

Strain-Dependent Induction of Allergic Sensitization Caused by Peanut Allergen DNA Immunization in Mice¹

Xiu-min Li,^{2*} Chih-Kang Huang,* Brian H. Schofield,[†] A. Wesley Burks,[‡] Gary A. Bannon,[‡] Kawn-Hyoung Kim,[§] Shau-Ku Huang,[§] and Hugh A. Sampson*

To investigate the potential application of allergen gene immunization in the modulation of food allergy, C3H/HeSn (C3H) mice received i.m. injections of pAra h2 plasmid DNA encoding one of the major peanut allergens, Ara h2. Three weeks following pDNA immunization, serum Ara h2-specific IgG2a, IgG1, but not IgE, were increased significantly in a dose-dependent manner. IgG1 was 30-fold higher in multiply compared with singly immunized mice. Ara h2 or peanut protein injection of immunized mice induced anaphylactic reactions, which were more severe in multiply immunized mice. Heat-inactivated immune serum induced passive cutaneous anaphylaxis, suggesting that anaphylaxis in C3H mice was mediated by IgG1. IgG1 responses were also induced by intradermal injection of pAra h2, and by i.m. injection of pOMC, the plasmid DNA encoding the major egg allergen protein, ovomucoid. To elucidate whether the pDNA immunization-induced anaphylaxis was a strain-dependent phenomenon, AKR/J and BALB/c mice also received multiple i.m. pAra h2 immunizations. Injection of peanut protein into these strains at weeks 3 or 5 following immunization did not induce reactions. Although IgG2a was increased significantly from week 2 in AKR/J mice and from week 4 in BALB/c mice and remained elevated for at least 6 wk, no IgG1 or IgE was detected. These results indicate that the type of immune responses to pDNA immunization in mice is strain dependent. Consequently, models for studying human allergen gene immunization require careful selection of suitable strains. In addition, this suggests that similar interindividual variation is likely in humans. *The Journal of Immunology*, 1999, 162: 3045–3052.

Deoxyribonucleic acid vaccines employing plasmid DNA (pDNA)³-encoding specific Ags are a novel means of generating immune responses. Intradermal (i.d.) and i.m. injections of naked pDNA are the most commonly used methods to deliver DNA and induce a prolonged immune response. DNA vaccine has been shown to be effective in eliciting protective immunity against various viral (1–3), bacterial (4, 5), and parasitic diseases (6). Hsu et al. (7) reported that i.m. injection of Brown Norway rats with pDNA-encoding house dust mite allergen (Der p 5) prevented the induction of IgE synthesis, histamine release, and airway hyperresponsiveness following challenge with aerosolized allergen. Raz et al. (8) showed that i.d. immunization with pDNA-encoding β -galactosidase induced a predominant IgG2a response, and reduced β -galactosidase-induced specific IgE Ab levels by 66–75% in BALB/c mice. These two studies suggest that immunization

with pDNA-encoding allergens may have potential for developing a new form of allergen immunization therapy.

Peanuts are highly allergenic and cause allergic reactions in both children and adults (9, 10), and are the most frequent cause of fatal food-allergic reactions (11, 12). The prevalence of peanut allergies has increased in the past decades (13). At the present time, the only available therapy for peanut allergy is strict avoidance (14), although accidental ingestions remain common (15). In view of the prevalence and severity of peanut allergy, research has been directed toward developing new therapeutic approaches for controlling peanut allergy. Traditional desensitization therapy (immunotherapy) was shown to reduce reactions to oral peanut challenge in a minority of patients, but frequent systemic anaphylactic reactions during immunotherapy limit the application of this approach (16, 17). Three major protein fractions have been identified in peanut, Ara h I (63.5 kDa), Ara h2 (17 kDa), and Ara h3 (14 kDa), and more than 95% of peanut-allergic patients are sensitive to both Ara h1 and Ara h2 (18, 19). The characterization of the major peanut allergens has made further novel therapeutic approaches to peanut allergy possible.

The current study was designed to investigate the potential application of allergen gene immunization to the modulation of peanut allergy. We utilized i.m. injections of C3H mice with pAra h2, a plasmid DNA encoding the Ara h2 peanut allergen. We report in this study that both IgG2a and IgG1 responses were induced by pAra h2 immunization, and that systemic anaphylaxis developed following the first injection of pAra h2-immunized mice with crude peanut extract (PN) or purified Ara h2 protein. We also demonstrate that IgG1 was most likely the reagenic Ab in the induction of this anaphylaxis. The induction of anaphylactic reactions is strain specific because pAra h2-immunized AKR and BALB/c mice did not exhibit anaphylactic reactions following peanut protein injection. These strains showed only a significant increase in IgG2a, but not IgG1 or IgE.

*Department of Pediatrics, Mount Sinai School of Medicine, New York, NY 10029; [†]Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, MD 21205; [‡]Department of Pediatrics, University of Arkansas School of Medicine, Little Rock, AR 72205; and [§]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD 21224

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² Address correspondence and reprint requests to Dr. Xiu-Min Li, Pediatric Allergy and Immunology, Mount Sinai School of Medicine, Box 1198, One Gustave L. Levy Place, New York, NY 10029-6574. E-mail address: Xiu-min_li@smtpink.mssm.edu

³ Abbreviations used in this paper: pDNA, plasmid DNA; CA, conalbumin; i.d., intradermal; PCA, passive cutaneous anaphylaxis; PN, crude peanut extract.

Materials and Methods

Mice and reagents

Female C3H/HeSn (H-2^K), BALB/c (H-2^d), and male AKR (H-2^K) mice, 6 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained on peanut protein-free chow under specific pathogen-free conditions.

PN and Ara h2 protein were prepared as previously described (20). Ara h2 cDNA was generated as previously described (21). Conalbumin (CA), Con A, DNP-BSA, and ovomucoid were purchased from Sigma (St. Louis, MO). Abs for ELISAs were purchased from The Binding Site (San Diego, CA).

Plasmid DNA preparation

The plasmid DNA-based gene construct, pAra h2, was generated by using a TA cloning kit (Invitrogen, San Diego, CA). Briefly, PCR-amplified Ara h2 coding region gene segment with the addition of a Kozak consensus translation codon was ligated into a pCR3.1-Uni expression vector containing CMV promoter. The pOMC was also generated using the same vector, pCR 3.1-Uni, encoding the ovomucoid, a major allergen from egg. The plasmid DNA pcDNA3 (pcDNA) (Invitrogen) was used as a mock DNA control since its backbone is identical to pAra h2 and pOMC, with the exception of the cloning site. The pDNA was prepared and purified by BioServe (Laurel, MD), and resuspended in endotoxin-free water.

DNA immunization and Ag administration

Mice were anesthetized by i.p. injection with a mixture of ketamine (45 mg/g) and xylazine (10 mg/g), and each mouse was then injected i.m. with 15 μ g of naked pDNA diluted in PBS to a final volume of 50 μ l. In the dose-dependent study, mice received one injection (single immunization) or three daily injections, followed by a fourth injection 1 wk later (multiple immunization). Control mice received mock DNA (pcDNA), or were untreated. Three weeks after the initial pDNA immunization, mice were injected i.p. with 1 mg/mouse of PN or Ara h2-purified protein, or an irrelevant Ag, CA.

Measurement of serum Ag-specific Abs

Blood was obtained weekly from each group of mice following the initial pDNA immunization. After centrifugation, the sera were collected and stored at -80°C until analyzed. The levels of Ag-specific IgE, IgG1, and IgG2a Abs were measured by ELISA, as described previously (22). Immulon II plates (Dynatech Laboratories, Chantilly, VA) were coated with 10 μ g/ml purified Ara h2 protein in coating buffer (Sigma). After overnight incubation at 4°C , plates were washed three times with PBS/0.05% Tween-20 and blocked with 1% BSA-PBS for 1 h at 37°C . After three washings, serum samples (1/5 or 1/10 dilutions in 1% BSA-PBS) were added to the plates and incubated overnight at 4°C . Plates were then washed, and 100 μ l of goat anti-mouse IgE or IgG1, or IgG2a Abs (0.3 μ g/ml) were added to the plates for detection of IgE, IgG1, and IgG2a Abs, respectively. The plates were incubated for 2 h at 37°C . After three washings, 100 μ l of donkey anti-goat IgG Ab conjugated with peroxidase (0.3 μ g/ml) was added for 1 h at 37°C . Plates were developed with tetramethylbenzidine (TMB) (Bio-Rad, Hercules, CA) for 30 min at 22°C , stopped by the addition of 1 N H₂SO₄, and read at 450 nm. The levels of IgE, IgG1, and IgG2a Abs were calculated by comparison with a reference curve generated by using mouse mAbs, anti-DNP IgE, IgG1, and IgG2a (Accurate Scientific, Westbury, NY). All analyses were performed in duplicate, and discrepant values (coefficient of variation >10%) were repeated to ensure a high degree of precision. Values less than 4 ng/ml were regarded as undetectable in this assay.

Assessment of hypersensitivity responses

Signs of systemic anaphylaxis became apparent in C3H mice 10 to 15 min following i.p. PN injection and peaked at 20–40 min. Symptoms of anaphylaxis were evaluated by a scoring system 40 min after challenge. This scoring system was modified slightly from previous descriptions (23, 24), and scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes, pilar erecti, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, cyanosis around the mouth and the tail; 4, no activity after prodding, or tremor and convulsion; 5, death.

Detection of vascular leakage

At the time of peanut protein Ag injection, C3H mice from each group received 100 μ l of 0.5% Evan's blue dye by tail vein injection, immediately followed by i.p. peanut injection. Thirty to forty minutes after dye/Ag

administration, the mice's feet were examined for signs of vascular leakage (visible blue color).

Determination of plasma histamine levels

Five to eight minutes following peanut injection, 0.3–0.5 ml of blood from each mouse was collected into chilled tubes containing 30–40 μ l of 7.5% potassium-EDTA. After centrifugation (1500 rpm) for 10 min at 4°C , the plasma was collected and frozen at -80°C until used. The levels of histamine were determined using an enzyme immunoassay kit (Immunotech, Westbrook, ME), as described by the manufacturer. The concentration of histamine was calculated by comparison with a standard curve provided by the manufacturer.

Histologic studies

Mast cell degranulation during systemic anaphylaxis was assessed by histologic examination of ear tissues. Samples were collected immediately after anaphylaxis-related death or 40 min after challenge from surviving mice. Tissues were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3), at room temperature for 30 min, then stored at 4°C until processing into 3 μ m paraffin or glycol methacrylate, toluidine blue-stained sections. A degranulated mast cell was defined as a toluidine-positive cell with five or more distinct stained granules completely outside of the cell (25). One section from each of three sites of each mouse ear was examined by light microscopy at $\times 400$ by an observer unaware of their identities. A total of 200–400 mast cells was classified in each ear sample. In some instances, Wright's stained blood smears were also prepared from mice experiencing anaphylactic shock.

Passive cutaneous anaphylaxis (PCA) test

Sera were obtained from 4 to 6 pAra h2 multiply immunized C3H mice and pooled. The PCA test was modified slightly from previous descriptions (26, 27). Briefly, the abdomens of naive C3H mice were carefully shaved 1 day before i.d. injection of 30 μ l of heated (56°C for 3 h) and unheated undiluted sera. Control mice received equal amounts of pooled sera from mock DNA-immunized mice or an equal amount of PBS. Injections were repeated 24 h later. Three hours after the second injection, mice were injected i.v. with a mixture of 100 μ l of 0.5% Evan's blue dye and 1 mg PN protein. Thirty minutes following the dye/PN injection, the mice were sacrificed, the skin of the belly was inverted, and PCA reactions were examined by visible blue color. A reaction was scored as positive if the bluing of the skin at the injection sites was >0.5 cm in diameter.

Quantitation of cytokines

Spleens were removed from each group of mice at 3 wk after pDNA immunization. Cells were isolated and suspended in complete culture medium (RPMI 1640 plus 10% FBS, 1% penicillin/streptomycin, and 1% glutamine). Cell suspensions were cultured in 24-well plates (4×10^6 /well/ml) in the presence or absence of PN (50 μ g/ml) (Ara h2 comprises 15–20% of total peanut protein) or Con A (2 μ g/ml). The supernatants were collected after 24-, 48-, and 72-h culture. Levels of cytokines, IFN- γ , IL-4, and IL-5 were determined by ELISA, according to the manufacturer's instructions (PharMingen, San Diego, CA) and as previously described (22).

Statistical analysis

The statistical significance of the data was determined by ANOVA or *t* test. A *p* value of <0.05 was considered significant.

Results

Isotype profile of Ag-specific Abs induced by pDNA immunization in C3H mice

Three weeks after the initial pDNA immunization of C3H mice, significantly increased levels of Ara h2-specific IgG2a as well as IgG1 were present in pAra h2-immunized mice (Fig. 1), but not in pcDNA (mock DNA)-immunized mice. The level of IgG2a was 10-fold higher than IgG1. The dose-dependent study showed that IgG2a in the multiply immunized group was twofold higher than in the singly immunized group. The titer of IgG1 in the multiply immunized group was 30-fold higher than that in singly immunized group. No Ara h2-specific IgE was detectable in either singly or multiply immunized mice (data not shown). In addition, multiple i.d. injections of pAra h2 produced significant increase of Ara h2-specific IgG1 (data not shown).

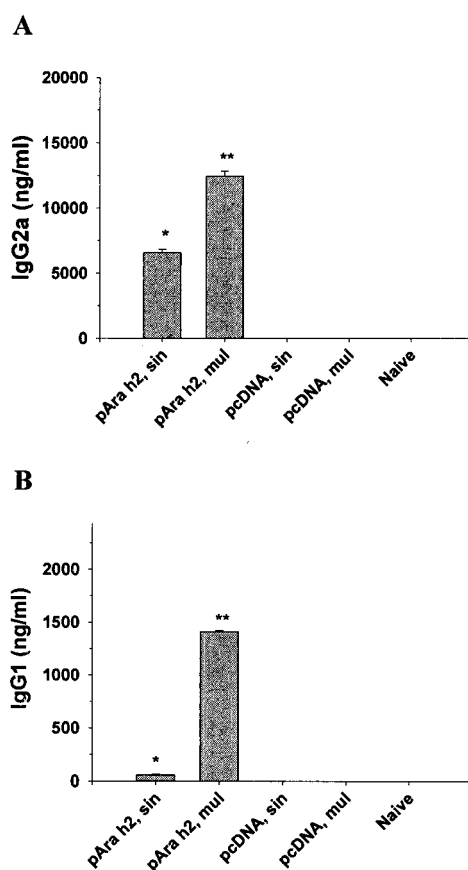


FIGURE 1. Levels of Ara h2-specific Abs in C3H mice. **A**, Levels of Ara h2-specific IgG2a. **B**, Levels of Ara h2-specific IgG1. Sera from different groups of mice ($n = 4-7$) as indicated were obtained 3 wk after pDNA immunization. The levels of Ara h2-specific IgG2a and IgG1 were determined by ELISA, and calculated by comparison with a reference curve generated using mouse mAb, anti-DNP Abs. *, $p < 0.05$ versus pcDNA, sin; **, $p < 0.001$ versus pcDNA, mul.

Induction of anaphylactic reactions by PN injection of pAra h2-immunized C3H mice

The initial experiment was designed to investigate whether pAra h2 immunization could prevent peanut-induced hypersensitivity, as reported for different Ags by others (7, 8). In this study, mice were immunized with pDNA 3 wk before peanut protein Ag sensitization. Surprisingly, i.p. injection of either PN or Ara h2 protein into the mice immunized with pAra h2 resulted in anaphylactic reactions. The severity of the reactions was evaluated and scored as shown in Fig. 2. Anaphylactic reactions in the multiply pAra h2-immunized group were more severe than in singly immunized mice, with a mortality rate of 60%, indicating an association between the increased level of IgG1 and the severity of the anaphylactic reactions. No anaphylactic reactions were observed in mock DNA-immunized mice following peanut injection or in the pAra h2-immunized mice following injection with an irrelevant Ag CA. Thus, the anaphylactic reactions in this model were Ag specific and dose dependent.

Increased vascular permeability following PN injection of pAra h2-immunized C3H mice

Increased vascular permeability is a hallmark of systemic anaphylaxis. To further characterize the anaphylaxis, vascular leakage was assessed by PN/Evan's blue injection. Extensive Evan's blue extravasation was evident in mouse feet of pAra h2-immunized

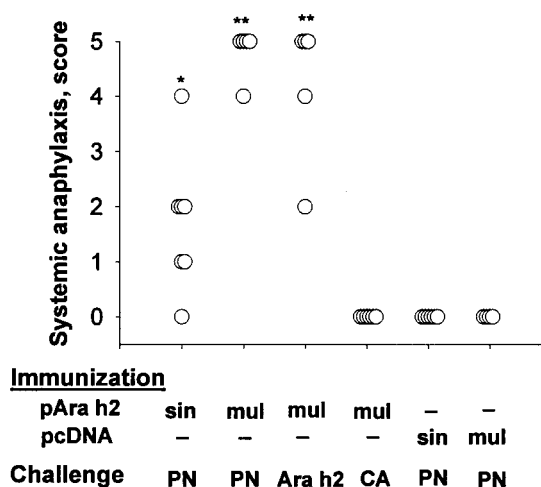


FIGURE 2. Peanut-induced anaphylaxis in C3H mice. Three weeks following the initial pDNA immunization, mice ($n = 4-7$) in each group received an i.p. injection of PN, or Ara h2, or CA. The severity of anaphylaxis was scored 20–40 min after i.p. Ag administration, as described in *Materials and Methods*. *, **, $p < 0.001$ versus pcDNA; **, $p < 0.01$ versus pAra h2, sin.

mice (Fig. 3). In addition, peripheral blood smears showed extensive platelet aggregation in pAra h2-immunized mice following PN administration (data not shown).

Elevated plasma histamine following PN injection of pAra h2-immunized C3H mice

Following PN administration, plasma histamine was increased significantly in the pAra h2-immunized group when compared with control groups (Fig. 4). Moreover, the histamine levels in pAra h2 multiply immunized mice were significantly greater than in singly immunized mice. These results indicate that histamine is most likely one of the mediators of anaphylaxis in this model.

Mast cell degranulation in C3H mice

Histologic analysis of mouse ear tissue showed a significant increase in the number of degranulated mast cells in pAra h2-immunized mice following PN injection when compared with control mice (Fig. 5, *A* and *B*). Consistent with the findings of elevated levels of plasma histamine, the percentage of degranulated mast cells in mice given multiple pAra h2 immunizations was markedly greater than in singly immunized mice (Fig. 5*C*). These data demonstrate that mast cell degranulation and consequent histamine release are involved in the induction of anaphylaxis in pAra h2-immunized C3H mice following PN injection.

PCA reactions in C3H mice

The virtual absence of IgE and the high levels of IgG1 induced by pAra h2 immunization, together with the association between the level of IgG1 and the severity of anaphylactic reactions (Figs. 1 and 2) suggested that peanut-induced anaphylactic shock in the C3H mouse model is IgG1 mediated. To further evaluate this hypothesis, PCA testing was performed as described in *Materials and Methods*. PCA reactions were induced by heat-inactivated and nonheated sera from pAra h2-immunized C3H mice (Table I). In contrast, no PCA reactions were found in peanut-injected mice that received mock pDNA immune sera or PBS. These results demonstrate that IgG1, but not IgE, was the reagenic Ab in this model.



FIGURE 3. Peanut Ag-induced vascular leakage in C3H mice. *A*, pAra h2 multiply immunized showing marked bluing. *B*, pcDNA multiply immunized mouse. Slight bluing of the skin is due to intravascular Evan's blue dye in cutaneous blood vessels. *C*, Naive mouse not injected with Evan's blue dye.

Isotype profile of Ag-specific Abs induced by pOMC immunization of C3H mice

To determine whether the induction of Ara h2-specific IgG1 in pAra h2-immunized C3H mice is specific to peanut allergen, C3H mice were multiply immunized with pOMC, the plasmid DNA encoding the major egg allergen protein, ovomucoid. The Ab responses were measured kinetically after immunization. Similar to pAra h2-immunized C3H mice, both IgG1 and IgG2a Ab levels were markedly increased 2 wk after immunization (Fig. 6). At 3 wk, the level of ovomucoid-specific IgG1 levels in the multiply immunized group was about 32-fold greater than that in the singly immunized group, whereas IgG2a levels in multiply immunized mice were threefold greater than in singly immunized mice. Challenge of pOMC-immunized mice with ovomucoid also resulted in severe anaphylactic reactions (data not shown). These results demonstrate that pDNA immunization-induced IgG1 Ab responses in C3H mice are not unique to pAra h2.

Strain-dependent reactions to peanut protein injection following pAra h2 DNA immunization

Since the results described above differed from those of the two previous studies of allergen gene immunization, in which different rodent models were used (7, 8), we hypothesized that the consequences of allergen gene immunization may be strain dependent. To evaluate this possibility, we employed AKR and BALB/c mice, utilizing the same multiple pDNA immunization protocol used in C3H mice. In contrast to C3H mice, peanut protein injection of AKR or BALB/c mice at 3 or 5 wk following pAra h2 DNA immunization did not elicit any sign of anaphylaxis (Table II).

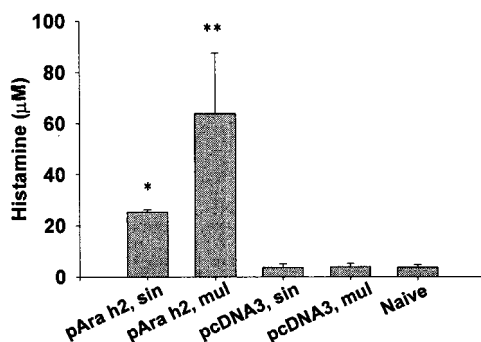


FIGURE 4. Plasma histamine levels following PN injection of C3H mice. Five to eight minutes after PN injection, plasma from different groups of mice, as indicated ($n = 4-5$), was obtained. The level of histamine was measured by ELISA, and calculated by comparison with a standard curve. *, $p < 0.01$ versus pcDNA, sin; **, $p < 0.001$ versus pcDNA, mul.; **, $p < 0.01$ versus pAra h2, sin.

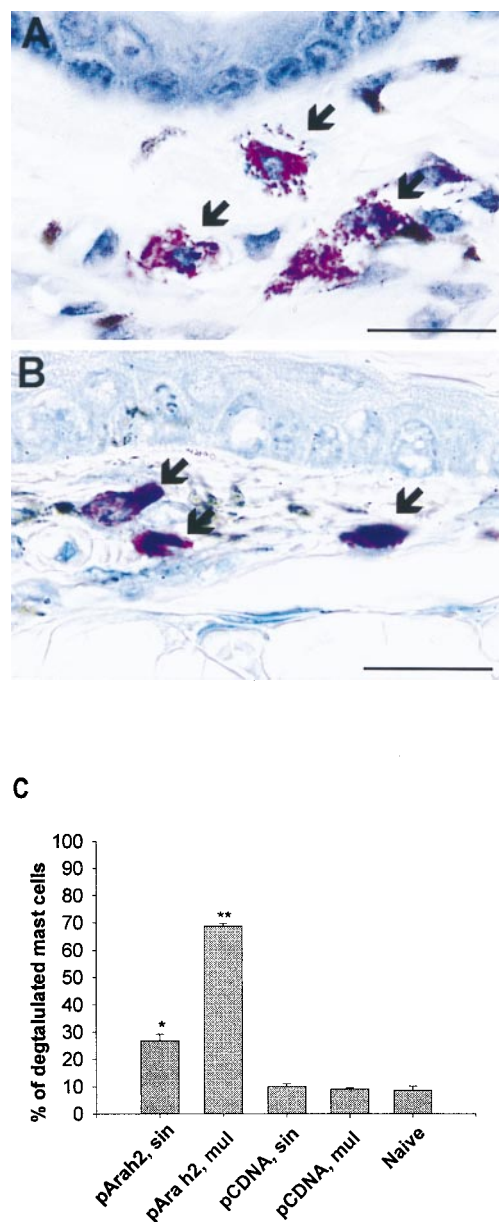


FIGURE 5. Mast cell degranulation. *A*, Shows degranulated and *B*, non-degranulated mast cells in ear samples of pAra h2 and mock DNA multiply immunized mice, respectively (bar = 50 [μm]). *C*, Shows the percentage of degranulated mast cells in ear samples of pAra h2- and mock DNA-immunized mice (200–400 mast cells were analyzed). *, $p < 0.01$ versus pcDNA, sin; **, $p < 0.001$ versus pcDNA, mul.; **, $p < 0.01$ versus pAra h2, sin.

Kinetics and isotype profile of Ag-specific Abs induced by pDNA immunization of AKR, BALB/c, and C3H mice

To elucidate the immunologic mechanisms underlying these different types of responses in AKR, BALB/c, and C3H mice, we examined the kinetics of the Ara h2-specific IgG2a, IgG1, and IgE Abs from week 1 through week 6 following multiple doses of pDNA immunization (Fig. 7). In AKR mice, IgG2a was markedly increased at 2 wk and reached a peak at 5 wk. In BALB/c mice, no IgG2a Ab was present until week 4; the peak level was found at week 6. No IgG1 or IgE Ara h2-specific Abs were detected following pAra h2 immunization at any time point in either AKR or BALB/c mice. Although BALB/c mice presented a similar pattern of IgG2a responses as AKR mice, the responses occurred slightly

Table I. PCA reactions in C3H mice^a

Donor Immunization	Heat Inactivation	Diameter (cm) (mean $\pm$ SE)	Positive Reaction	
			n/total	%
pAra h2	+	2.67 $\pm$ 0.21	6/6	100
pAra h2	—	2.75 $\pm$ 0.17	6/6	100
pcDNA	—	0.14 $\pm$ 0.06	0/5	0
PBS	—	0.12 $\pm$ 0.05	0/5	0

^a Naive C3H mice in each group ($n = 5-6$) as indicated received heated or non-heated pAra h2 immune sera, mock DNA (pcDNA) immune sera, or PBS followed by PN/Evan's blue dye administration. PCA reactions were scored as described in *Materials and Methods*.

later and were weaker. In contrast to the IgG isotype profile in AKR and BALB/c mice, both IgG2a and IgG1 were increased significantly in C3H mice at week 3, and peaked at week 3 for IgG2a and at week 4 for IgG1. No significant decrease in the level of either IgG2a or IgG1 was observed thereafter. Furthermore, the levels of IgG2a in C3H mice were significantly lower than that in AKR mice. These findings demonstrate that the variability of Ab responses to pDNA immunization is primarily strain dependent.

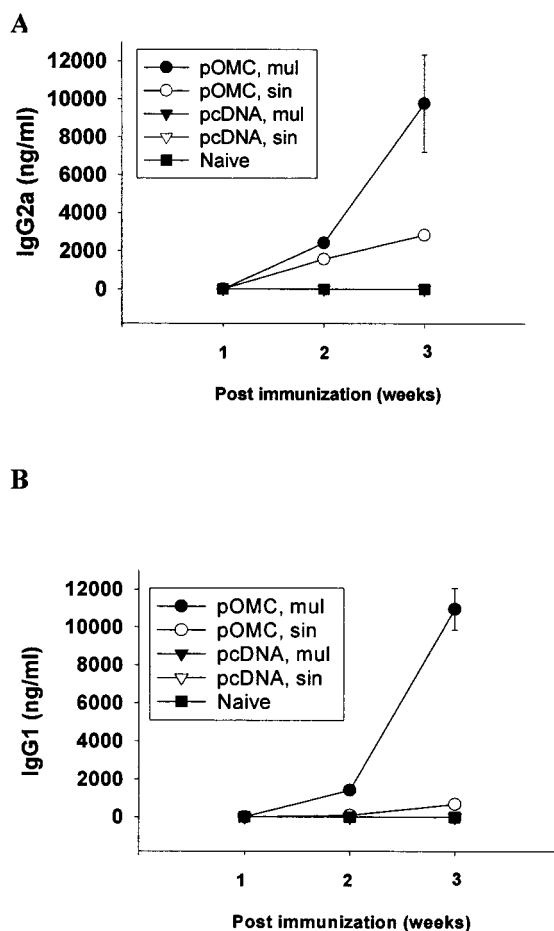


FIGURE 6. Ovomucoid-specific Abs induced by pOMC in C3H mice. Sera from different groups of mice ($n = 5$) as indicated were obtained at weekly intervals from 1–3 wk after the initial pDNA immunization. The levels of ovomucoid-specific IgG2a and IgG1 were determined by ELISA, and calculated by comparison with a reference curve generated using mouse mAb, anti-DNP Abs.

Table II. Anaphylactic reactions to PN injection in different strains of mice following pAra h2 DNA immunization^a

Strain	3 wk		5 wk	
	n/total	%	n/total	%
C3H	10/10	100	5/5	100
AKR	0/10	0	0/8	0
BALB/c	0/10	0	0/6	0

^a Mice ($n = 5-10$) in each group as indicated received i.p. injection of PN at 3 or 5 wk following pDNA multiple immunization. The incidence of anaphylaxis in each group of mice was calculated and described as morbidity rate.

T cell cytokine profiles induced by pAra h2 immunization

To determine whether the different Ab responses of these three strains were related to differential production of T cell cytokines, cytokines produced by spleen cells were measured 3 wk following multiple pAra h2 immunization. Since cytokine production in culture following PN stimulation revealed that levels of IFN- γ peaked at 72 h, IL-4 increased significantly at 24 h, but did not decrease significantly thereafter, and IL-5 was not detected at any time point, Table III depicts supernatant cytokine levels after 72 h of culture. Levels of IFN- γ were markedly increased in Con A-stimulated cultures from all three strains. Levels of IFN- γ in PN-stimulated cultures were also significantly higher than unstimulated cultures from all three strains ($p < 0.001$ in C3H; 0.01 in AKR; 0.05 in BALB/c). C3H spleen cells produced approximately twice

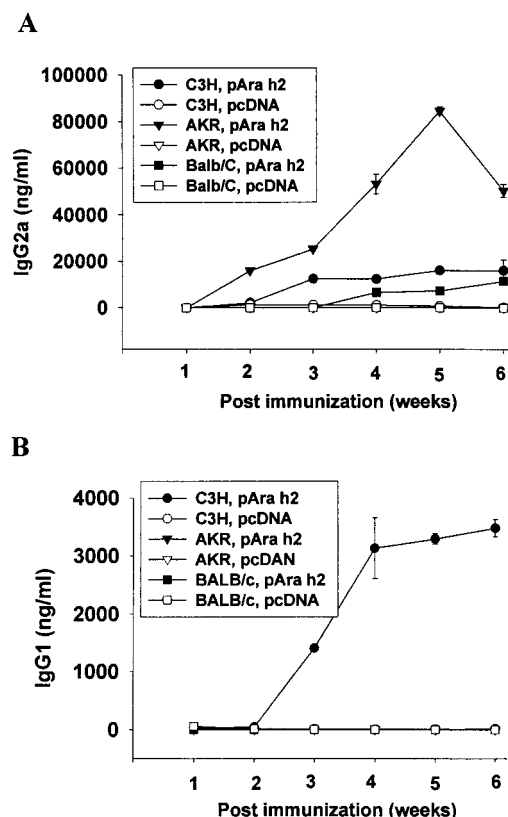


FIGURE 7. Kinetics of isotype profile of Ara h2-specific Abs induced by pAra h2 immunization of AKR, BALB/c, and C3H mice. A, Levels of Ara h2-specific IgG2a. B, Levels of Ara h2-specific IgG1. Sera from different groups of mice ($n = 5$) were obtained at weekly intervals following multiple pDNA immunization. The levels of Ara h2-specific IgG2a and IgG1 were determined by ELISA, and calculated by comparison with a reference curve generated by using mouse mAb, anti-DNP Abs.

Table III. Cytokine secretion by spleen cells from different strains of mice following pAra h2 immunization^a

Strain	IFN- γ (pg/ml)			IL-4 (pg/ml)			IL-5 (pg/ml)		
	PN	Con A	Control	PN	Con A	Control	PN	Con A	Control
C3H	749 $\pm$ 101	>6000	<7.8	115 $\pm$ 5	389 $\pm$ 21	56 $\pm$ 1	<7.8	564 $\pm$ 10	<7.8
AKR	397 $\pm$ 69	>6000	133 $\pm$ 15	105 $\pm$ 7	207 $\pm$ 2	70 $\pm$ 4	<7.8	131 $\pm$ 14	<7.8
BALB/C	360 $\pm$ 57	>6000	296 $\pm$ 4	68 $\pm$ 2	712 $\pm$ 51	52 $\pm$ 3	<7.8	385 $\pm$ 12	<7.8

^a Spleen cells from the mice ($n = 2-3$) at 3 wk after pAra h2 multiple immunization were cultured in the presence or absence of Con A or PN. Levels of IFN- γ , IL-4, and IL-5 in 72-h culture supernatants were measured by ELISA and calculated by comparison with a standard curve. The lower and upper levels of the assay in this experiment were 7.8 and 6000 pg/ml.

as much PN-induced IFN- γ as AKR and BALB/c cells. Although Con A stimulation significantly increased ($p < 0.01$) IL-4 secretion in cultures from all three strains, PN stimulation resulted in similar significantly increased ($p < 0.02$) levels of IL-4 production by the cells from C3H and AKR, but not from BALB/c. Levels of IL-5 were increased significantly in Con A-stimulated cultures ($p < 0.01$) from all three strains. However, IL-5 was not detectable in PN-stimulated cultures from any of the three strains. These results indicate that Th1/Th2 cytokine production in splenic cells does not reflect the differential expression of pAra h2-induced IgG1 or IgG2a between the three strains in these experiments.

Discussion

To investigate the potential application of allergen gene immunization for the modulation of peanut allergy, our first experiments were aimed at determining whether pDNA immunization could prevent peanut-induced hypersensitivity, as reported for other Ags (7, 8). C3H mice were immunized with pAra h2, a plasmid DNA encoding a major PN allergen. Unexpectedly, following the first i.p. injection of peanut protein at week 3, the mice exhibited anaphylactic symptoms. These anaphylactic reactions were both Ag specific and dose dependent. The marked vascular leakage, hemoconcentration in major organs, and marked platelet aggregation in peripheral blood smears suggested that death in these mice resulted from hypovolemic shock. Mast cell degranulation and plasma histamine levels in pAra h2-immunized mice were markedly increased, particularly in multiply immunized mice. Increased serum histamine levels were associated with the severity of the anaphylactic reactions. These data suggest that histamine released from mast cells is a major mediator of anaphylactic shock in this model.

It has been demonstrated that IgE plays an important role in mediating type 1 hypersensitivity in response to Ag in humans (28, 29) as well as in animal models (30, 31). However, recent studies have shown that IgE is not required for Ag-induced anaphylaxis in mice. Oettgen et al. (32) generated IgE-deficient mice with a homozygous null mutation of C ϵ gene. These mice made no IgE, but produced IgG2a, IgG1, and IgM after OVA sensitization, and exhibited anaphylactic shock following OVA challenge. It has also been reported that sensitization of several mouse strains with BSA, horse γ -globulin, and lysozyme induced Ag-specific IgG1, but not Ag-specific IgE responses in these models. Death from anaphylactic shock was induced by i.v. Ag challenge. Immune serum could induce anaphylactic death, and heating did not destroy its activity (33). Oshiba et al. demonstrated that immediate hypersensitivity and airway hyperresponsiveness could be passively transferred by allergen-specific IgE and IgG1, but not IgG2a or IgG3 (34). These studies indicate that IgG1 Abs can play as important a role as IgE in the induction of immediate hypersensitivity in mice. It has also been demonstrated that IgG was able to trigger mast cell

degranulation and histamine release via IgG Fc γ receptors on the mast cells (35–37).

In the present study, we demonstrated that anaphylactic reactions induced by peanut protein injection of pAra h2 DNA-immunized C3H mice are also IgG1 mediated. First, no significant levels of Ara h2-specific IgE Ab were present following pAra h2 immunization. Lack of IgE response following plasmid DNA immunization appears to be a general phenomenon following DNA immunization. Second, the IgG1 levels were increased significantly in pAra h2-immunized mice with markedly greater (30-fold) levels in multiply immunized mice than that in singly immunized mice. In addition, IgG1 levels directly reflected the severity of anaphylactic reactions. Finally, PCA reactions were not reduced by heat inactivation of sera from pAra h2-immunized mice.

Serum IgG2a was also markedly increased in this model, and was in fact even higher than IgG1 following pAra h2 immunization. Lack of any protective effect of IgG2a following peanut protein injections in C3H mice may be due to the fact that IgG1 levels were so high that IgG1 binding to mast cells could not be blocked by competitive IgG2a binding on the mast cells.

In recent years, DNA vaccination has emerged as a novel therapeutic approach for the control of infectious diseases and allergic disorders. Raz and Spiegelberg et al. (8, 38, 39) demonstrated that i.d. injections of plasmid DNA-encoding β -galactosidase in BALB/c mice induced a Th1 response and the generation of IgG2a Abs, but almost no IgG1 or IgE Abs. Similar results were reported by Hsu et al. (7), who immunized Brown Norway rats with pDNA-encoding Der p 5, which produced IgG2a, but not IgE Abs, and inhibited Ag challenge-induced airway hyperresponsiveness and histamine release. More recently, Slater et al. (40) reported that BALB/c mice primed with latex allergen Hev b5 and boosted with pDNA encoding the allergen Hev b 5 exhibited a 23% decrease in the specific IgE Ab titer within 10 days following immunization. One possible mechanism of these pDNA-elicited Th1 responses includes the induction of IL-12 and IFN- α/β secretion by accessory cells induced by noncoding immunostimulatory DNA sequences containing CpG motifs in the backbone of the pDNA (41–43).

Immunization with pDNA, however, does not always induce a Th1 response. Th1- or Th2-like responses can be driven by several factors. Feltquate et al. (44), for example, showed that the nature of pDNA-induced immune responses can be dependent on the method of inoculation. Immunization with pDNA by i.d. or i.m. injection produced a Th1-like immune response with mostly IgG2a Abs. However, the same DNA delivered i.d. or i.m. by gene gun produced a Th2-like response and predominantly IgG1 Abs. In the present study, neither the method nor route of pDNA inoculation was the determining factor because i.m. injection of pAra h2 was used in all animals studied. Moreover, IgG1 was also produced by i.d. injection of C3H mice with pAra h2.

In our model, the nature of the Ag encoded in the plasmid DNA was also not the major factor in determining the isotype profile of Ab responses to pDNA immunization. In C3H mice, i.m. injection of pOMC, a plasmid DNA-encoding ovomucoid protein, resulted in the same type of allergic reactions as i.m. injection of pAra h2. In addition, the resulting Ab isotype was not dose dependent. Although single or multiple immunizations with pAra h2 induced significant differences in the levels of IgG1, the Ab isotype profile was not altered.

The present study demonstrates that the outcome of pDNA immunization in mice is strain dependent. AKR and BALB/c strains exhibited a different Ab isotype than C3H mice. In these strains, only IgG2a Ab was induced. The absence of anaphylactic reactions following PN protein administration in these strains is attributable to the absence of reagenic IgG1 or IgE Ab. In addition, although AKR and BALB/c mice showed a similar pattern of Ab responses to pAra h2 immunization, the level of specific IgG2a was significantly higher in AKR mice. The IgG2a responses in BALB/c mice appeared 2–3 wk later than AKR mice. These findings show that the Ab isotype, as well as the levels and kinetics of Ab responses, is strain dependent.

It is not clear why the pAra h2-immunized C3H mice developed both IgG2a and IgG1 responses, whereas AKR and BALB/c mice developed only IgG2a responses. It has been well documented that Th1/Th2 cytokines are responsible for the production of different Ab isotypes in mice, e.g., IL-4 drives IgE and IgG1, and IFN- γ favors IgG2a (45–48). However, our results cannot be explained solely by the Th1/Th2 cytokine pattern. In this study, production of IFN- γ , but not IL-4, by PN-stimulated splenic cells was higher in C3H mice than AKR and BALB/c mice 3 wk after the initial pAra h2 immunization. However, C3H mice at this time point had high levels of Ag-specific IgG1, while AKR and BALB/c strains did not. These results suggest that although the IgG2a response in C3H mice may be related to IFN- γ production, the IgG1 response may not be solely the consequence of IL-4 production. Previous studies have shown that anti-IL-4 Abs induce little or no inhibition of IgG1 synthesis either in vitro (49) or in vivo (50). Taken together, these findings suggest that substantial IgG1 responses may be induced by an IL-4-independent mechanism, and that other non-Th1/Th2 pathways may also be important.

Finally, there is no clear linkage of these strain-dependent Ab responses following pDNA immunization to MHC haplotype since C3H and AKR mice share the same haplotype (H-2^K). It has been shown that C3H mice are easily sensitized to many Ags using oral or i.p. sensitization and exhibit anaphylaxis following challenge (25). BALB/c mice, one of the most commonly used strains in allergy and DNA vaccine studies, have been reported to be less susceptible to sensitization by several Ags (51, 52) than some other strains. AKR mice that have been used as a model for IgE-mediated allergic responses (22, 53) were found to produce a significant IgG2a response following DNA immunization in the present study. The AKR strain may therefore be a suitable model for investigating the therapeutic potential of allergen gene immunization.

In conclusion, C3H mice were sensitized by peanut allergen gene immunization, which resulted in a peanut-specific IgG1 response. Subsequent administration of PN protein caused severe, often fatal anaphylactic reactions. AKR and BALB/c mice were not sensitized by peanut allergen gene immunization because these strains synthesized only anti-Ara h2 IgG2a Ab. Careful consideration should be given to selection of a suitable mouse strain in attempts to develop models of human disease. In addition, it is possible that further understanding of murine strain-related allergic reactions may assist in our understanding of the genetic basis of susceptibility to food and other allergies in man.

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References

- Donnelly, J. J., A. Friedman, D. Martanez, D. L. Montgomery, J. W. Shiver, S. L. Motzel, J. B. Ulmer, and M. A. Liu. 1995. Preclinical efficacy of prototype DNA vaccine-enhanced protection against antigenic drift in influenza-virus hemagglutinin. *Nat. Med.* 1:583.
- Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccine-protective immunizations by parental, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* 90:11,478.
- Robinson, H. L. 1997. DNA vaccines for immunodeficiency viruses. *AIDS* 11(Suppl.1):A:s109.
- Tascon, R. E., M. J. Colston, S. Ragon, E. Stavropoulos, D. Gregory, and D. B. Lowrie. 1996. Vaccination against tuberculosis by DNA injection. *Nat. Med.* 2:888.
- Huygen, K., J. Content, O. Denis, D. L. Montgomery, A. M. Yawman, R. R. Deck, C. M. Dewitt, I. M. Orme, S. Baldwin, C. D'Souza et al. 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. *Nat. Med.* 2:893.
- Xu, D., and F. Y. Liew. 1995. Protection against leishmaniasis by injection of DNA encoding a major surface glycoprotein, gp63, of 1-major. *Immunology* 84:173.
- Hsu, C.-H., K.-Y. Chua, M.-H. Tao, Y.-L. Lai, H.-D. Wu, S.-K. Huang, and K.-H. Hsieh. 1996. Immunoprophylaxis of allergen-induced immunoglobulin E synthesis and airway hyperresponsiveness in vivo by genetic immunization. *Nat. Med.* 2:540.
- Raz, E., H. Tighe, Y. Sato, M. Corr, J. A. Dudler, M. Roman, S. L. Swain, H. L. Spiegelberg, and D. A. Carson. 1996. Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization. *Proc. Natl. Acad. Sci. USA* 93:5141.
- Sampson, H. A., and C. C. McCaskill. 1985. Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. *J. Pediatr.* 107:669.
- Atkins, F. M., S. S. Steinberg, and D. D. Metcalfe. 1985. Evaluation of immediate adverse reactions to foods in adults. II. A detailed analysis of reaction patterns during oral food challenge. *J. Allergy Clin. Immunol.* 75:356.
- Yunginger, J. W., K. G. Sweeney, W. Q. Sturmer, L. A. Giannandrea, J. D. Teigland, M. Bray, P. A. Benson, J. A. York, L. Biedrzyck, and D. L. Squillace. 1988. Fatal food-induced anaphylaxis. *JAMA* 260:1450.
- Sampson, H. A., L. Mendelson, and J. P. Rosen. 1992. Fatal and near fatal anaphylactic reactions to food in children and adolescents. *N. Engl. J. Med.* 327:380.
- Sampson, H. A. 1992. Food allergy and the role of immunotherapy. *J. Allergy Clin. Immunol.* 90:151.
- Sampson, H. A., N. Buckley, S.-K. Huang, A. W. Burks, and G. A. Bannon. 1998. Characterization of major peanut allergens. *J. Allergy Clin. Immunol.* 101:S240.
- Sicherer, S. H., A. W. Burks, and H. A. Sampson. 1998. Clinical features of acute allergic reactions to peanut and tree nuts in children. *Pediatrics* 102:E6.
- Oppenheimer, J. J., H. S. Nelson, B. Allan, F. Chrostensen, J. Lahr, A. Bock, and D. Leung. 1992. Treatment of peanut allergy with rush immunotherapy. *J. Allergy Clin. Immunol.* 90:256.
- Nelson, H. S., J. Lahr, R. Rule, A. Bock, and D. Leung. 1997. Treatment of anaphylactic sensitivity to peanut by immunotherapy by injection of aqueous peanut extract. *J. Allergy Clin. Immunol.* 99:744.
- Burks, A. W., L. W. Williams, R. M. Helm, C. Connaughton, G. Cockrell, and T. O'Brien. 1991. Identification and characterization of a major peanut allergen, Ara h I, in patients with atopic dermatitis and positive peanut challenges. *J. Allergy Clin. Immunol.* 88:172.
- Burks, A. W., L. W. Williams, C. Connaughton, G. Cockrell, T. O'Brien, and L. W. Williams. 1992. Identification and characterization of second major peanut allergen, Ara h II, with the use of the sera of the patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* 90:962.
- Kopper, R., S. Maleki, R. Helm, H. Sampson, S.-K. Huang, G. Cockrell, A. W. Burks, and G. A. Bannon. 1998. Rapid isolation of peanut allergen and their physical and biological characterization. *J. Allergy Clin. Immunol.* 101:S240.
- Stanley, J. S., N. King, A. W. Burks, S.-K. Huang, H. Sampson, G. Cockrell, R. M. Helm, C. M. West, and G. A. Bannon. 1997. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h2. *Arch. Biochem. Biophys.* 342:244.
- Li, X.-M., B. H. Schofield, Q.-F. Wang, K. H. Kim, and S.-K. Huang. 1998. Induction of pulmonary allergic responses by antigen-specific Th2 cells. *J. Immunol.* 160:1378.
- McCaskill, A. C., C. S. Hosking, and D. J. Hill. 1984. Anaphylaxis following intranasal challenge of mice with ovalbumin. *J. Immunol.* 51:669.
- Poulsen, O. M., J. Hau, and J. Kollerup. 1987. Effect of homogenization and pasteurization on the allergenicity of bovine milk analyzed by a murine anaphylactic shock model. *Clin. Allergy* 17:449.
- Snider, D. P., J. S. Marshall, M. H. Perdue, and H. Liang. 1994. Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein antigen and cholera toxin. *J. Immunol.* 143:647.

26. Saloga, J., H. Renz, G. Lack, K. L. Bradley, J. L. Greenstein, G. Larsen, and E. W. Gelfand. 1993. Development of transfer of immediate cutaneous hypersensitivity in mice exposed to aerosolized antigen. *J. Clin. Invest.* 91:133.
27. Poulsen, O. M., and J. Hau. 1987. Murine passive cutaneous anaphylaxis test (PCA) for the "all or none" determination of allergenicity of bovine whey protein and peptide. *Clinical Allergy* 17:75.
28. Martin, T. K., S. J. Galli, I. M. Katona, and J. M. Drazen. 1989. Role of mast cells in anaphylaxis. *J. Clin. Invest.* 83:1375.
29. Ishizaka, T., H. Tomioka, and K. Ishizaka. 1971. Degranulation of human basophil leukocytes by anti-IgE antibody. *J. Immunol.* 160:705.
30. Dobrowicz, D., V. Flamand, K. K. Brigman, B. H. Koller, and J.-P. Kinet. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. *Cell* 75:969.
31. Naito, K., M. Hirama, K. Okumora, and C. Ra. 1996. Recombinant soluble form of the human high-affinity receptor for IgE prevents anaphylactic shock in mice. *J. Allergy Clin. Immunol.* 97:973.
32. Oettgen, H. C., T. R. Martin, A. Wtmslaw-Boris, C. Deng, J. M. Drazen, and P. Leder. 1992. Active anaphylaxis in IgE deficient mice. *Nature* 22:769.
33. Lei, H.-Y., S. H. Lee, and S.-H. Leir. 1996. Antigen-induced anaphylactic death in mice. *Int. Arch. Allergy Immunol.* 109:407.
34. Oshiba, A., E. Hamelmann, K. Takeda, K. L. Bradley, J. E. Loader, G. L. Larsen, and E. W. Gelfand. 1996. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J. Clin. Invest.* 97:1398.
35. Fox, P. C., L. K. Basciano, and R. P. Siranian. 1982. Mouse mast cell activation and desensitization for immunaggregate induced histamine release. *J. Immunol.* 129:314.
36. Katz, H. R., M. B. Baizman, C. S. Gartner, H. C. Scott, A. C. Benson, and K. F. Austen. 1992. Secretory granule mediator release and generation of oxidative metabolites of arachidonic acid via Fc-IgG receptor bridging in mouse mast cells. *J. Immunol.* 148:868.
37. Hirayama, N., T. Hirano, G. Kohler, A. Kurata, K. Okumora, and Z. Ovary. 1982. Biological activities of antitritonphenyl and antinitrophenyl mouse monoclonal antibodies. *Immunology* 97:613.
38. Spiegelberg, H. L., E. R. Orozco, M. Roman, and E. Raz. 1997. DNA immunization: a novel approach to allergen-specific immunotherapy. *Allergy* 52:964.
39. Spiegelberg, H. L., H. Tighe, M. Roman, L. Beck, and E. Raz. 1998. Down-regulation of IgE antibody formation by allergen gene immunization. *ACI Int.* 10:52.
40. Slater, J. E., Y. J. Zhang, A. Arthur-Smith, and A. Colberg-Poley. 1997. A DNA vaccine inhibits IgE responses to the latex allergen Hev b 5 in mice. *J. Allergy Clin. Immunol.* 99:S504.
41. Sato, Y., M. Roman, H. Tighe, D. Lee, M. P. Corr, M. D. Nguyen, G. J. Silverman, M. Lotz, D. A. Carson, and E. Raz. 1996. Immuno-stimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* 273:352.
42. Yamamoto, S., T. Yamamoto, T. Kataoka, E. Kuramoto, O. Yano, and T. Tokunaga. 1992. Unique palindromic sequences in synthetic oligonucleotides are required to induce INF and INF-mediated natural killer activity. *J. Immunol.* 148:4072.
43. Raz, E., M. Roman, E. Martin-Orozco, and D. A. Carson. 1997. Immunostimulatory DNA sequences (ISS) are a Th1 promoting adjuvant. *J. Allergy Clin. Immunol.* 99:S365.
44. Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson. 1997. Different T help cells and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* 158:2278.
45. Snapper, C. M., and W. E. Paul. 1987. Interferon- γ and B cell stimulatory factor 1 reciprocally regulate Ig isotype production. *Science* 236:944.
46. Howard, M., and W. E. Paul. 1983. Regulation of B cell growth and differentiation by soluble factors. *Annu. Rev. Immunol.* 1:307.
47. Mosmann, T. R., and R. L. Coffman. 1989. Th1 and Th2 cells: different patterns of lymphokine secretion leading to different functional properties. *Annu. Rev. Immunol.* 7:145.
48. Beck, L., and H. L. Spiegelberg. 1989. The polyclonal and antigen-specific IgE and IgG subclass response of mice injected with ovalbumin in alum or complete Freund's adjuvant. *Cell. Immunol.* 123:1.
49. Coffman, R. T., B. W. Seymour, D. A. Leberman, D. D. Hiraki, J. A. Christiansen, B. Shrader, H. M. Cherwinski, H. F. J. Savelkoul, F. D. Finkelman, M. W. Bond, and T. R. Mosmann. 1988. The role of help T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 102:5.
50. Coffman, R. T., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, and W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE responses of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.
51. Harada, M., R. Misaki, H. Fukushima, M. Nagata, and S. Makino. 1989. Strain difference and model of inheritance of the susceptibility to passive cutaneous anaphylaxis mediated by allogeneic IgE antibody in the mouse. *Immunol. Invest.* 18:723.
52. Zuany, A. C., B. B. Vargaftig, J. Maclof, and M. Pretolani. 1994. Strain-dependency of leukotriene C₄ generation from isolated lungs of immunized mice. *Br. J. Pharmacol.* 112:1230.
53. Li, X.-M., R. K. Chopra, T.-Y. Chou, B. H. Schofield, M. Wills-Karp, and S.-K. Huang. 1996. Mucosal IFN γ -gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 157:3216.