION

House dust mite allergens from the species *Dermatophagoides pteronyssinus* are major causative factors associated with various allergic diseases such as asthma [1]. Among these allergens, Der p 1 is the most immunodominant antigen, since the prevalence of anti-Der p 1 IgE antibodies in allergic patients is higher than 80% [2,3]. The potent allergenicity of Der p 1 is not fully understood but recent data provide evidence that it is associated with its cysteine proteinase activity [4,5]. Although the biological properties of Der p 1 are well characterized, only few studies have been performed to examine immune responses after allergen immunization in a mouse model of house dust mite-induced airway changes [6–9]. Indeed, mice injected with adjuvanted Der p 1 and exposed to house dust mite aerosols developed Th2-dominant

immune responses characterized by a specific IgE production, eosinophil recruitment in bronchoalveolar lavage fluids, and airway hyperresponsiveness. Th2 cells play a pivotal role in allergy, notably by secreting cytokines of type IL-4, which are required to drive the production of allergen-specific IgE, and IL-5, which regulates the development of eosinophilia. Deviation of the allergic immune responses from Th2- to Th1-like should provide an effective therapeutic approach for treating this chronic disease, as Th1 cells secrete IL-2 and IFN- γ , which suppress IgE synthesis through direct effects on B cells [10,11].

The production of recombinant allergens provides promising tools for allergy diagnosis as well as for the development of novel vaccines for the treatment of allergic diseases [12–14]. In addition, protein-based vaccines do not present the risk of DNA integration, making this approach safer for clinical applications. We recently produced at high yield a recombinant precursor form of Der p 1, secreted as ProDer p 1 in insect and CHO cells

Abbreviations used: diC14-amidine, 3-tetradecylamino-*N*-tert-butyl-*N'*-tetradecylpropionamide.

[15,16]. Recombinant ProDer p 1 displayed IgG and IgE binding characteristics very similar to those of Der p 1.

It is now well established that Th1 or Th2 responses can be modulated *in vivo* depending on the adjuvant used for immunization [17]. Aluminum salts, the only adjuvants approved for use in humans, elicit essentially Th2 immune responses characterized by the production of specific IgG1 antibodies and cytokines IL-4 and IL-5 [18]. Immunizations using allergens formulated with alum induce specific IgE synthesis [8,9,19]. In contrast, Freund's complete adjuvant, monophosphoryl lipid A, saponins (QS21), or BCG can be used as adjuvant for the induction of Th1 immune responses, but their use is limited to animal models [20–22]. In this respect, novel Th1-inducer adjuvants are highly desirable [23].

Here we evaluated the potential of cationic lipid diC14-amidine as an adjuvant for a house dust mite vaccine. It has been suggested that cationic liposomes amplify immune responses and particularly cell-mediated immunity [24,25]. DiC14-amidine has substantial transfection activity and is currently used as a gene delivery vehicle [26,27]. The immunogenicity of recombinant ProDer p 1 adjuvanted with diC14-amidine was evaluated in a murine model of house dust mite allergy. The adjuvant effect of this cationic lipid was compared with that of aluminum hydroxide.

RESULTS

ProDer p 1 is Complexed with DiC14-amidine Mainly through Ionic Interactions

To demonstrate the existence of a complex between the allergen and the cationic lipid, we incubated ProDer p 1 with Rh-PE-labeled diC14-amidine liposomes (allergen/cationic lipid ratio 1/4) in a buffer containing 7% HBS-20 and 11% PBS and centrifuged the formulation at 20,000g for 15 min. A pellet was clearly visualized after centrifugation, whereas, under the same experimental conditions, the individual components did not pellet. ELISA and fluorescence measurements used to detect ProDer p 1 and diC14-amidine, respectively, clearly showed that more than 90% of the ProDer p 1 and diC14-amidine were associated with the pellet, suggesting that the components formed a complex (Fig. 1).

The ProDer p 1–diC14-amidine complexes were formed at pH 7.2–7.3. Under these conditions and according to the experimental isoelectric point of the recombinant allergen (*pI* 4.8, data not shown), ProDer p 1 is negatively charged. By contrast, the cationic lipid is positively charged at this pH value, suggesting that ProDer p 1 could be associated with the cationic lipid through ionic interactions. We incubated the recombinant allergen with the cationic lipid under different ionic strength conditions (from 0 to 75 mM NaCl in a buffer containing 7% HBS-20 and 11% PBS) and evaluated the complex formation by sucrose density gradient ultra-

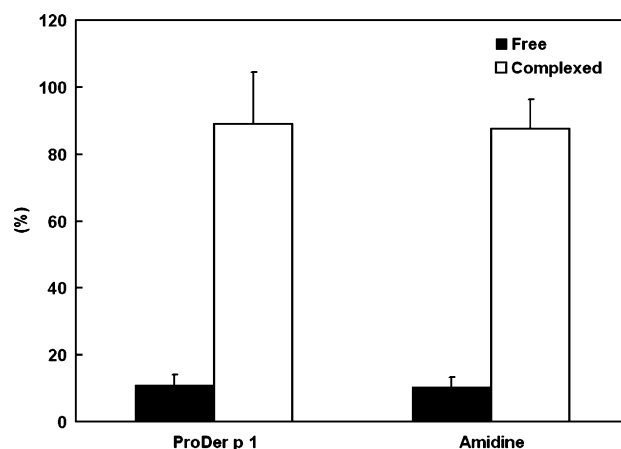


FIG. 1. Complexation of ProDer p 1 with diC14-amidine. ProDer p 1 was incubated with Rh-PE-labeled diC14-amidine liposomes (allergen/cationic lipid ratio 1/4) and the mixture was centrifuged at 20,000g for 15 min. The presence of ProDer p 1 and diC14-amidine in the supernatant and the pellet was detected by ELISA and fluorescence, respectively. The pellet was resuspended in 5% Triton X-100 (w/v) before any analysis.

centrifugation. The yield of complex formation decreased with increasing NaCl concentrations, the proportion of the non associated allergen being correlated with increasing ionic strength (Fig. 2). Yield of complex formation is maximal in the absence of NaCl.

Sialic acids present in ProDer p 1 likely participate in the global negative charge of ProDer p 1 and, consequently, could play a role in the complexation of the allergen with diC14-amidine [16]. Removal of sialic acids by treatment of ProDer p 1 with neuraminidase reduced drastically the complexation of the allergen with the amidine (Fig. 3). More than 70% free ProDer p 1 was indeed detected in the supernatant of centrifuged complexes. These results confirmed the contribution of ionic interactions in the formation of ProDer p 1–diC14-amidine complex.

ProDer p 1 Formulated with DiC14-amidine Prevents IgE Synthesis and Induces the Production of IgG2a and IFN- γ in Naïve Mice

We evaluated the immunogenicity of the ProDer p 1–diC14-amidine complexes in naïve Balb/C mice. We compared the specific immune responses with that induced after injection with ProDer p 1 adjuvanted with alum. As expected, we detected no specific responses in mice injected with alum or diC14-amidine alone (data not shown). Mice immunized by four injections of ProDer p 1 adjuvanted with alum revealed typical Th2-biased immune response characterized by high titers of anti-ProDer p 1 IgG and IgG1 as well as large amounts of specific IgE antibodies (Figs. 4A, 4B, and 4D). In contrast, the anti-ProDer p 1 IgG2a response was absent (Fig. 4C). It must be pointed out that control mice immunized with unadjuvanted ProDer p 1 developed also a weak but

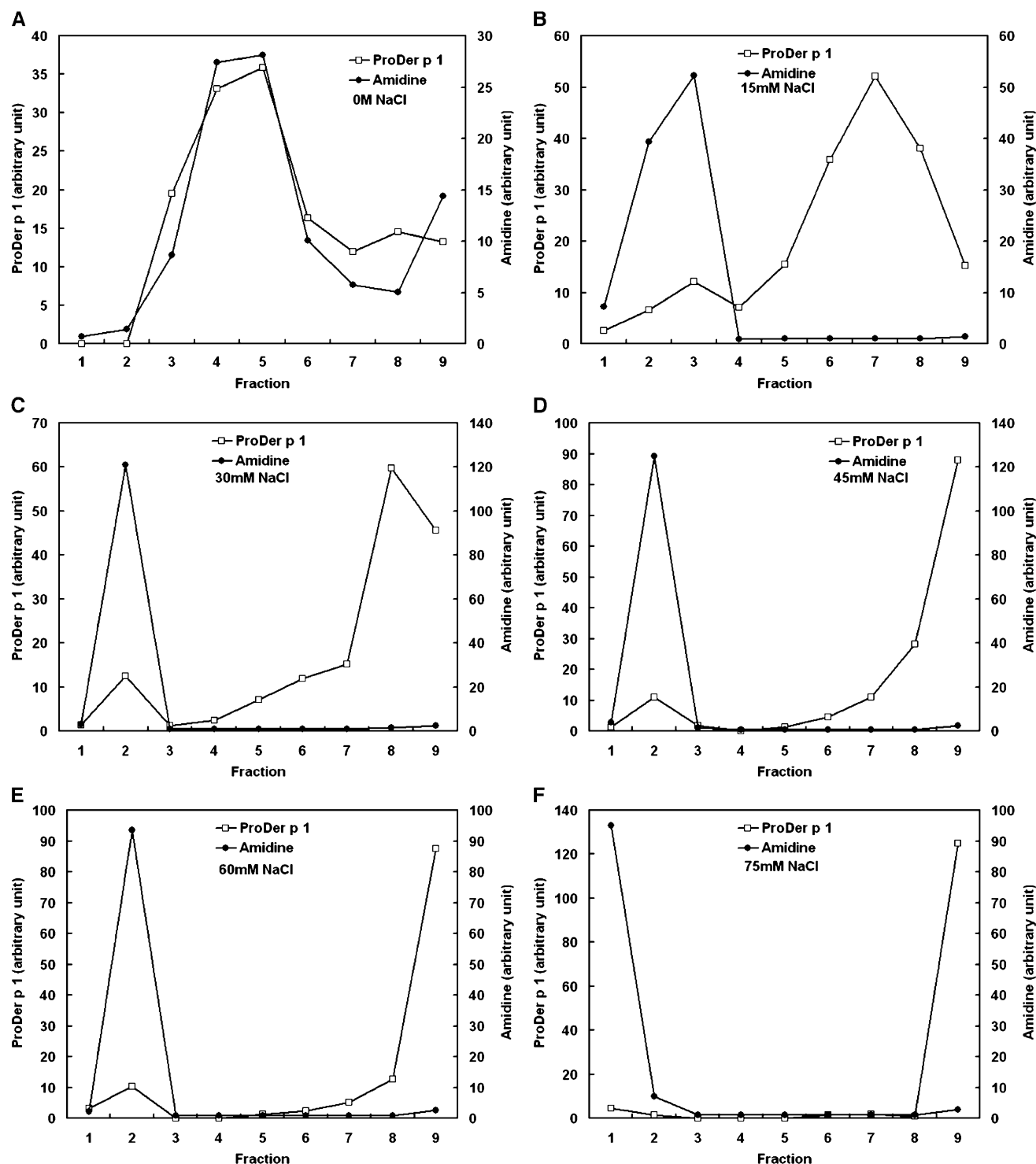


FIG. 2. Effects of ionic strength on the ProDer p 1-diC14-amidine complex formation. Rh-PE-labeled diC14-amidine-ProDer p 1 complexes were prepared as described under Materials and Methods and under different NaCl concentrations (0 to 75 mM). Complexes were centrifuged on a 30–20% continuous sucrose gradient. Liposome-associated rhodamine fluorescence (filled circles) and ProDer p 1 ELISA detection (open circles) were measured along the gradient. Fraction 1 represents the top of the gradient and fraction 9 the bottom.

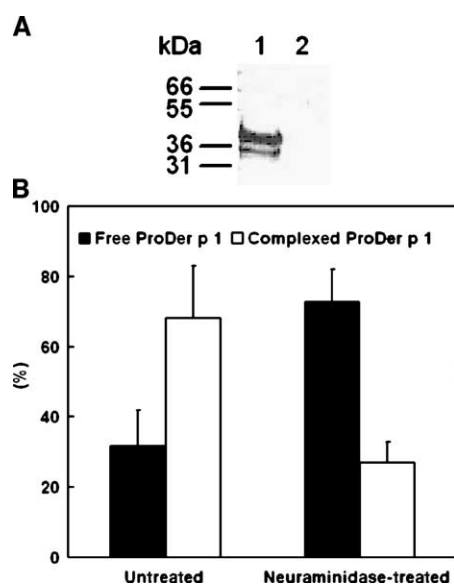


FIG. 3. Influence of sialic acids on the allergen–amidine complex formation. (A) ProDer p 1 was treated with *Vibrio cholerae* neuraminidase for 17 h. Removal of sialic acids was evidenced by lectin blotting revealed with the *Maackia amurensis* agglutinin. Lane 1, untreated ProDer p 1; lane 2, ProDer p 1 digested with neuraminidase. Wild-type ProDer p 1 migrated on SDS–PAGE as two glycosylated forms of 41 and 35 kDa [16]. (B) Wild-type and desialylated ProDer p 1 were incubated with Rh-PE-labeled diC14-amidine liposomes (allergen/cationic lipid ratio 1/4) and the mixture was centrifuged at 20,000g for 15 min. The presence of ProDer p 1 and diC14-amidine in the supernatant and the pellet was detected by ELISA and fluorescence, respectively. The pellet was resuspended in 5% Triton X-100 (w/v) before any analysis.

unique Th2-biased immune response (data not shown). ProDer p 1 formulated with the cationic lipid was less immunogenic than alum-adjuvanted ProDer p 1 (Fig. 4A, $P < 0.001$). However, ProDer p 1 complexed with diC14-amidine induced significant production of anti-ProDer p 1 IgG2a (Fig. 4C, $P < 0.001$) and, strikingly, prevented the development of specific IgE antibodies (Fig. 4D, $P < 0.001$). Moreover, production of specific IgG1 antibodies following this treatment was reduced compared with that induced after alum-adjuvanted ProDer p 1 vaccinations (Fig. 4B, $P < 0.001$). Immune responses generated by ProDer p 1 formulated with diC14-amidine in the presence of 150 mM NaCl were weaker than those induced by a complex formed under low-salt conditions (Fig. 4A), but the Th1 polarization was maintained (Figs. 4B, 4C, and 4D). Taken together, these data indicated that immune responses induced by the allergen–cationic lipid complex are Th1 oriented and the complex formation between ProDer p 1 and diC14-amidine influences the intensity but not the polarization of the immune response.

To confirm that diC14-amidine could constitute an adjuvant that promotes Th1-biased immune responses, we stimulated spleen cells from ProDer p 1-vaccinated

mice with ProDer p 1. Splenocytes from allergen-vaccinated mice proliferated in a dose-dependent manner following native ProDer p 1 stimulation and the stimulation index values were comparable between groups (data not shown, $P > 0.05$). We harvested supernatants from cultured stimulated lymphocytes and assayed them for IFN- γ and IL-5 (Fig. 4E). ProDer p 1 adjuvanted with alum elicited a typical Th2 cytokine profile characterized by high production of IL-5 and no secretion of IFN- γ . Strikingly, we observed a significant shift in cytokine profile from Th2 to Th1 when diC14-amidine was used as adjuvant ($P < 0.05$). The use of the cationic lipid dramatically enhanced IFN- γ secretion and concomitantly reduced IL-5 production. These results confirmed that diC14-amidine induced preferentially the development of ProDer p 1-specific Th1 immune response.

Vaccinations with ProDer p 1–DiC14-amidine Complexes Prevent the Development of Allergic Airway Inflammation in an Experimental Allergy Model

To investigate whether the Th1-biased immune response induced by immunization of mice with the ProDer p 1–diC14-amidine complex has any effect on the development of a subsequent allergic response, we challenged vaccinated mice with alum-adjuvanted Der p 1 and subsequently exposed them to aerosolized house dust mite extracts. We used mice pretreated with diC14-amidine or with saline alone as negative controls.

Pretreatment with the ProDer p 1–amidine complex was highly effective in reducing drastically the ProDer p 1-specific IgE antibody responses. The specific IgE antibody level after the allergic challenge was significantly weaker compared to that of the animals pretreated with amidine or NaCl (Fig. 5D, $P < 0.05$). Remarkably, allergen-specific IgG2a antibody levels persisted after the injections of alum-adjuvanted Der p 1 and exposures of house dust mite extract aerosols (Fig. 5C). The specific IgG and IgG1 levels were not significantly different between groups after the challenge (Figs. 5A and 5B, $P = 0.26$ for IgG compared with amidine treatment, $P = 0.31$ for IgG1 compared with saline treatment).

We also analyzed the effects of ProDer p 1–amidine pretreatment on the eosinophilic inflammation of Der p 1-immunized and challenged mice using house dust mite extract aerosols. As shown in Fig. 5E, aerosol challenge to mice pretreated with amidine alone or NaCl resulted in an important airway eosinophilia. Strikingly, vaccination of mice with ProDer p 1 complexed with diC14-amidine reduced drastically the eosinophilic inflammation of the airways following the allergen challenge ($P < 0.001$ compared with other groups).

Next, we analyzed the IL-5 and IFN- γ secretion by ProDer p 1-restimulated splenocytes in each group of mice (Fig. 5F). We detected IFN- γ production only in the animal group preimmunized with the allergen–cationic

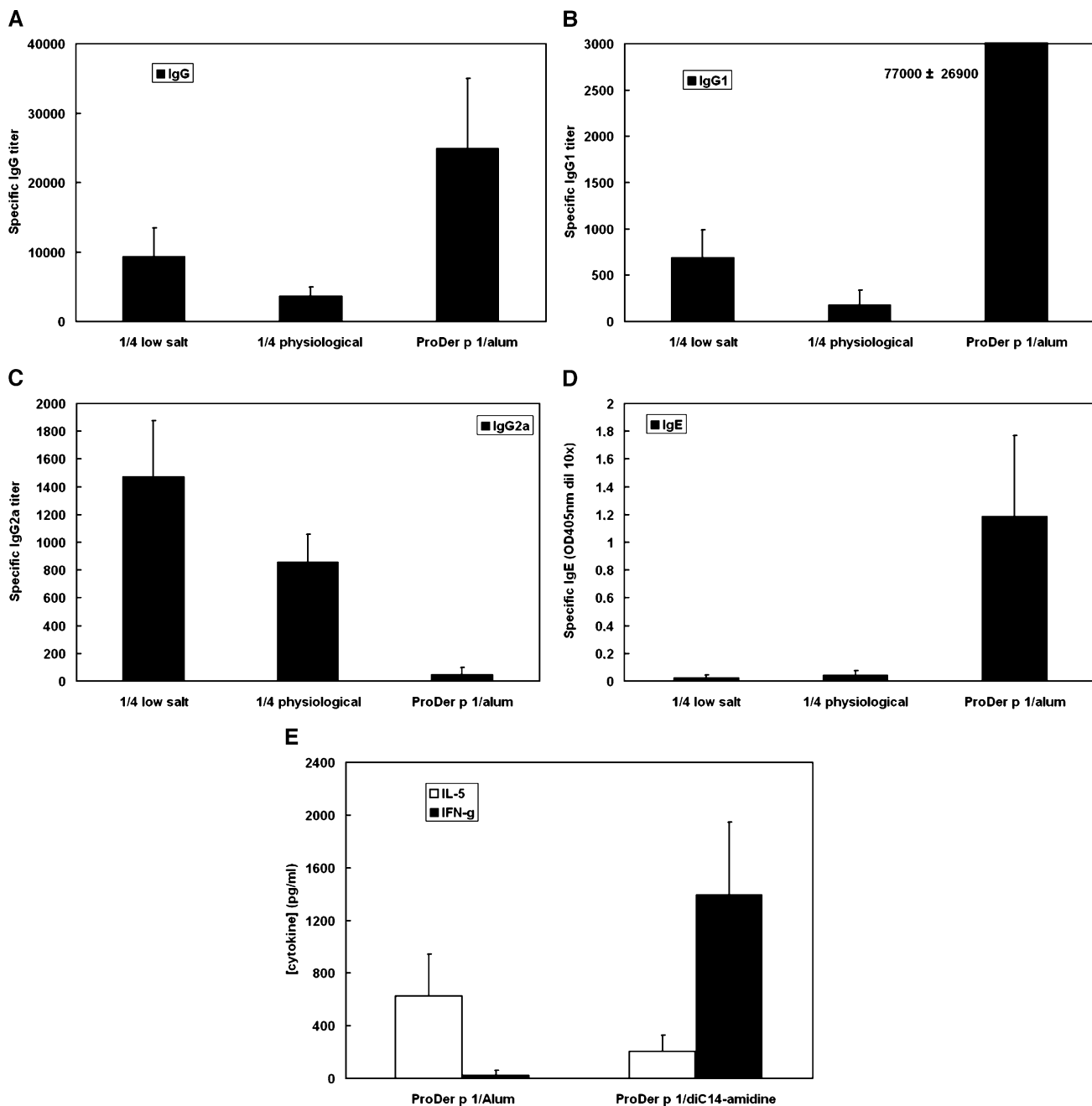


FIG. 4. Immune responses induced by ProDer p 1–diC14–amidine vaccinations. Naïve Balb/C mice were vaccinated with two different ProDer p 1–diC14–amidine complexes (allergen/cationic lipid ratio 1/4), a physiological and a low-salt complex formulated in the presence or absence of 150 mM NaCl, respectively. The respective immune responses were compared with that induced by immunization with alum-adsjuvanted allergen. Specific anti-ProDer p 1 (A) IgG, (B) IgG1, and (C) IgG2a titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD_{415nm} value. (D) For IgE titers, results are expressed as OD_{415nm} values for a 1/10 dilution of serum. (E) Splenocytes were stimulated with 20 µg/ml ProDer p 1, and culture supernatants were collected after 72 h. The production of IL-5 and IFN-γ in supernatants was measured by ELISA. Results are expressed as means ± SD from 10 mice.

lipid complex in comparison to the pretreated control groups. We observed no statistically significant differences in IL-5 production between all groups ($P > 0.05$).

These results suggest that the prophylactic effect of ProDer p 1–diC14–amidine in this model can be due to the production of IFN-γ.

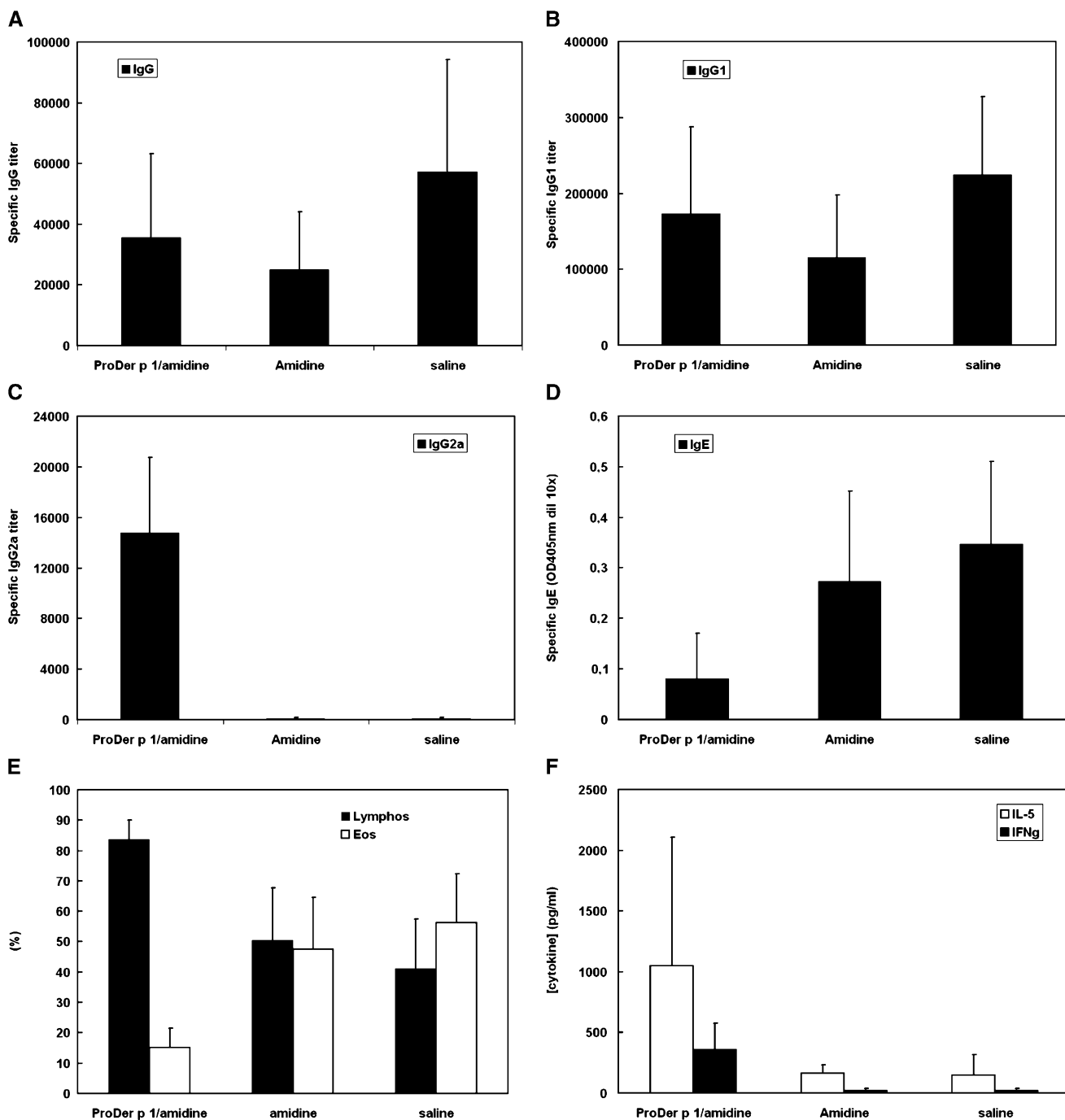


FIG. 5. Effects of ProDer p 1-diC14-amidine vaccinations on allergen challenge-induced immune responses. Naïve Balb/C mice were vaccinated with ProDer p 1-diC14-amidine complexes (allergen/cationic lipid ratio 1/4) or with the cationic lipid or saline alone. Animals were then challenged with alum-adjuvanted Der p 1 and subsequently exposed to aerosolized house dust mite extracts. 48 h after the last aerosol, challenged mice were sacrificed, and individual sera and spleens were collected. Specific anti-ProDer p 1 (A) IgG, (B) IgG1, and (C) IgG2a titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD_{415nm} value. (D) For IgE titers, results are expressed as OD_{415nm} values for a 1/10 dilution of sera. (E) Percentage of eosinophils and lymphocytes in bronchoalveolar lavage fluids after challenge with aerosolized house dust mite extracts. Values are expressed as the means $\pm$ SD from ten mice. (F) Splenocytes from challenged mice were stimulated with serial dilutions of ProDer p 1 as relevant antigen. The production of IL-5 and IFN- γ in the culture supernatants was measured by ELISA. Results are expressed as means $\pm$ SD from 10 mice.

DISCUSSION

Allergic diseases are characterized by strong Th2-biased immune responses, inducing specific IgE production. Therefore, inhibition of the function of allergen-specific Th2 cells is an attractive target for allergen based therapies. One way to improve the efficiency of recombinant allergen-based prophylactic or therapeutic vaccines against allergy is the coadministration of an appropriate adjuvant to induce Th1-biased immune responses in naïve individuals or to shift Th2 responses toward Th1 in the allergic context. Consequently, the development of novel "Pro-Th1" adjuvants could be of considerable interest. In the present study, we evaluated in mice the immunogenicity of recombinant ProDer p 1, an enzymatically inactive precursor form of the major house dust mite allergen Der p 1. Considering that the proteolytic activity of Der p 1 amplifies its allergenicity [4,5], ProDer p 1, which is enzymatically inactive [15,16], could be considered as a hypoallergenic variant of Der p 1. Since recombinant ProDer p 1 was shown to be poorly immunogenic in the absence of adjuvant (unpublished results), animals were vaccinated with adjuvanted proteins. Two ProDer p 1 formulations were used: the recombinant allergen was adjuvanted with alum, a well-known "Pro-Th2" adjuvant used as control, or was combined with the cationic lipid diC14-amidine. DiC14-amidine is an efficient gene delivery vector due to its ability to destabilize the endosomal membranes and to allow an efficient DNA release into the cell cytoplasm [26,27]. In addition, diC14-amidine could constitute a putative potent adjuvant as reported for other cationic lipids like DC-Chol, DOTAP, Vaxfectin, stearylamine, or DDAB [24,28–32]. Moreover, vaccination of DNA incorporated into mannan-coated diC14-amidine was shown to elicit Th1-biased immune responses [33].

Our results demonstrated that immunizations with alum-adjuvanted ProDer p 1 elicited typical Th2-biased immune responses characterized by high titers of specific IgG1 and IgE, absence of IgG2a, and high IL-5 secretion as well as weak IFN- γ production by splenocytes. Strikingly, ProDer p 1 can induce Th1-biased immune responses when administered with diC14-amidine. Indeed, although specific IgG1 response was always present, the specific IgE production was totally abolished, and specific IgG2a antibodies could be detected. These results indicate that diC14-amidine is a Th1 adjuvant able to prevent the development of specific IgE. The cytokine pattern of T cell proliferation confirmed the Th1-inducer character of diC14-amidine since the drastic reduction of IL-5 production was accompanied by an enhanced IFN- γ secretion. This important IFN- γ production could play a critical role in the induction of cell-mediated immunity as previously reported for a DNA vaccine combined with this cationic lipid [33].

Previous experiments clearly indicated that the polarization of the immune response induced by ProDer p 1–amidine or ProDer p 1–alum formulations was not dependent on the route of injection but on the adjuvant used. Indeed intramuscular immunization with alum-adjuvanted ProDer p 1 elicited a typical Th2 response that was similar in intensity with that measured after intraperitoneal vaccination. ProDer p 1–amidine administered intravenously was shown to induce a Th1-biased immune response characterized by the presence of specific IgG2a antibodies and the absence of anti-Der p 1 IgE (unpublished results).

Strikingly, ProDer p 1–amidine immunizations induced sufficient ProDer p 1-specific Th1 cells to prevent the subsequent Th2-biased immune response against natural Der p 1 and lung inflammation following repeated airway challenges, despite the strength of the allergen challenge (four sensitizations with Der p 1–alum followed by seven challenges with aerosolized house dust mite extracts). To the best of our knowledge, our study is the first demonstration that the formulation of an allergen with diC14-amidine could represent a good vaccine candidate against allergy.

Although the adjuvant effect of diC14-amidine could not be attributed exclusively to its capacity to form a complex with ProDer p 1, several arguments support the notion that the complex formation, even in small amounts, is important for the development of a Th1-biased immune response. First, the intensity of the Th1 immune response is well correlated with the complex formation between the cationic lipid and the recombinant allergen (compare Figs. 2 and 4). Second, unadjuvanted ProDer p 1 induced a weak but unique Th2-biased immune response in naïve mice. Third, injection of the recombinant allergen and amidine in the mouse muscle but at two different places elicited a Th2-biased immune response similar to that obtained with unadjuvanted ProDer p 1 (unpublished results).

Liposomes have been used to introduce a large variety of molecules into cultured cells. During the process of liposome formation, water and solute molecules are trapped into the liposome internal compartment. The yield of entrapment is low and requires the separation of free and encapsulated material. A significant advantage of cationic lipid–protein complexes is that they are formed by simple mixing. Complex formation is fast, yield is high, and no separation of free and lipid-associated material is needed.

ProDer p 1–diC14-amidine complex is currently being evaluated as a therapeutic vaccine against house dust mite allergy. This vaccination could shift the Th2 immune responses induced by Der p 1 sensitization and airway exposure toward Th1.

The mechanism by which the allergen–amidine complex can modulate the Th2-biased allergic response remains to be elucidated. A recent study showed that

cationic but not neutral or anionic liposomes interacted with a very high percentage of both the human and the murine dendritic cells [34]. ProDer p 1-amidine complexes were shown to be easily taken up by murine dendritic cells, and intracellular localization of ProDer p 1 was already evidenced after 10 min incubation (data not shown). DiC14-amidine-associated ProDer p 1 could thus be presented by the MHC class I. This allergen presentation would trigger the activation of CD8⁺ T cells producing a high level of IFN- γ and consequently modulate the Th2 response.

MATERIALS AND METHODS

Antigens. Natural Der p 1 was isolated from whole *D. pteronyssinus* culture by immunoaffinity chromatography. Briefly, whole mite cultures were extracted several times with PBS, pH 7.2. The supernatant obtained after ultracentrifugation was applied to a monoclonal anti-Der p 1-affinity chromatography column prepared using purified 4C1 antibodies (Indoor Biotechnology, Charlottesville, VA, USA) and equilibrated with PBS, pH 7.2. Unbound material from the column was eluted with PBS, and Der p 1 was eluted after reequilibration of the column with 5 mM glycine buffer, pH 10, containing 50% ethylene glycol. The fractions were neutralized directly at pH 7.2 using concentrated phosphate buffer and those containing purified Der p 1 were pooled and stocked at -20°C. Recombinant ProDer p 1 was purified from CHO spent culture medium [16].

Endotoxin levels were determined using the *Limulus* assay (*Limulus* amoebocyte lysate QCL 1000; BioWhittaker). The antigens used for the vaccinations contained less than 0.2 U/10 μ g.

In some experiments, 100 μ g ProDer p 1 was treated with 0.2 U of neuraminidase (from *Vibrio cholerae*; Roche) in 2 ml of 50 mM sodium acetate, pH 5.5, containing 4 mM CaCl₂ for 17 h at 37°C. Following incubation, the desialylated protein was dialyzed against PBS, pH 7.2. The removal of sialic acids after the enzymatic treatment was confirmed by lectin blotting. Briefly, digested or untreated ProDer p 1 migrated onto 12% SDS-PAGE and was transferred to nitrocellulose sheets using a semidry transfer cell. The presence of sialic acids was evidenced using the specific digoxigenin-conjugated MAA lectin (*Maackia amurensis* agglutinin; Roche). The complexes were detected after incubation with an alkaline phosphatase-conjugated anti-digoxigenin monoclonal antibody (Roche) according to the manufacturer's instructions.

Animals. Female Balb/C mice (6 weeks of age) were obtained from Harlan (AD Horst, The Netherlands).

Liposome preparation. DiC14-amidine (3-tetradecylamino-*N*-tert-butyl-*N'*-tetradecylpropionamidine) was synthesized as described [26]. DiC14-amidine liposomes were prepared as follows: 20 mg of diC14-amidine was dissolved in chloroform in a glass tube and the solvent was evaporated in a rotary evaporator under a stream of N₂. The resulting lipid film was further dried under vacuum overnight. Four milliliters of the HBS-20 buffer (20 mM Hepes, 150 mM NaCl, pH 7.3) was prewarmed to 55°C and added to the lipid film to a final lipid concentration of 5 mg/ml. After 10 min incubation at 55°C, the tube was vortexed for 1 min and the pH of the resulting liposome suspension was adjusted to 7.3 using small amounts of HCl. The liposomes were then extruded seven times through a 0.4- μ m polycarbonate filter (Osmonics) while the extruder block was heated at 55°C.

Formulation of ProDer p 1 in diC14-amidine liposomes. All solutions used in the diC14-amidine/ProDer p 1 preparation were autoclave-sterilized and warmed to 37°C immediately before use. ProDer p 1 was diluted in water to a concentration of 0.1 mg/ml. The diC14-amidine liposomes were diluted in water to a concentration of 0.4 mg/ml in a 4-ml polystyrene tube (Falcon). A volume of ProDer p 1 solution (usually 0.5–1 ml) was added into an equal volume of the liposome solution while the tube was shaken gently. The diC14-amidine/ProDer p 1 mixture was

allowed to stand for 15 min at room temperature. Under these conditions, the diC14-amidine/ProDer p 1 ratio was 4/1 (w/w) and the final buffer composition was 7% HBS-20 and 11% PBS.

In some experiments, diC14-amidine liposomes were labeled with Rh-PE [N-(lissamine rhodamine B sulfonyl) phosphatidylethanolamine; Avanti Polar Lipids (Alabaster, AL, USA)]. Rhodamine-labeled diC14-amidine liposomes were prepared as previously described except that Rh-PE was added (1% mol of total lipid) to the diC14-amidine in chloroform. The liposome suspensions were stored at 4°C and heated at 60°C for 15 min (with intermittent brief vortexing) before use.

Sucrose gradient ultracentrifugation. Two hundred microliters of diC14-amidine/ProDer p 1 complex was mixed with an equal volume of 80% sucrose (prepared in HBS-20) and transferred to a 4-ml ultracentrifugation tube. A 30 to 2% continuous sucrose gradient was formed and the tubes were centrifuged overnight at 35,000 rpm at 20°C in an SW-60 Beckman rotor. Liposome and ProDer p 1 were centrifuged separately as controls. After centrifugation, the tubes were quickly frozen by immersion in liquid nitrogen and then cut into nine equal slices. Each slice was transferred into an Eppendorf tube and allowed to thaw at room temperature. One hundred microliters of a buffer containing 30 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% (w/v) reduced Triton X-100 was added into each fraction. Rh-PE (568 nm excitation and 605 nm emission) was measured in a Jobin-Yvon spectrofluorimeter using 100 μ l of each fraction. ProDer p 1 was detected in each fraction using an anti-Der p 1 ELISA kit.

Der p 1 ELISA. Recombinant ProDer p 1 was detected with an ELISA kit using Der p 1-specific monoclonal antibodies 5H8 and 4C1 (Indoor Biotechnologies). The Der p 1 standard (UVA 93/03) used in the assay was at a concentration of 2.5 μ g/ml.

Immunizations. Groups of 10 Balb/C mice were immunized four times at 1-week intervals with 5 μ g of recombinant ProDer p 1 adjuvanted with alum (ratio allergen/adjuvant 1/20, intraperitoneal injection). In one group, the ProDer p 1-diC14-amidine solution (allergen/cationic lipid ratio 1/4) was administered intramuscularly (50 μ l containing 2.5 μ g of ProDer p 1 for each tibialis muscle). As control, one group of mice was immunized with alum or diC14-amidine alone. Mice were bled 7 days after the last injections and sera were collected.

In another set of experiments, a group of 10 Balb/C mice was vaccinated five times at weekly intervals with 5 μ g ProDer p 1 formulated with diC14-amidine. As controls, animals were immunized with diC14-amidine alone or with saline. Vaccinated animals were subsequently sensitized at weekly intervals for 4 weeks with 5 μ g of natural Der p 1. To induce airway inflammation, mice were airway allergen challenged 10 days after the last sensitization by exposure to aerosolized crude *D. pteronyssinus* extract over a 30-min period for 7 consecutive days.

Bronchoprovocation. Mice were placed in a Plexiglas chamber (13 $\times$ 19 $\times$ 37.5 cm) and exposed to aerosolized crude *D. pteronyssinus* extract over a 30-min period for 7 consecutive days. The concentration of crude mite extract was 300 μ g/ml. The aerosols were generated by an ultrasonic nebulizer (Syst' AM). The output of the nebulizer was 0.5 ml/min and the mean particle size of the aerosol was between 1 and 5 μ m.

Measurement of Der p 1-specific IgG, IgG1, and IgG2a. Sera were assayed for anti-Der p 1 IgG, IgG1, and IgG2a antibodies by ELISA. Immunoplates were coated with ProDer p 1 (500 ng/well), for 16 h at 4°C. Plates were washed five times with TBS-Tween (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 80) and saturated for 1 h at 37°C with 150 μ l of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 h at 37°C. Plates were washed five times with TBS-Tween buffer and antigen-bound antibodies were detected with the second antibody (goat anti-mouse IgG; Promega, USA) coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer). The enzymatic activity was measured using the *p*-nitrophenyl phosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{415nm} was measured in a Bio-Rad Novapath ELISA reader.

Mouse antibody subclass was determined using immunoplates coated as described above and IgG1- or IgG2a-specific biotin-labeled monoclonal

antibodies (rat anti-mouse, dilution 1/7000 in TBS-Tween buffer and 1% BSA; Biosource) as second antibodies. Phosphatase alkaline-conjugated streptavidin (1/1000 dilution; Amersham) was added to each well. Assay of the enzymatic activity proceeded as described above.

In all cases, ELISA titers were identified as the reciprocal of the dilution giving a signal corresponding to 50% of the maximal OD_{415nm} value.

Measurement of Der p 1-specific IgE. Immunoplates were coated with rat anti-mouse IgE (10 ng/well), for 16 h at 4°C. Plates were washed five times with TBS-Tween and saturated for 1 h at 37°C with 150 µl of the same buffer supplemented with 1% BSA. Serial dilutions of sera in saturation buffer were incubated for 1 h at 37°C. ProDer p 1 was then added at 500 ng/ml in saturation buffer. Bound ProDer p 1 was detected by addition of biotinylated anti-Der p 1 monoclonal antibody 4C1 (Indoor Biotechnologies). Plates were washed five times with TBS-Tween buffer and antibody-bound antigen was detected with addition of streptavidin coupled to alkaline phosphatase (dilution 1/7500 in TBS-Tween buffer). The enzymatic activity was measured using the *p*-nitrophenyl phosphate substrate (Sigma) dissolved in diethanolamine buffer (pH 9.8). OD_{415nm} was measured in a Bio-Rad Novapath ELISA reader.

Proliferation and cytokine assays. The Der p 1-specific T cell proliferative responses and the level of IFN-γ and IL-5 in the supernatants were measured as previously described [35].

Briefly, spleen cells from immunized mice were stimulated with serial dilutions of ProDer p 1. After 72 h, cells were pulsed with 1 µCi/well [³H]thymidine (Amersham) for 16 h. Cells were harvested and [³H]thymidine uptake was measured by scintillation counting. Culture supernatants from restimulated splenocytes were assayed for the presence of IFN-γ and IL-5 using ELISA kits supplied by PharMingen.

Bronchoalveolar lavage. The bronchoalveolar lavages as well as the differential cell counts were performed as described previously [35]. Briefly, the lungs from mice were washed, the lavage fluid was centrifuged, and the cells were counted in a Thoma hemocytometer. Cytospin preparations from 50-µl aliquots were stained with May-Grünwald Giemsa stain for differential cell counts.

Statistical analysis. Group data were compared using Student's *t* test. Differences were considered significant for *P* values <0.05.

ACKNOWLEDGMENTS

We thank Louis Delhaye for excellent animal handling. This work was supported in part by GlaxoSmithKline (Rixensart, Belgium) and by the Walloon Region (Direction Générales des Technologies, de la Recherche et de l'Energie) of Belgium. Michel Vandenbranden is a Research Associate of the Belgian National Fund for Scientific Research.

RECEIVED FOR PUBLICATION AUGUST 4, 2004; ACCEPTED DECEMBER 18, 2004.

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