

Original article

Various factors (allergen nature, mouse strain, CpG/recombinant protein expressed) influence the immune response elicited by genetic immunization

Background: Genetic immunization is a very promising therapeutic approach for allergy treatment. In the present study we investigate the influence of the nature of the allergen, the mouse strain, and the relative amount of CpG to expressed recombinant protein on immune responses using two major peanut allergens, Ara h 1 and Ara h 4.

Methods: The cDNA of Ara h 1 and of an isoform of Ara h 4 were cloned and inserted in pcDNA3. Antigen specific IgG1, IgG2a and IgE were followed after genetic immunization with 100 µg of these clones in mouse strain SKH-Hr1 or BALB/c and with 1 µg of the clones + 99 blank plasmid in SKH-Hr1.

Results: Genetic immunization in SKH-Hr1 with Ara h 1 elicited a classical Th1 type response, but Ara h 4 elicited a mixed Th1/Th2 response with high IgG1 and even IgE in some mice. In BALB/c both plasmids produced a high IgG1 level. Decreasing the amount of plasmid injected did not change the immune response profile. However, increasing the amount of CpG administered relative to the recombinant Ara h 4 protein expressed reversed the Th1/Th2 response pattern in SKH-Hr1 mice.

Conclusions: Immune responses after genetic immunization are strongly influenced by the nature of the allergen, the mouse strain, and the ratio of CpG to recombinant protein expressed.

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Key words: allergy; genetic immunization; peanuts.

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Accepted for publication 28 February 2003

Food allergy poses a considerable public health problem especially in children because of the potential severity of allergic hypersensitivity. In the US, food allergies are the most common cause of anaphylaxis treated in hospital emergency rooms (1). Peanuts and tree nuts together are the leading cause of fatal and near-fatal reactions (2). At the present time there are no safe and effective therapies for food allergies except avoidance. Unfortunately, accidental ingestion of peanut containing food is not rare and results in many deaths each year (3).

Intramuscular or intradermal injection of plasmid DNA encoding antigens, called genetic immunization, induces a strong T helper cell type 1 (Th1) response in contrast to the Th2-type response after conventional immunization with protein (4). The major reason for this Th1-type response is thought to be the presence of immunostimulatory sequences (ISS) in the plasmid DNA vector used for immunization (5). These ISS

contain unmethylated CpG motifs flanked by two 5' purines and two 3' pyrimidines, which bind to toll-like receptor 9 on macrophages and dendritic cells (6), thereby stimulating the production of Th1-biasing cytokines (7).

In this paper using DNA plasmids encoding for peanut allergen Ara h 1 (pAra h 1) and an isoform of Ara h 4 and Ara h 3 (pAra h 4), we found that the Th1/Th2 bias of the resulting immune response after genetic immunization was influenced by multiple factors, including the nature of the allergen protein, the mouse strain, and the relative ratio of the quantity of ISS to the recombinant protein expressed. Genetic immunization in SKH-Hr1 mice with pAra h 1 resulted in the expected Th1 biased response with a high level of IgG2a and no detectable IgE. In contrast, immunization with pAra h 4 in the same mouse strain resulted in a Th2 bias with a high level of IgG1 and even the presence of IgE. In BALB/c, administration of both plasmids elicits a strong IgG1 response. The Th2 immune response profile for pAra h 4 in SKH-Hr1 mice was reversed by increasing the amount of administered CpG relative to the expressed recombinant protein.

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Material and methods

DNA purification from λ gt11 cDNA library

A λ gt11 cDNA library constructed with RNA from developing seeds of F78-1339 (*A. hypogaea*) as described (8) was a kind gift of Albert Abbott (Clemson University, Clemson, SC, USA). The titer of the cDNA library, $1.8/10^8$ pfu/ml was obtained as described (9). Fifty thousand pfu were diluted in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin) and added to 500 μ l of *E. coli* MRA previously grown until $OD_{600} = 0.5$ and resuspended in 10 mM $MgSO_4$. After 15 min, at 37°C, we added 7.5 ml of LB M/M Top agarose: (LB broth, maltose 0.2%, $MgSO_4$ 10 mM, high melting point agarose 0.4%, low melting point agarose 0.3%) and plated on 150 mm NZY Agarose plates (yeast extract 0.5%, N-Z-Amine A 1%, Casaminoacids 0.1%, NaCl 100 mM, $MgSO_4$ 8 mM, high melting point agarose 0.7%). After an overnight (ON) incubation, at 37°C, 5 ml SM buffer was added on each plate. Plates were shaken at room temperature (room temperature) for 1 h. Then, we added 2 ml chloroform for each plate and incubated it for 1 h at 37°C with shaking. The slurry was collected and then centrifuged at 15 000 *g* for 15 min. The supernatant was loaded on gradient 40/5% glycerol in TMG (10 mM Tris-HCl pH 7.5, $MgSO_4$ 5 mM, gelatin 0.01%) and then centrifuged at 28 000 rpm for 2 h. For each 10 plates, the pellet was resuspended in 5 ml TMG buffer. DNase I was added to a final concentration of 1 μ g/ml, and RNase was added to a final concentration of 5 μ g/ml. The suspension was then incubated at 37°C for 30 min. Enzymes were inactivated by the addition of EDTA (final concentration 20 mM) and SDS (final concentration 0.5%) with a 15 min incubation at 70°C. Then, proteinase K was added (final concentration 50 μ g/ml), and the solution was incubated at 60°C for 1 h, followed by inactivation at 70°C for 1 h. The solution was extracted with phenol/chloroform until the interface was clear, and the DNA was precipitated by ethanol.

Amplification and cloning of Ara h 1 and Ara h 4

Amplification by PCR was done on purified DNA from λ gt11 cDNA using the Expand High Fidelity PCR System (Boehringer). Primers used were pair H1F1 (ATG AGA GGG AGG GTT TC TCC A) and H1R1 (TCA GTT AAA AGC CTT CAA AAT) priming, respectively, at the N- and C-terminus of Ara h 1 (Genbank L34402) and pair H4F1 (ATG GCT AAG CTT CTT GAG CTT TCT TTT TGC) and H4R1 (TTA AGC CAC ACG CCT CGG AGA CTG CTG AAA) priming respectively at N- and C-terminus of Ara h 4 (Genbank AF086821). The typical program used was 94°C for 10 min (one cycle); then 94°C for 1 min, 55°C for 1 min, 72°C for 1 min (25 cycles) and finally 72°C for 10 min (one cycle). Cloning was done in pcDNA3.1/V5-His using pcDNA3.1/V5-His TOPO TA Expression Kit (Invitrogen) as described by the supplier. Briefly 2 μ l of each amplification reaction was directly added to 1 μ l of pcDNA3.1/V5-His TOPO vector, 1 μ l of salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and 2 μ l of H_2O for 30 min at room temperature. Then, 2 μ l of the cloning reaction was transformed in TOP10 chemically competent cells provided by the supplier. Transformed cells were spread on ampicillin (50 μ g/ml) plates, and incubated overnight at 37°C.

Positive clones were first selected for the presence of an insert of the right size by PCR on 20 isolated colonies using primer H1F1 and H1R1 or H4F1 and H4R1. Positive clones after the first selection were tested for an insert in the correct orientation for expression by PCR using T7F promoter (5' of the cloning site) primer and H1R1 or H4R1. Positive clones after the second selection

were confirmed by another PCR using primers H1F1 or H4F1 and BGH Reverse primer (3' of the cloning site).

Selected clones were amplified on selective medium and plasmids were purified (Plasmid Mini Kit, Qiagen), and then sequenced (Lone Star Labs Inc., Houston, TX).

Immunization

All animal experiments were approved by the Institutional Review Board for Animal Studies (Baylor College of Medicine). Groups of mice were injected intradermally in the skin 1 cm from the base of the tail (10) with a total of 100 μ g plasmid DNA in two separate 25 μ l PBS injections, except as otherwise specified. Plasmids were prepared using the Endofree Plasmid Giga Kit (Qiagen, Valencia, CA). Sequential serum samples were obtained by tail incision bleed and were assayed fresh or were stored frozen (-20°C) until assayed. The IgG subclass response patterns were analyzed by the Mann-Whitney rank sum test for each group of mice.

Immunoassays for the detection of specific IgG1, IgG2a and IgE anti-peanut extract

Ninety-six-well plates were coated passively with 1 μ g/ml of peanut extract diluted in phosphate buffer 50 mM, pH 7.4, for 18 h at room temperature. After washing, the plates were saturated with phosphate buffer 50 mM, pH 7.4, NaCl 0.15 M, BSA 0.1% (EIA buffer), for 18 h at room temperature. Sera were serially diluted in EIA buffer and incubated overnight at 4°C, in 96-well plates previously coated with peanut extract. The plates were washed five times with PBS-Tween (0.1%). Bound Abs were detected with HRP-conjugated goat anti-mouse IgG1 (SBA, Birmingham, AL), or rat anti-mouse IgG2a (Serotec, England) or rat anti-mouse IgE (clone LO-ME-3) (Serotec, England). Second antibodies were added for 2 h at room temperature. After five washes with PBS-Tween (0.1%) reactions were developed with the TMB liquid substrate system for ELISA (Sigma, St Louis, MI, USA). Reactions were stopped by addition of 1 volume of sulfuric acid 2N. The resulting yellow end product was read at 450 nm with an SLT microplate reader (TELAC Inc., Research Triangle Park, NC). The background was determined using preimmune sera. Mice sera immunized with peanut extract in alum were used as a positive control. Naive mice sera were used as a negative control.

Results

Cloning and characterization of Ara h 1 and Ara h 4

Ara h 1 and Ara h 4 were cloned by PCR using a polymerase with proof reading activity and N- and C-terminal oligonucleotides. Nucleotide sequences were deduced from the published sequence of Ara h 1 (clone p41b, Genbank L34402) (11) and Ara h 4 (Genbank AF086821) (12). Ara h3 (Genbank AF093541) (13) and Ara h 4 are two isoforms that are members of the 11S protein storage family, 92% homologous in amino acids (Fig. 1). We chose to use the Ara h 4 sequence because the cDNA was complete. Each amplification produced only one band at the predicted size (data not shown). The amplification products were cloned in the pcDNA3 vector and both sense and anti-sense strands were sequenced. We found one type of Ara h 1 clone, 100%

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Ara h 412 MAKLELSFCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRISEGGYIETWNPNN
Ara h 3 -----RQQPEENACQFQRLNAQRPDNRISEGGYIETWNPNN
Ara h 4 MAKLELSFCFCFLVLGASSISFRQQPEENACQFQRLNAQRPDNRISEGGYIETWNPNN
          *****

Ara h 412 QEFECAGVALSRLVLRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPSTYEPAQQGR
Ara h 3 QEFECAGVALSRLVLRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPPRHYEEPHTQGR
Ara h 4 QEFECAGVALSRLVLRNALRRPFYSNAPQEIFIQQGRGYFGLIFPGCPSTYEPAQQGR
          *****

Ara h 412 RSQSQRPPRRLQGEDQSQQQDSDHVKVHRFDEGDLIAVPTGVAFWLYNDHDTDVAVSLT
Ara h 3 RSQSQRPPRRLQGEDQSQQQDSDHVKVHRFDEGDLIAVPTGVAFWLYNDHDTDVAVSLT
Ara h 4 RYQSQRPPRRLQGEDQSQQQDSDHVKVHRFNEGDLIAVPTGVAFWLYNDHDTDVAVSLT
          * *****

Ara h 412 DTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQSRSRRSLPSPYSPQSPRQEEREFS
Ara h 3 DTNNNDNQLDQFPRRFNLAGNTEQEFLRYQQQSRSRRSLPSPYSPQSPRQEEREFS
Ara h 4 DTNNNDNQLDQFPRRFNLAGNHEQEFLRYQQQSRSRRSLPSPYSPHSPRPREEREFR
          *****

Ara h 412 PRGQHSRRERAGQEEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGENSEEEGAIVT
Ara h 3 PRGQHSRRERAGQEEENEGGNIFSGFTPEFLEQAFQVDDRQIVQNLRGETESEEGAIVT
Ara h 4 PRGQHSRRERAGQEEDEEGGNIFSGFTPEFLEQAFQVDDRQIVQNLWGENESEEEGAIVT
          *****

Ara h 412 VRGGLRILSPDRKRGADDEEYDEDEYDEEDRRRGRGSRGRGNGIETICTASVKKNI
Ara h 3 VRGGLRILSPDRKRGADDEEYDEDEYDEEDRRRGRGSRGRGNGIETICTASAKKNI
Ara h 4 VRGGLRILSPDGRGADDEEYDEDEYDEEDRRRGRGSRGGGNGIETICTACVKKNI
          *****

Ara h 412 GRNRSPDIYNPQAGSLKTANDLNLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYAL
Ara h 3 GRNRSPDIYNPQAGSLKTANDLNLILRWLGPSAEYGNLYRNALFVAHYNTNAHSIIYRL
Ara h 4 GGNRSPHIYDPQRWFTQNC HDLNLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYAL
          *****

Ara h 412 RGRAHVQVVDNSGNRVYDEELQEGHVLVVPQNFAVAGKSQSDNFEYVAFKTDSPRSIANL
Ara h 3 RGRAHVQVVDNSGNRVYDEELQEGHVLVVPQNFAVAGKSQSENFEYVAFKTDSPRSIANL
Ara h 4 RGRAHVQVVDNSGNRVYDEELQEGHVLVVPQNFAVAGKSQSENFEYVAFKTDSPRSIANF
          *****

Ara h 412 AGENSVIDNLPEEVVANSYGLPREQARQLKNNNPFFKFFVPPSQSPRRA
Ara h 3 AGENSVIDNLPEEVVANSYGLPREQARQLKNNNPFFKFFVPPSQSPRAVA
Ara h 4 AGENSFIDNLPEEVVANSYGLPREQARQLKNNNPFFKFFVPPFQQSPRAVA
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Figure 1. Sequence comparison between allergens Ara h 3, Ara h 4 and clone Ara h 412. Alignment has been done using CLUSTAL W (26). Homology between three sequences is indicated by *. Linear IgE binding epitopes of Ara h 3 (13) are underlined. Essential amino acids for IgE binding in each epitope are in italics. The proteolysis sequence consensus is in bold. Cysteine residues implicated in conserved disulfide bridge are in gray.

homologous to clone p41b of Ara h 1 and 97% homologous in nucleotide or amino acids to clone p17 (Genbank L38853). None of our clones were 100% homologous to the published isoform sequences for Ara h 4 or Ara h 3, and the clone we selected for further use, Ara h 412, is another isoform of the 11S family, 97% homologous in amino acids to Ara h 3 and 94% to Ara h 4 (Fig. 1). The plasmids used for the genetic immunization studies were named, respectively, pAra h 1 and pAra h 4.

ID Injection of 100 µg of pAra h 1 or pAra h 4 in SKH-HR1 mice

Genetic immunizations used 100 µg of either pAra h 1 or pAra h 4 injected intradermally in the tail of SKH-HR1 mice ($n = 4$ for pAra h 1, $n = 5$ for pAra h 4). After

4 weeks, a second injection, or boost, of 100 µg was done. The mice were bled at 4 weeks, before the boost injection, and again at 8 weeks, 4 weeks after the boost injection. The IgG1 and IgG2a responses were monitored in the sera by ELISA using plates coated with peanut extract or purified Ara h 1 protein. At 8 weeks after the first injection, both groups of mice showed IgG1 and IgG2a responses (Fig. 2). In the response to pAra h 1, the IgG2a responses were consistently higher than in the IgG1 responses ($P < 0.026$, Mann-Whitney rank sum test). In contrast, pAra h 4 injected mice showed consistently higher IgG1 responses than mice injected with pAra h 1 (pAra h 1 mice), and the IgG2a signals were usually less than the IgG1 signal, though the latter difference did not reach statistical significance. The same profile types were obtained 4 weeks after the first injection but with a lower

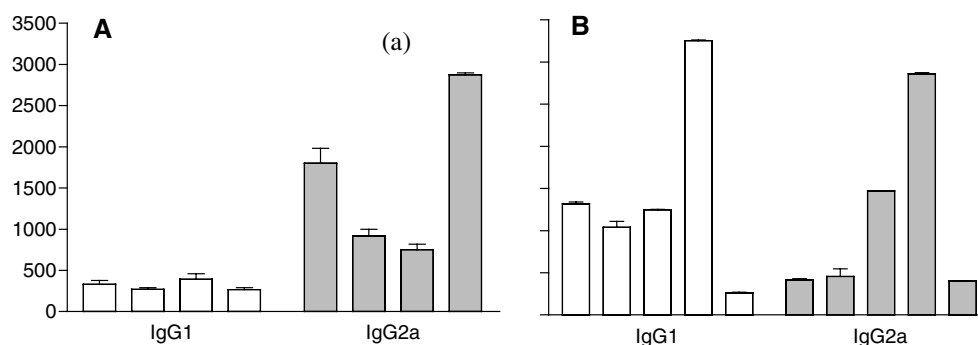


Figure 2. IgG1 and IgG2a response after 100 µg ID administration of pAra h 1 or pAra h 4 in SKH-Hr1 mice. Groups of SKH-Hr1 mice were injected ID in the tail with 100 µg in PBS of pAra h 1 (A) and pAra h 4 (B). A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 15 000°. The enzyme activity was stopped after 20 min. (a) in the pAra h 1 immunized mice, the IgG2a responses were consistently higher than the IgG1 responses ($P < 0.026$, Mann-Whitney rank sum test).

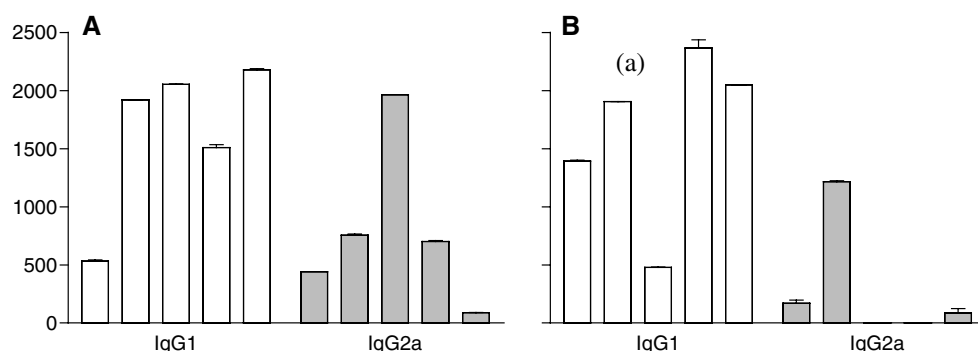


Figure 3. IgG1 and IgG2a response after ID administration of 100 µg of pAra h 1 or pAra h 4 in BALB/c mice. Groups of BALB/c mice were injected ID in the tail with 100 µg in PBS of pAra h 1 (A) or pAra h 4 (B). A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 15 000°. The enzyme activity was stopped after 20 min. (a) the IgG1 response to pAra h 4 was consistently higher than the IgG2a response in these mice ($0.032 < P < 0.056$, Mann-Whitney rank sum test).

intensity, and the same pattern for Ara h 1 immunized mice was also obtained with plates coated with purified Ara h 1 protein (data not shown). IgE was detected in pAra h 4 mice with high IgG1 levels, e.g., mouse number 4 (Fig. 2), but not in pAra h 1 mice.

ID Injection of 100 µg of pAra h1 or pAra h 4 in BALB/c mice

The protocol of immunization and bleeding for groups of BALB/c mice was the same as that above. Like the SKH-Hr1 mice, both groups had IgG1 and IgG2a responses. The IgG1 response was equivalent between the pAra h 1 ($n = 5$) and pAra h 4 groups ($n = 5$), but the IgG2a response was lower in the pAra h 4 group (Fig. 3). The IgG1 response to pAra h 4 was consistently higher than the IgG2a response in these mice ($0.032 < P < 0.056$). IgG2a was detectable in the number 3 and 4 mice of pAra h 4 group at a lower dilution (1/5000). As with the SKH-Hr1 mice, the ratio of IgG2a/IgG1 was higher in the

pAra h 1 group than in the pAra h 4 group. Despite the high level of IgG1, no IgE could be detected in either group.

ID injection of 1 µg of pAra h 1 or pAra h 4 in SKH-Hr1 mice

Two groups of SKH-Hr1 mice were injected with 1 µg of plasmid pAra h 1 ($n = 3$) or pAra h 4 ($n = 4$). Immune response could not be detected at a higher dilution than 1/500. The profiles for both groups were similar to the profile obtained with 100 µg. Nevertheless the ratio IgG2a/IgG1 was lower with 1 µg of pAra h 1 than with 100 µg. No IgE could be detected in either group (Fig. 4).

ID injection of 1 µg of pAra h 1 or pAra h 4 plus 99 µg of blank plasmid in SKH-Hr1 mice

Two groups of SKH-Hr1 mice were injected with 1 µg of plasmid pAra h 1 ($n = 4$) or pAra h 4 ($n = 5$) plus

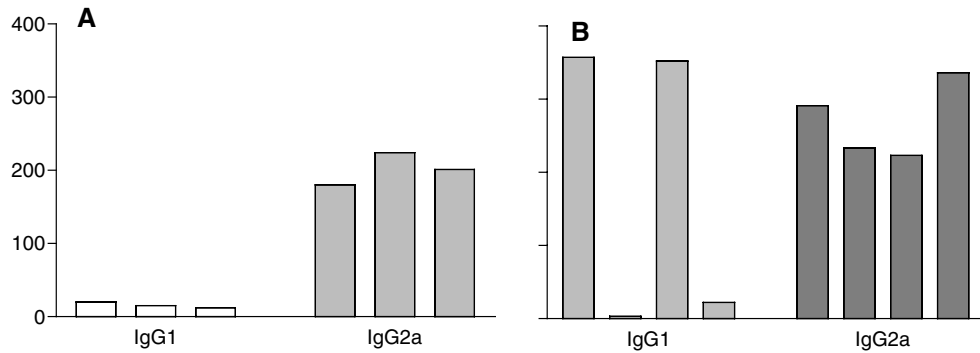


Figure 4. IgG1 and IgG2a response after ID administration of 1 mg of pAra h 1 or pAra h 4 in SKH-Hr1 mice. Groups of SKH-Hr1 mice were injected ID in the tail with 1 μ g in PBS of pAra h 1 (A) and pAra h 4 (B). A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 500^e. The enzyme activity was stopped after 20 min.

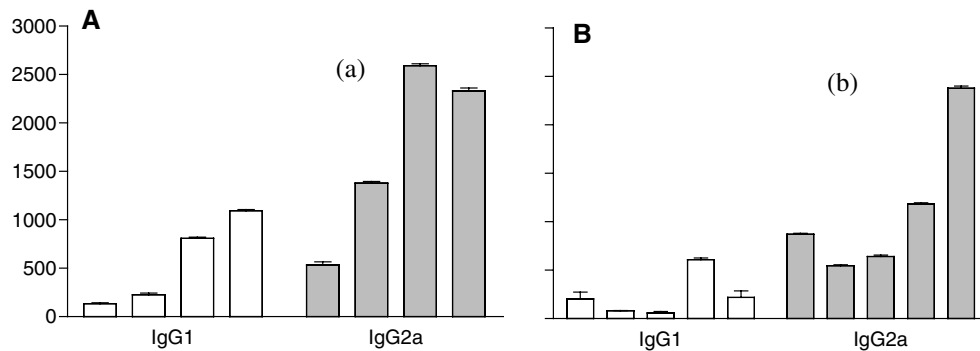


Figure 5. IgG1 and IgG2a response after ID administration of 1 μ g of pAra h 1 or pAra h 4 + 99 μ g of blank plasmid in SKH-Hr1 mice. Groups of SKH-Hr1 mice were injected ID in the tail with 1 μ g in PBS of pAra h 1 (A) and pAra h 4 (B) + 99 μ g of blank plasmid. A second administration 4 weeks after the first one was done. Mice were bled 4 weeks after each administration. The figure illustrates the results obtained with the second bleed at a dilution of 1 : 5000^e. (a) the relative responses of IgG1 and IgG2a for pAra h 1 showed a statistically significant predominance of IgG2a ($P < 0.05$, Mann-Whitney rank sum test). (b) the relative responses of IgG1 and IgG2a for pAra h 4 showed a statistically significant predominance of IgG2a ($P < 0.01$, Mann-Whitney rank sum test).

99 μ g of a blank plasmid in order to increase the ratio of CpG to recombinant protein expressed. pcDNA3 and blank plasmid (pCMVi) contain respectively 26 and 23 CpG. For both groups the magnitude of the immune response was lower than with 100 μ g but higher than with 1 μ g alone, showing the adjuvant effect of DNA (Fig. 5). The profile obtained in the pAra h 1 group was similar to that obtained after the 100 μ g injection. The individual mice in both groups showed similar profiles. The IgG2a/IgG1 ratio was very close for both groups. The relative responses of IgG1 and IgG2a for both pAra h 1 and pAra h 4 showed a statistically significant predominance of IgG2a ($P < 0.05$ and $P < 0.01$, respectively). No IgE could be detected in either group.

Discussion

In this paper, we investigated various issues linked to genetic immunization with plasmid DNA encoding allergens. In our results, we showed that administration of pAra h 4 elicited a mixed Th1–Th2 immune response with detectable IgE in some mice or at least a Th1 response with a large amount of IgG1. In contrast, administration of pAra h 1 elicited a more classical Th1 response for genetic immunization with low amounts of IgG1. Previous studies have found that IgE can be detected after genetic immunization, as well. In our laboratory, we previously showed that administration of a plasmid encoding bovine beta-lactoglobulin (BLG) could elicit BLG specific IgE in some mice (14) that was correlated

with the quantity of IgG1. In their experiments, Raz et al. also detected very small amounts of IgE (4).

Immune responses to proteins are known to be highly dependent on the nature of the antigen. In 1998, Lee et al. showed that for genetic immunization, using plasmids encoding the hemagglutinin and the nucleoprotein of influenza virus, the magnitude of the immune response and the degree of Th1 bias from CpG sequences depended on the antigen nature (15). In contrast to our results, the ratio IgG2a/IgG1 always favored IgG2a. Isoforms of major allergens of birch pollen, Bet v 1a and Bet v1d, on the other hand, induced different immune responses after genetic immunization (16). The humoral response was similar, but the cytokine profile and proliferation test showed different results. In contrast to our results, they could not detect any IgE, despite a high IgG1 level. The effect of CpG motifs *in vivo* depends also on a variety of parameters like the nature of the antigen and the immunization modality (17).

Immunization with plasmid DNA does not always induce a Th1 dominated response. The nature of plasmid DNA induced response is also dependent on the method of inoculation (18). DNA delivered by a gene gun produced a Th2-like response, while immunization by ID or IM with the same plasmid produced a Th1-like response.

Immune responses after genetic immunization are also influenced by the cellular location of expressed antigen (19). Plasmid DNA encoding secreted ovalbumin generated 50- and 100-fold higher levels of IgG1 than the cytoplasmic or membrane bound ovalbumin form. These results were confirmed by administration of a plasmid encoding for BLG, which elicited a large amount of specific IgG1 (14). BLG is naturally secreted in cow's milk and was found to be partially secreted by transfected cells and could even be detected in the sera after genetic immunization (20). The proteins belonging to the 11S globulin family, like Ara h 3 or Ara h 4, are storage proteins and so are unlikely to be secreted.

The immune response also depends on the mouse strain. Administration of plasmid encoding Ara h 2 in C3H mice induced an IgG2a and IgG1 response while in BALB/c or AKR mice it stimulated only an IgG2a response (21). In contrast, in our experiments we have a much higher IgG1 response in BALB/c than in SKH-Hr1 for each allergen. BALB/c has been shown to be Th2-biased for many other antigens (22).

The high IgG1 response to pAra h 4 is significant because in some cases it has been reported that

anaphylactic reactions can be mediated by an isotype other than IgE (23). Indeed, IgE knock-out mice produced IgG2a, IgG1 and IgM after ovalbumin (OVA) sensitization but no IgE, and yet exhibited an anaphylactic reaction after OVA challenge. Oshiba et al. demonstrated that immediate hypersensitivity could be passively transferred not only by allergen-specific IgE, but also by IgG1, and it was not transferred by IgG2a or IgG3 (24). These studies demonstrate that IgG1 antibodies can play a role as important as IgE in the induction of immediate hypersensitivity in mice.

The immune response to allergens after genetic immunization is the result of two contradictory influences. The nature of the allergen itself promotes a Th2 response, while the presence of ISS sequences in the plasmid strongly stimulate cytokines promoting a Th1 response. For most antigens, the Th1 bias is predominant using genetic immunization, but with allergens, the relative strength of the Th2 bias of the antigen and the Th1 bias of the ISS sequences results in a more variable, mixed response. As a result, it is not surprising that we can modulate the immune response by changing the ratio ISS to the recombinant protein expressed.

Ara h 3 and Ara h 4 were cloned independently by two different groups, Rabjohn et al. (13) and Kleber-Janke et al. (12), respectively. Both proteins belong to the 11S protein storage family, which is a multigene family (25). The Ara h 3 clone was incomplete and so we used primers from the Ara h 4 sequence, because our goal was to obtain a complete cDNA for genetic immunization. From our source material, none of our cDNA clones had 100% homology to published Ara h 3 or Ara h 4 isoform sequences. However, all our clones contain a consensus cleavage site, Arg-Gly, and two cysteine residues implicated in a conserved disulfide bridge.

Further experiments will be required to determine if there is a difference in the protective effect of preventive genetic immunization with pAra h 1 and pAra h 4. It would also be interesting to better understand the properties of the expressed allergen of pAra h 4 that cause it to elicit a strong Th2 biased response.

Acknowledgments

This study was supported by the Department of Veterans Affairs and the Huffington Foundation, Houston, Texas.

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