

Expression and immunogenicity of the major house dust mite allergen Der p 1 following DNA immunization

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Abstract

The mite protein Der p 1 is a major trigger of allergy and atopic asthma world-wide, and thus, a good vaccine candidate for allergy prevention. Since it is a cysteine protease, the catalytic effects of Der p 1 vaccination may be unpredictable. One approach to reduce this risk is to vaccinate with DNA encoding enzymatically inactive forms of Der p 1. Here we show that Der p 1 DNA without its native pre-pro sequences potently induced Der p 1-specific antibodies, as long as its pre-sequence was substituted by another leader sequence. Without any pre-pro sequence, the same DNA fragment was well expressed but failed to induce significant level of anti-Der p 1 antibodies, without further boosting by protein.

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1. Introduction

From an epidemiological point of view, Der p 1, a protein synthesized by *Dermatophagoides pteronyssinus* mites, is one of the top candidates for an allergy vaccine: house dust mites are major indoor triggers of atopic allergy; the majority of allergic individuals are sensitized to one or more species that are common in their environment; *D. pteronyssinus* is one of the three most common species of mites in temperate regions; the majority of mite sensitive patients respond to group I and group II allergens, and Der p 1 is one of the prevalent antigens in this mixture [1–4]. The amino acid sequence is highly conserved among group I allergens of different mite species. Exposure and initiation of an allergic process by a major mite allergen may facilitate sensitization to other less prevalent allergens [5].

Der p 1 protein is a cysteine protease; its full-length cDNA encodes a precursor of 320 amino acid residues, including an 18 amino acid signal peptide, a pro-enzyme region of 80 amino acid, and a mature enzyme of 222 amino acid residues [6]. Like its homologues papain and actinidin, Der p 1 has a two-domain structure. The left domain (approximately corresponding to amino acid sequence 1–116) consists of three α -helix and the right domain (approximately corresponding

to amino acid sequence 114–222) is largely composed of β -pleated sheets [7].

The synthetic pathway of Der p 1 has imposed limitations to the production of large quantities of recombinant protein for immunotherapy or vaccination [8]. The recombinant allergen is trapped as a zymogen inside inclusion bodies. Der p 1 maturation does not occur naturally in bacterial or insect systems, and therefore the protein has to be solubilized and renatured. Removal of the pro-sequence by autocatalysis was problematic and often resulted in protein degradation [9,10].

We propose that genetic immunization with Der p 1 gene is a feasible alternative strategy to develop useful prophylactic and therapeutic reagents for mite allergy [11–18]. In this case, DNA sequences encoding the T cell epitopes could be exploited for the vaccine design [19]. However, as indicated by the published data, numerous important human T cell epitopes that have been mapped were located throughout the Der p 1 molecule [20,21]. Given the complexity and polymorphic nature of the MHC genes, vaccine design using the full Der p 1 gene or large gene fragments would offer a broader spectrum of protective immunity in the highly heterogeneous human populations.

Since Der p 1 is a cysteine protease with demonstrated proteolytic activity [6,22–24], prolonged endogenous synthesis in a vaccinated host may have unpredictable effects. Therefore, DNA encoding enzymatically inactive immunogenic forms of the allergen may represent a feasible and safer

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Der p 1 vaccine. It has been reported that the pro-enzyme peptide of cysteine proteases serves as an intramolecular chaperon, and is essential for correct folding of the mature protein [25]. It has been shown that incorrectly folded Der p 1 lacked enzymatic activity [8,9]. Therefore, we rationale that excluding the pre-pro-region of Der p 1 in the DNA construct is a feasible strategy to produce immunogenic but enzymatically inactive Der p 1 in vivo.

In this study, we evaluated IgG reactivity to native Der p 1 and antigen expression in vivo after immunization with Der p 1 gene or gene fragments. Various DNA constructs encoding either Der p 1 full-length gene or gene fragments, with or without its own pre-pro sequences, were evaluated.

2. Materials and methods

2.1. Animals

Six to eight-week-old female BALB/cJ mice were purchased from Laboratory Animals Centre (Sembawang, Singapore). Mice were housed at the National University of Singapore Animal Holding Unit and cared for under institutional guidelines for the care and use of laboratory animals. Immunization experiments were performed with groups of 4–10 animals. For DNA immunizations mice were anesthetized with a cocktail of Hypnorm plus Midazolam provided by the Animal Holding Unit, National University of Singapore.

2.2. Constructs preparation

Fig. 1 shows the Der p 1 variant expression constructs that were produced: Der p 1 full-length cDNA inserted into pcDNA3; Der p 5 L/Der p 1_(1–116) and leaderless Der p 1_(1–116), each encoding the N-terminal half of Der p 1, inserted into pEGFP to assess expression or into pCIneo for immunization; Der p 5 L/Der p 1_(1–222) and Der p 1_(1–222) each encoding for the full-length mature protein inserted also into pEGFP or into pCIneo for the same purposes.

The construction of plasmid pcDNA3-pre-pro-Der p 1 was carried out by insertion of the PCR amplified full-length cDNA fragment of Der p 1, using oligonucleotide primers (5'-CCCGGATCCAACATGAAAATTGTTTGGCC-3' and 5'-GCGCTCGAGTTTAGAG AATGACAACATATGG-3') and the λ gt10RecL3a Der p 1 cDNA as DNA template, into the *Bam*HI and *Xho*I sites of mammalian expression vector pcDNA3 (Invitrogen™ Life technologies).

PCR was used to amplify the sequences of Der p 1 DNA coding for the N-terminal domain (residues 1–116) and the full-length mature protein (residues 1–222). The sequences of the synthetic oligonucleotide primers (Operon, Qiagen, Alameda, CA) specific for PCR amplification of Der p 1 are 5'-AATCCGGAACATAACGCCTGCAG TATCAAT-3', 5'-AAGGATCCATAGTTTGAGATACCGAAACGTTG-3' and 5'-CCTCCGGATCAAACATATTGCCAAATTTACC-

CA-3'. Each individual primer was engineered to include a unique restriction endonuclease site at its 5' terminus for compatible cloning of the PCR fragment into the pEGFP-N3 (Clontech, Palo Alto, CA) expression vectors. The PCR amplification and cloning procedure were performed with standard protocols as previously described [26]. PCR fragment were cloned into pCR2.1 vector for sequence confirmation by automatic sequencing (ABI PRISM™ sequencing kit).

In order to link the Der p 5 signal peptide sequence to the Der p 1 fragments, the pCR2.1-Der p 1 recombinant constructs were digested with *Bsp*EI and *Xho*I restriction endonucleases. The 64-basepairs synthetic oligonucleotides corresponding to the Der p 5 leader (sense sequence 5'-TCGAGATCAATCATGAAATTCATCAT TGCTTTCTTTGTTGCCA CTTTGGCAGTTATGACTGRTT-3' and anti-sense sequence 5'-CCGGAAA CAGCCTAAACTGCCAAAGTGGCAACAAAGAAAGCAAT GATGAAT T TCATGATTGATC-3') were then inserted into the *Bsp*EI and *Xho*I sites of the 4.7 kb vector to generate pCR2.1-Der p 5 L/Der p 1_(1–116), and pCR2.1-Der p 5 L/Der p 1_(1–222). The resulting plasmids were sequenced using ABI PRISM™ sequencing kit.

To generate the pEGFP-N3-based expression vectors, the inserts encoding the Der p 5 L/Der p 1_(1–116), and Der p 5 L/Der p 1_(1–222) fusion proteins were released from plasmids pCR2.1-Der p 5 L/Der p 1_(1–116) and pCR2.1-Der p 5 L/Der p 1_(1–222) by digestion with *Xho*I, *Bam*HI and *Eco*RI. Subsequently, the agarose gel purified DNA fragments were extracted from the gel using the QIAEX II kit and inserted into the *Xho*I-*Bam*HI gap of pEGFP-N3 vector. The ligated plasmid DNA was then transformed into the *E. coli* strain DH5 α . Kanamycin resistant transformants were selected by screening for pDer p 5 L/Der p 1_(1–116)-EGFP and pDer p 5 L/Der p 1_(1–222)-EGFP DNA, respectively. Subsequent construction of pCIneo-based Der p 5 L/Der p 1 variant constructs was carried out by PCR amplification of the encoding sequences using three specific synthetic oligonucleotide primers and the DNA templates pDer p 5 L/Der p 1_(1–116)-EGFP and pDer p 5 L/Der p 1_(1–222)-EGFP. The sequence of these specific primers corresponding to the Der p 5 signal peptide sequence, the C-terminal sequence of the N-terminal domain of Der p 1 and the C-terminal sequence of the C-terminal domain of Der p 1 domain are 5'-GCCTCGAGCCACCATGAAATTCATCATTGCT-3', 5'-GCGGAT CCGCGGCCGCT TAGAGAAT GACAACA TAT GG-3' and 5'-CCGCGGCCGCTAATAGT TTGAGATACCGAAA-CGTTG-3', respectively. The PCR products were sub-cloned into pCR2.1 vector to generate plasmid constructs termed pCR2.1-Der p 5 L/Der p 1_(1–116)ter and pCR2.1-Der p 5 L/Der p 1_(1–222)ter. The expression constructs pCIneo-Der p 5 L/Der p 1_(1–116) and pCIneo-Der p 5 L/Der p 1_(1–222) were produced by insertion of the Der p 5 L/Der p 1 encoding sequences released from pCR2.1-Der p 5 L/Der p 1_(1–116)ter and pCR2.1-Der p 5 L/Der p 1_(1–222)ter into the *Xho*I and *Not*I sites of the pCIneo (Promega, Madison, WI). Similar PCR amplification

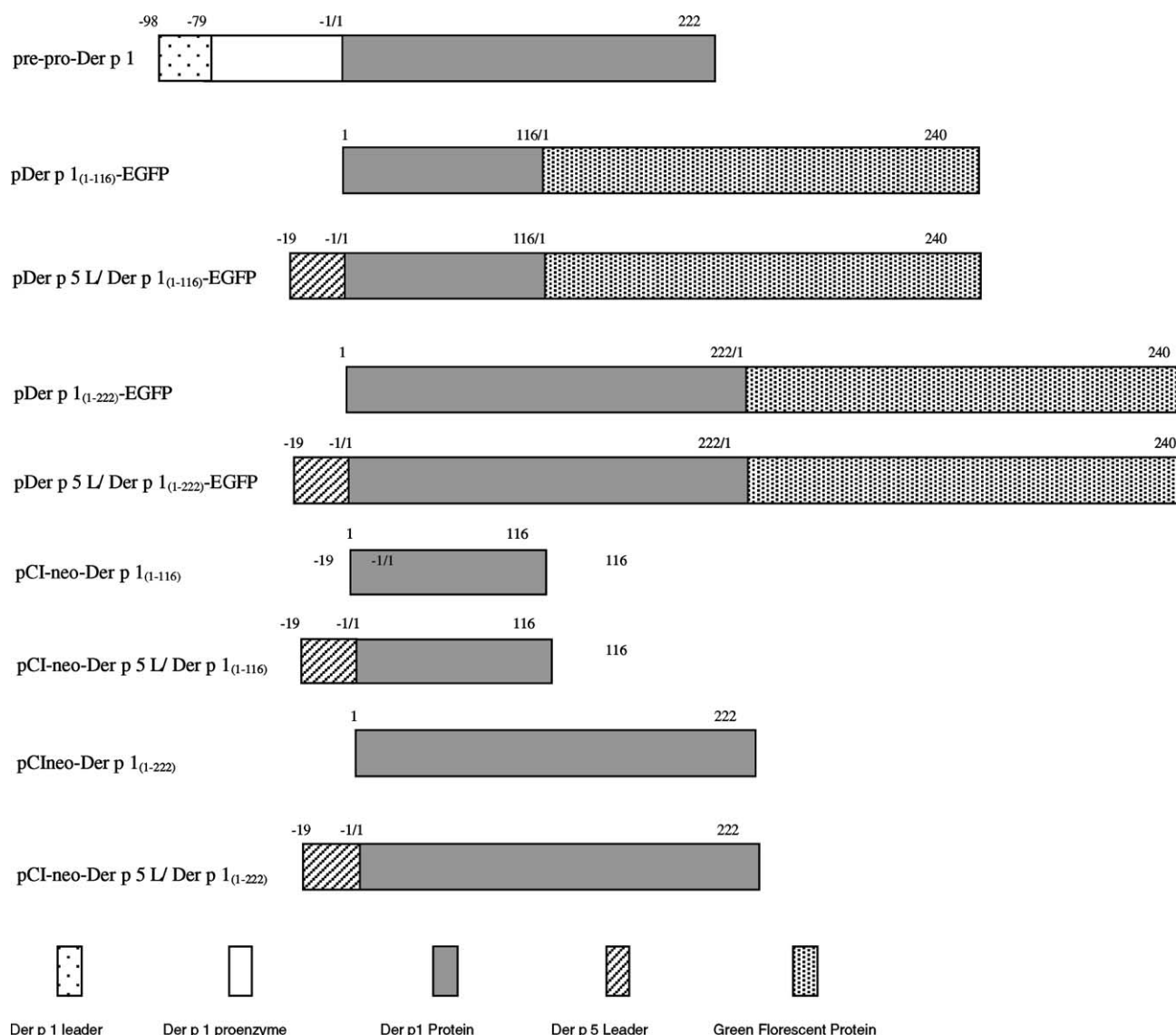


Fig. 1. Schematic representation of the constructs used for immunization.

strategy was employed to generate pCR2.1-Der p 1₍₁₋₁₁₆₎ and pCR2.1-Der p 1₍₁₋₂₂₂₎ using specific oligonucleotide primers 5'-GCCTCGAGCCACCAGACTAACGCCTGCA-GTATCATG GAAAT-3', 5'-GCGGATCCGCGGCCGCT-TAGAGAATGACAACATATGG-3', 5'-CCGCGGCCGCT-AATAGTT TGAGATACCGAAACGTTG-3', the DNA templates pCR2.1-Der p 5 L/ Der p 1₍₁₋₁₁₆₎ter and pCR2.1-Der p 5 L/ Der p 1₍₁₋₂₂₂₎ter. The *XhoI*-*BstXI* fragments encoding for the Der p 1₍₁₋₁₁₆₎ and Der p 1₍₁₋₂₂₂₎ fusion proteins released from the vectors pCR2.1-Der p 1₍₁₋₁₁₆₎ and pCR2.1-Der p 1₍₁₋₂₂₂₎ were used to replace the *XhoI*-*BstXI* fragments of pDer p 5 L/ Der p 1₍₁₋₁₁₆₎-EGFP and pDer p 5 L/ Der p 1₍₁₋₂₂₂₎-EGFP and resulted in pDer p 1₍₁₋₁₁₆₎-EGFP and pDer p 1₍₁₋₂₂₂₎-EGFP. To generate pCIneo-Der p 1₍₁₋₁₁₆₎ and pCIneo-Der p 1₍₁₋₂₂₂₎ the *BstXI*-*NotI* fragments from pCIneo-Der p 5 L/ Der p 1₍₁₋₁₁₆₎ and pCIneo-Der p 5 L/ Der p 1₍₁₋₂₂₂₎ and the

XhoI-*BstXI* fragments from pDer p 1₍₁₋₁₁₆₎-EGFP and pDer p 1₍₁₋₂₂₂₎-EGFP were linked together with the *XhoI* and *NotI* sites of pCIneo, respectively.

2.3. Mice immunization

BALB/cJ mice were immunized under anesthesia, by intramuscular injection of 50 μ l of naked DNA (1 μ g/ μ l) in the left quadriceps using a 1 ml insulin syringe and a 27 G needle. For immunization with electroporation, the front of the upper left leg was shaved, and DNA was injected as above, in the anterior tibialis, followed by immediate electroporation with an ECM 830 apparatus (BTX, Genetronics, San Diego, CA). Electroporation was performed with a 0.5 cm 2-needle array inserted 2 mm deep in parallel to the muscle fibers, that delivered four pulses of 20 ms, 80 V each, with a 200 ms interval. Animals received pEGFP-N3-Der p 1 or

pCIneo-Der p 1 constructs with or without electroporation. Serum was collected before (day 0) and sequentially after immunization.

2.4. Purification of native Der p 1 and Der p 1 recombinant peptides

Spent Mite Media (a gift from CSL Limited, Melbourne, Australia) was resuspended to 10% (w/v) in a 0.5 M NaCl in 10 mM Tris–HCl pH 7.5 solution containing 1 mM PMSF and 1 mM EDTA and the suspension was stirred at 4 °C overnight. The next day the suspension was centrifuged at 12,100 g for 15 min and the supernatant was collected for purification, using a protocol that was previously described [27]. Briefly, A monoclonal antibody (4C1) sepharose affinity column was equilibrated with five bed volumes of the suspension buffer and the supernatant of spent mite medium was applied to the column, Der p 1 was eluted with 5 mM glycine in 50% ethylene-glycol pH 10 and the fractions were collected in tubes containing 100 µl/ml fraction of 0.2 M Na₂HPO₄ pH 7 neutralizing buffer. The peak samples were pooled, dialyzed immediately against 50 mM NH₄HCO₃, and lyophilized. The samples were resuspended in PBS and quantitated by comparison with a protein standard using a colorimetric assay (BioRad, Hercules, CA).

E. coli clones producing Der p 1 recombinant peptides GST-(1–116) and GST-(114–222) were grown in LB medium containing 100 µg/ml of ampicillin and induced for 3 h with 0.5 mM IPTG (Calbiochem, LaJolla, CA). The bacteria were pelleted and resuspended in TBS with 1 mM PMSF, 20 µg/ml of Dnase I, 20 µg/ml of Lysozyme and Tween 20 to 1% of the cell suspension. The bacteria were disrupted by sonication on ice, and the supernatant was collected after centrifugation. The peptides were purified by passage over a column of glutathione agarose beads pre-equilibrated in TBS, and elution of the bound material with 10 mM reduced glutathione in 50 mM Tris–HCl pH 8 buffer. The concentration of recombinant peptide was quantified by a colorimetric assay (BioRad, Singapore) and purity was assessed by SDS–PAGE. For Western analysis, aliquots of the eluted peak were run on 7.5% SDS–PAGE, transferred to Hybond C nitrocellulose membranes (Amershampharmacia Biotech AB, Malaysia) and probed with monoclonal antibodies 6BC, specific for 1–116 peptide, and 1BB specific for 114–222 peptide, produced for this purpose.

2.5. Protein expression in cryosections of muscle

Muscle samples from the injection site were collected at 1, 6 h, and 1, 2, 4, 7, 10, 14, 21, and 28 days after injection. The samples were embedded in O.C.T compound (Tissue-Tek, Sakura, Torrance, CA) snap frozen in liquid nitrogen and stored at –20 °C. Ten micrometers cryosections were cryostatistically sectioned and mounted with fluorescence quenching medium (DAKO). Fluorescent images were captured by confocal microscopy.

For immunostaining, 5 µm sections were cut and mounted onto silanized slides, fixed in cold acetone, treated with 0.3% hydrogen peroxide, washed, and blocked with 5% normal goat serum. Replica slides were then stained with a 0.3% dilution of irrelevant mouse antiserum, anti-Der p 1 antiserum or 0.1% dilution of ascites containing monoclonal anti-Der p 1 antibodies. The slides were developed with the VECTASTAIN ABC kit (Vector Laboratories Inc., Burlingame, CA) following manufacturer's instructions.

2.6. Detection of antisera specific for native Der p 1 and for recombinant Der p 1-derived peptides by ELISA

Maxisorp Immuno plates (Nalge Nunc Intl, Naperville, IL) were coated with 5 µg/ml of native Der p 1 in 0.1 M NaHCO₃ pH 8.3, overnight at 4 °C. The plates were then flicked to remove uncoated antibody and blocked for 1 h with a solution of 1% BSA in PBS/0.05% Tween 20 (washing buffer). The plates were washed three times with PBS/Tween 20. Dilutions of sera were added and incubated overnight at 4 °C. Dilutions of standard mouse IgG2a or mouse IgG1 (PharMingen, San Diego, CA) were added to each plate onto wells coated with anti-mouse kappa light chain clone 187.1 (PharMingen). The plates were washed three times and Biotin conjugated IgG2a- or IgG1-specific monoclonal antibody (clone LO-MG2a-7 and clone LO-MG1-2, respectively, Serotec, Oxford, England) were added for 1 h, followed by alkaline phosphatase conjugated ExtrAvidin (Sigma–Aldrich) and *p*-Nitrophenylphosphate substrate (Sigma–Aldrich). The Antibody Production Units of antigen-specific antibodies were determined by comparison of the plot of absorbance versus dilutions of the sample to that of the standard versus its concentration.

Similar methods were used for detection of peptide-specific antibodies, except that the plates were coated with 5 µg/ml of purified Der p 1-derived peptides GST-(1–116) or GST-(114–222) peptides. The amount of peptide coated on each well was comparable, as determined by binding of peptide-specific monoclonal antibodies. Replicas of the antisera were tested on plates coated with GST for specificity control.

3. Results

3.1. Immunogenicity of pcDNA3-pre-pro-Der p 1 plasmid

To determine whether naked Der p 1 cDNA inserted into an expression plasmid could induce anti-Der p 1 antibodies, pcDNA3 engineered pre-pro-mature Der p 1 plasmids were injected into the muscle of BALB/cJ mice. One injection was insufficient to elicit a detectable humoral response (data not shown); therefore the animals were boosted with similar protocol 1 or 2 weeks after priming (Fig. 2). Two

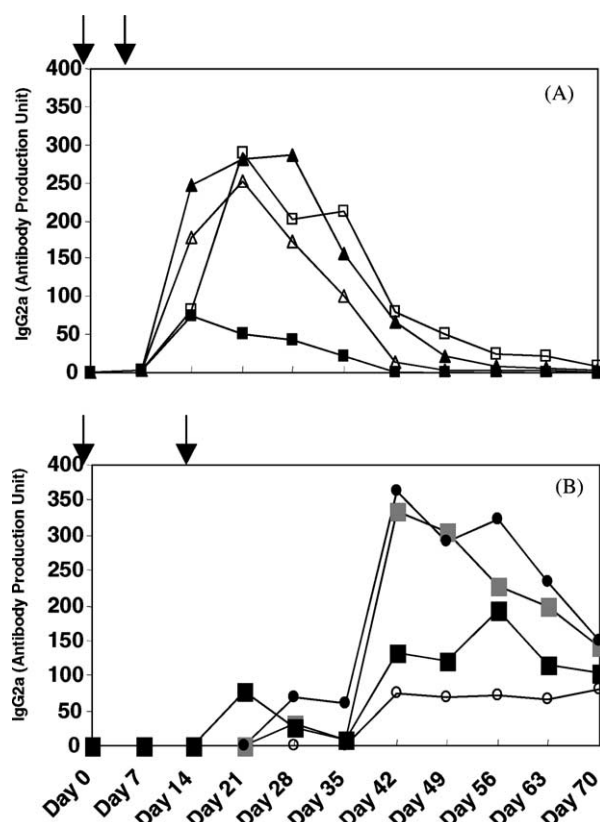


Fig. 2. Kinetics of the anti-Der p 1 antibody response induced by intramuscular injection of pcDNA3-pre-pro-Der p 1 plasmid. (A) Mice were injected on day 0 and boosted on day 7 (arrows). Sera were collected weekly and tested by ELISA on plates coated with native Der p 1. (B) Mice were injected on day 0 and boosted on day 14 (arrows). Sera were collected and tested as in A. Each line represents the Ig titer of an individual mouse serum. IgG1 anti-Der p 1 antibodies were not detected (<10 units).

injections of naked DNA given 1 week apart (Fig. 2A) induced a response peak by the third week after priming that had almost returned to baseline by day 56. Two injections of naked DNA given 2 weeks apart (Fig. 2B) induced two response peaks, the first of very low magnitude between 21 and 28 days after priming, returned to baseline by day 35. The second peak occurred during the sixth week (day 42 sample), and in this group anti-Der p 1 antibodies could still be detected by day 70. Spacing the first and second dose by 3 weeks produced similar results, a very low first antibody peak, followed by a secondary response of higher magnitude and even longer duration (data not shown). These results suggested that two intramuscular doses of whole length Der p 1 gene could produce antibodies with native Der p 1 specificity. For the following experiments the two doses were given 2 or 3 weeks apart.

The maximum antibody concentrations observed for naked DNA injection were low, ranging from 50 to 400 units of IgG2a in all the experiments. Other isotypes were tested but not detected (Fig. 2), indicative of predominant Th1 immunity.

3.2. Der p 1-specific antibodies are also induced by Der p 5 L/Der p 1 plasmids

To limit the possibility of production of enzymatically active form of Der p 1 in the vaccinated host, new constructs were made, where the pro-enzyme sequence, which is necessary for a right conformation of mature Der p 1, was deleted. It was also of interest to determine if engineering a different leader peptide into the expression vector could turn the plasmid into a more potent immunogen. Previous work had shown that Der p 5 cDNA could successfully induce a protective response to the house dust mite allergen Der p 5 [11]. Therefore, the autologous Der p 1 leader was replaced by the Der p 5 leader. Two plasmids were derived from the pCIneo vector: Der p 5 L/Der p 1_(1–222) encodes the whole mature Der p 1, and Der p 5 L/Der p 1_(1–116) encodes only the first 116 amino acids (Fig. 1). Sera from mice injected with this plasmid were collected sequentially and tested for the presence of Der p 1-specific antibodies. As shown in Fig. 3, the peak anti-Der p 1 IgG2a responses induced by the products of the pre-pro-Der p 1 plasmid and chimeric Der p 5 L/Der p 1_(1–222) DNA were comparable. These data suggest that the pro-enzyme sequence is not necessary for the production of Der p 1-specific antibody, and that substitution of the Der p 1 leader sequence with the Der p 5 leader sequence does not result in significant differences in immunogenicity.

For subsequent experiments, electroporation was incorporated to the immunization protocol to maximize in situ transfection of the muscle. Both the frequency of responders and the general anti-Der p 1 antibody titers increased (Fig. 4). Der p 5L/Der p 1_(1–222) plasmid and the second chimeric construct, Der p 5L/Der p 1_(1–116) are both

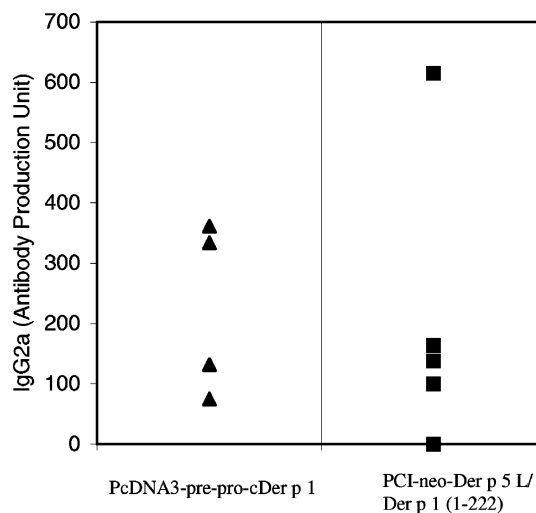


Fig. 3. Comparison of the anti-Der p 1 antibody response induced by intramuscular injection of the pcDNA3-pre-pro-Der p 1 plasmid (left panel) or the pCIneo-Der p 5 L/Der p 1_(1–222) construct (right panel). Sera were collected weekly. Each dot represents the peak Ig titer response of an individual sample. Statistically, there was no difference between the two groups, as determined by the Student's *t*-test.

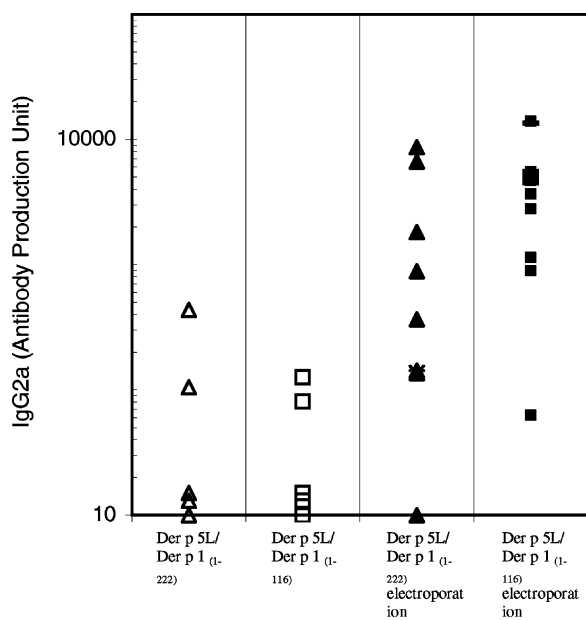


Fig. 4. Electroporation increases the frequency of responders and the titer of anti-Der p 1 antibodies. Each panel represents a group of animals intramuscularly injected with one of the two constructs with Der p 5 leader. The two left panels show the data from animals receiving naked plasmid by intramuscular injection alone. The two right panels show the data from animals receiving naked plasmid by intramuscular injection and electroporation. Each dot represents the serum of an individual mouse. The statistical significance of differences of means between different groups were $P < 0.05$ (Der p 5 L/Der p 1_(1–222) with or without electroporation) and $P < 0.01$ (Der p 5 L/Der p 1_(1–116) with or without electroporation), as determined by the Student's *t*-test.

immunogenic. The long-lasting antibody response induced by these two plasmids using naked DNA injection with electroporation is shown in Fig. 5. Anti-Der p 1 antibodies peaked between days 56 and 101 for both constructs. Detectable circulating antibodies persisted for at least 128 days after the last plasmid injection.

The levels of Der p 1-specific IgG2a and IgG1 antibodies in animals receiving the chimeric Der p 1 plasmids were compared. Control vector induced no detectable anti-Der p 1 antibody response. As shown in Fig. 6, both Der p 5L/Der p 1_(1–116) and Der p 5L/Der p 1_(1–222) immunization resulted in a skewed antibody response dominated by IgG2a antibodies, especially in mice receiving the Der p 5L/Der p 1_(1–222) plasmid. The Der p 5L/Der p 1_(1–116) induced larger amounts of IgG1.

3.3. Leaderless plasmids are poor anti-Der p 1 immunogens for humoral response

To understand whether the leader sequence was necessary at all to induce anti-Der p 1 antibody production, two leaderless plasmids encoding either the complete mature sequence of Der p 1, pCIneo-Der p 1_(1–222), or the first 116 amino acids, pCIneo_(1–116), were designed as described in Section 2 (Fig. 1). These plasmids were administered on

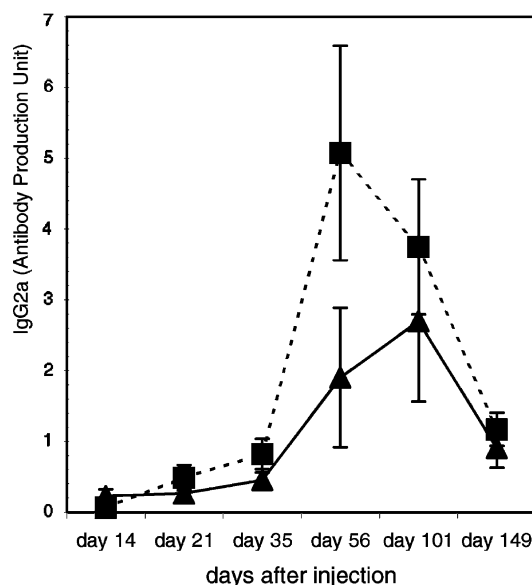


Fig. 5. Persistent anti-Der p 1 antibody response after Der p 1 DNA immunization. Mice received pCIneo-Der p 5 L/Der p 1_(1–116) (dashed line) or pCIneo-Der p 5 L/Der p 1_(1–222) (solid line) i.m. with electroporation on days 0 and 21. Serum samples were collected periodically and the titer of Der p 1-specific antibodies in serum was determined by ELISA. Data is shown as mean $\pm$ S.E.M.

days 0 and 21 with electroporation, and the results of this experiment are shown in Fig. 7. In the absence of a signal peptide, the anti-Der p 1 antibody response to pCIneo-Der p 1_(1–222) was very low (Fig. 7B), and for pCIneo-Der p 1_(1–116) it was below detection levels (Fig. 7A).

The lack of detection of a significant anti-Der p 1 response in the mice immunized by pCIneo-Der p 1_(1–116) could

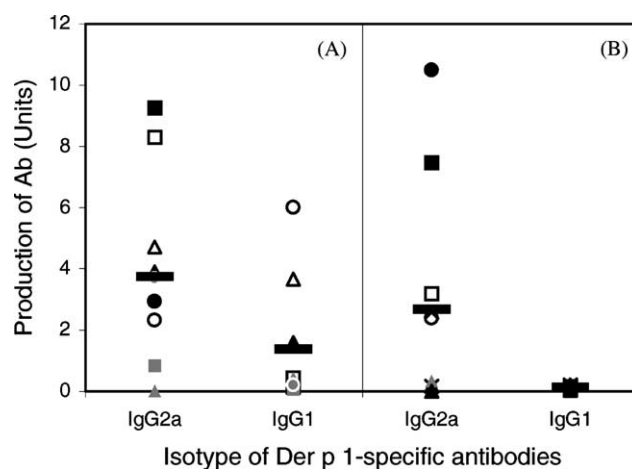


Fig. 6. Preferential induction of Der p 1-specific IgG2a. The mice received pCIneo-Der p 5 L/Der p 1_(1–116) (A) or pCIneo-Der p 5 L/Der p 1_(1–222) (B) with electroporation on days 0 and 21. Serum samples were collected on day 101 and assayed by ELISA. The statistical significance of differences of means between the concentration of Der p 1-specific IgG2a and IgG1 antibodies was $P < 0.02$, as determined by the Student's *t*-test.

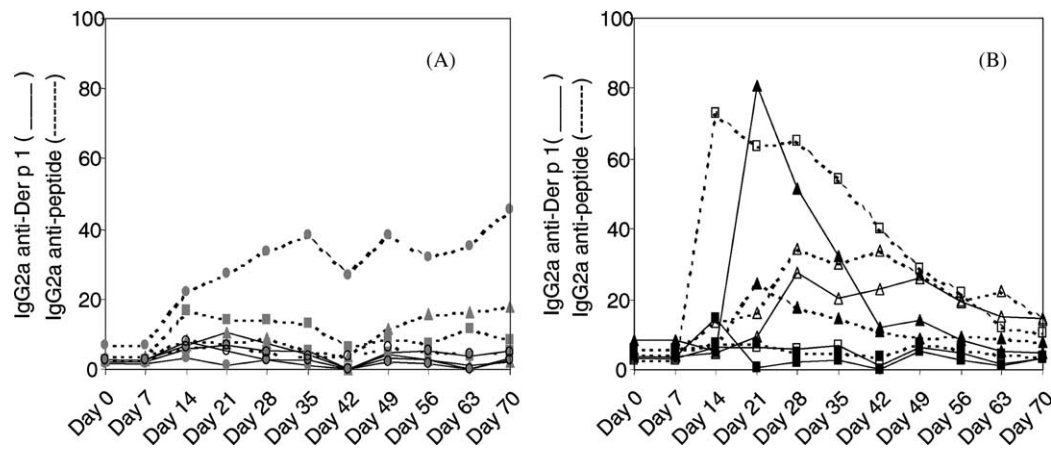


Fig. 7. Leaderless plasmids do not elicit a significant antibody response. Detection of IgG2a was assayed on Der p 1 coated plates (solid lines), or on 1–116 peptide coated plates (dashed lines). Mice received pCIneo-Der p 1_(1–116) (A) or pCIneo-Der p 1_(1–222) (B) with electroporation on days 0 and 21.

result from the absence of native epitopes in the expressed product of this leaderless plasmid. To explore this possibility, a recombinant peptide corresponding to the first half of Der p 1, amino acid sequence 1–116, was produced and used to coat plates. Anti-Der p 1 monoclonals specific for

the 1–116 domain were used to standardize the test. Fig. 7 shows that the anti-peptide response induced by leaderless plasmids was also insignificant.

These results indicate that a leader sequence is required to induce antibodies to Der p 1.

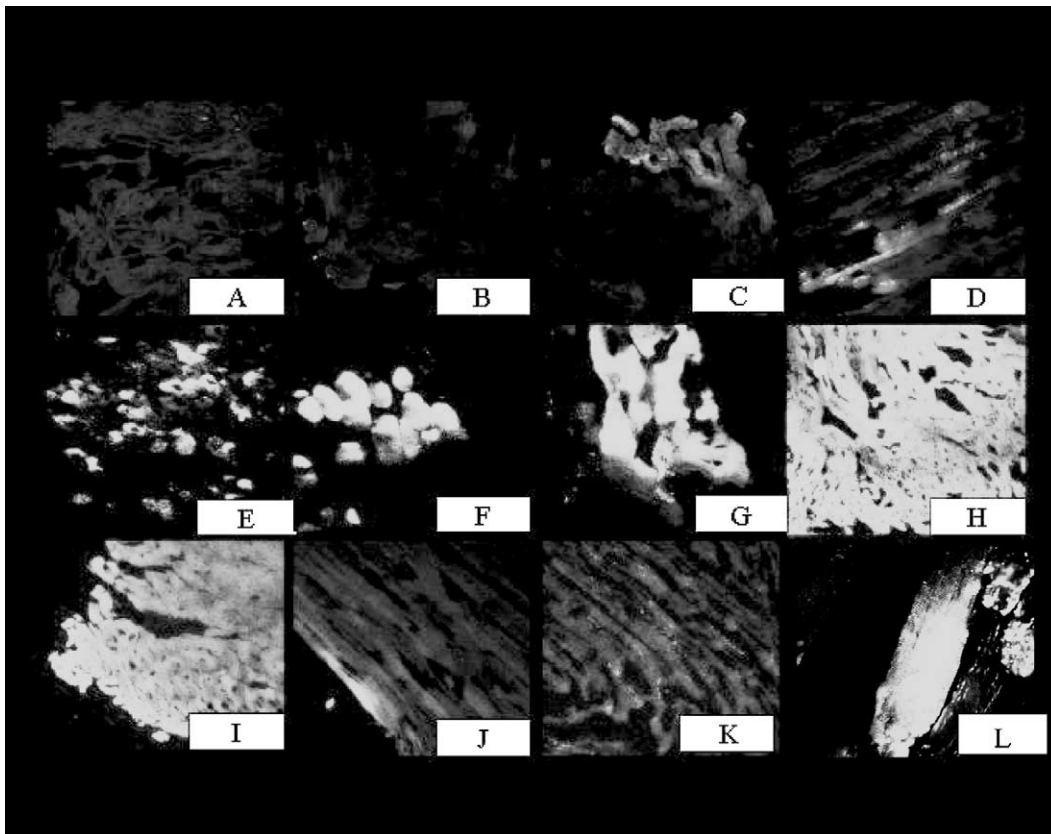


Fig. 8. Time course of pEGFP-Der p 1 expression in the muscle: cryosections from injected muscles were collected at different times after immunization with electroporation. Panel A: representative section of muscle from mice injected with PBS. Panels B to K: sections of muscle from mice injected with pDer p 5L/Der p 1_(1–116)-EGFP collected 1, 6, 24 h, days 2, 4, 7, 10, 14, 21, or 28 after immunization. Panel L: representative section of muscle from mice injected with leaderless pDer p 1_(1–116)-EGFP collected 4 days after injection.

3.4. Similar levels of antigen expression after injection of Der p 5 L/Der p 1 or leaderless Der p 1 constructs

Next we examined whether the lack of a prominent humoral response to leaderless Der p 1 constructs was due to a failure to express these proteins *in vivo*. pEGFP-N3 constructs encoding only Der p 1 amino acids 1–116, or the chimeric Der p 5 L/Der p 1_(1–116) were produced and injected as described above (Fig. 1). The Der p 1 sequence was inserted 5' of the GFP reporter sequence; therefore luminescence reported Der p 1 fragment translation. Cryosections of injected muscle were observed by confocal microscopy. In the absence of electroporation, naked plasmids resulted in very low frequency of dimly fluorescent cells (data not shown). Naked DNA injection and electroporation resulted in marked expression of pEGFP-Der p 5 L/Der p 1_(1–116). The kinetics of fluorescent signal were determined in muscle samples collected on days 1, 2, 4, 7, 10, 14, 21 and 28 after injection. Three animals were examined for every time point, and representative images are shown in Fig. 8.

Expression of Der p 5 L/Der p 1_(1–116)-GFP was consistent, widespread, and frequently intense. It could already be observed a few hours after immunization and continued to increase for a week. Expression was maintained at plateau levels for 7–14 days. Twenty-one days after immunization, expression was lower and barely detectable on day 28.

Surprisingly, the fluorescence emitted by muscle transfected with leaderless pEGFP-Der p 1_(1–116) was just as intense as that observed for animals injected with Der p 5 L/Der p 1_(1–116)-GFP (Fig. 8). These results suggest that the lack of antibody response to the leaderless construct is not due to the lack of protein expression.

4. Discussion

In this study, we demonstrate that DNA immunization with plasmids encoding the complete Der p 1 sequence or fragments of it induces antibodies against the native allergen. Constructs encoding the full-length mature protein (amino acids 1–222) or the globular predominantly α -helix domain (amino acids 1–116) were produced (Fig. 1). Each of the constructs was inserted into the pEGFP-N3 reporter plasmid to determine its expression, or into pCIneo for immunization.

It was first determined that plasmid immunization with DNA carrying the complete Der p 1 cDNA sequence, pcDNA3-pre-pro-Der p 1, can elicit antibodies with native Der p 1 specificity (Fig. 2). At least two immunizations were required to detect the response. A booster injection given 2–3 weeks after primary resulted in circulating anti-Der p 1 antibodies for over 2 months.

The design of the subsequent plasmids was based on several structural considerations. The pre-sequences of other cysteine proteases such as human cathepsin B and papain have been reported cytotoxic for *E. coli* in which they impaired protein synthesis [28,29]. Therefore, we designed

plasmids in which the Der p 1 signal peptide was replaced by that of another mite allergen, Der p 5. The pro-enzyme sequence was deleted to avoid the production of enzymatically active Der p 1 *in vivo*. In order to enhance immunity, we have tested these different constructs with different immunization protocols. Our results showed the antibodies induced by pro-region deleted, pre-region substituted pCIneo-Der p 5L/Der p 1 (1–222) were comparable to those induced by full-length Der p 1 DNA (Fig. 3).

Addition of electroporation to the immunization protocol greatly enhanced both the frequency of responders (90–100% of the mice for all experiments) and the titer of anti-Der p 1 antibodies. As shown for other DNA vaccines [30], electroporation enhances uptake of genes by permeabilization of the muscle fibers, and this results in a very high frequency of transfected bundles as well as higher intensity of expression. The enhancement of immunity apparently follows the increased availability of antigen. Either the Der p 5 L/Der p 1_(1–116) fragment or the Der p 5 L/Der p 1_(1–222) resulted in transfectant protein synthesis and in the induction of comparative antibody responses. Incidentally, the second half of Der p 1 sequence (114–222) encoding the C-terminal β -sheet domain was evaluated, but there was no detectable protein expression or antibody response (data not shown). This observation is currently under further investigation.

While the humoral response was still dominated by IgG2a, it is noteworthy that the enhancement of the overall level of humoral response by electroporation also induced detectable amounts of IgG1 anti-Der p 1, in particular when using the Der p 5L/Der p 1_(1–116) (Fig. 6). The reason for one plasmid inducing a greater isotype shift than another is not clear. It could be due to higher levels of secretory protein. Although IgG1 is usually referred to as a Th2 antibody, IL-12-dependent IgG1 production has also been demonstrated [31]. Whether the IgG1 anti-Der p 1 induced in this case is IL-4- or IL-12-driven needs to be clarified.

Immunization with leaderless constructs (Fig. 7) resulted in no detectable antibody induction. This was confirmed by testing the antisera not only on native Der p 1, but also recombinant peptides corresponding to the first 116 amino acids, GST-(1–116), or the second 109 amino acids domain, GST-(114–222). Even if the conformation of the leaderless product induced antibodies that did not recognize the native allergen, antibodies specific for continuous epitopes present in the denatured sequence could be detected in this assay. Der p 5 leader constructs induced high titers of anti-peptide antibodies and still no response could be detected when immunizing with the leaderless construct.

It was reasoned that the lack of humoral response was possibly due to the lack of expression of the leaderless plasmids. In order to address this issue, immunogenic Der p 5 L/Der p 1_(1–116) constructs or the leaderless Der p 1_(1–116) was each inserted into a reporter vector, pEGFP. Fluorescence was indicative of biosynthesis of the inoculated gene. Confocal examination revealed that muscle cryosections

transfected with Der p 5/Der p 1_(1–116) had bright green spots early after injection that extended to cover whole muscle bundles for extended periods of time. Surprisingly, the pictures looked quite similar for cryosections of muscles inoculated with Der p 1_(1–116). We conclude that the lack of antibody response was not due to lack of protein expression. With the leaderless constructs, we observed green fluorescence of similar intensity in the muscle samples, while there is no detectable antibody response. This could be possibly due to the different localization of the expressed protein. When the protein is expressed extracellularly, it can be phagocytosed by antigen presenting cells, and processed in lysosome, loaded onto MHC class II molecules, so as to stimulate the CD4⁺ T cells, which is critical for the antibody production. However, when the protein is expressed in the cytoplasm, most likely it will enter the endogenous antigen presenting pathway, in which case the processed peptides are loaded onto MHC class I molecules and have less chance to activate CD4⁺ T cells [32].

This finding raises the exciting possibility that leaderless constructs exclusively induce cellular immunity, which has to be further confirmed. If this immunity was characterized by the selective stimulation of IL-12/IL-18 producing cells and down-regulation of IL-4, IL-5 and IgE, leaderless constructs would make good candidates for the development of vaccines for allergic diseases.

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