

Chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen, Der p 1 for oral vaccination in mice

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Abstract

Our previous studies indicated that intramuscular (i.m.) immunisation with full length Der p 1 cDNA induced significant humoral response to the left domain (approximately corresponding to amino acids 1–116) but not to the right domain (approximately corresponding to amino acids 117–222) of Der p 1 allergen. This study explored the use of chitosan–DNA nanoparticles for oral immunisation to induce immune responses specific to both the left and right domains of Der p 1. DNA constructs pDer p 1 (1–222) and pDer p 1 (114–222) were complexed with chitosan and delivered orally followed by an i.m. injection of pDer p 1 (1–222) 13 weeks later. Such approach has successfully primed Th1-skewed immune responses against both domains of Der p 1. This strategy could be further optimised for more efficacious gene vaccination for full length *Der p 1*.

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1. Introduction

Following the seminal publication showing that direct transfer of plasmid DNA into mouse muscle cells led to the expression of encoded proteins [1], the modulation of immune responses by intramuscular (i.m.) injection of plasmid DNA containing house dust mite allergen genes was suggested for therapeutic applications [2–4]. Since then, most effort to deliver mite allergen genes has been through the parenteral (e.g. by intramuscular or intradermal) routes, with relatively few reports on mite gene delivery administered mucosally, and none via the oral route. In this report, we investigate the efficacy of delivering house dust mite allergen genes orally.

The main advantages presented by oral gene delivery are the enhanced patient compliance due to the non-invasive delivery method, low cost owing to the minimally trained personnel required, and elimination of the risk of needle stick injury or cross-contamination. These advantages and other

reports showing that orally delivered genes could induce mucosal immunity make oral delivery desirable for mass vaccination. However, oral administration has been largely ignored because traditional antigen-encoding DNA vaccines have been ineffective unless given with mucosal adjuvants. The poor efficacy of oral gene delivery is mainly due to the acid pH in the stomach that leads to depurination of the DNA, the harsh enzymatic environment present in the gastrointestinal tract and the poor permeability of both genes and gene carriers across the intestinal epithelium.

One of the recent attempts to overcome these obstacles has been the development of cationic polymers as gene delivery vehicles for oral administration [5–9]. Cationic polymeric carriers are less immunogenic than viral vectors, stable in storage and easy to produce in large quantities. Besides that, it has also been shown that protective systemic and mucosal immunity could be induced using these polymeric systems [9–15].

Of the cationic polymers studied thus far, chitosan has been reported to possess great potential as a gene carrier [16–18]. Chitosan is the deacetylated form of chitin, a natural polymer purified from the exoskeletons of crustaceans. Chitin is the second most abundant natural polysaccharide in

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nature. Chitosan is non-toxic, non-immunogenic, biodegradable, can form complexes with DNA easily and it is also able to open up the intercellular tight junctions in the small intestine [17,19,20]. It has been shown to effectively protect DNA from degradation [21,22]. Chitosan particles have also been demonstrated to be taken up by the Payer's patches [17,19,20,23,24]. Therefore, it is a valuable carrier for oral gene delivery. Roy et al. have demonstrated the effectiveness of orally delivered chitosan–DNA nanoparticles in inducing protective immunity in the peanut allergy mouse model [6].

Der p 1 has been regarded as the most important *Dermatophagoides pteronyssinus* allergen and is a major triggering factor for mite allergy world-wide. This 27 kDa glycoprotein is a cysteine protease present in faecal mite pellets. The three-dimensional structure of Der p 1 has been modelled by comparison to actinidin and papain. Mature Der p 1 has two globular domains, one predominantly α -helix, the other mostly β -sheet, connected by a flexible loop containing the catalytic cleft [25,26].

Epitope mapping of allergens has been used as a strategy for the design of DNA vaccines. However, several studies have shown that different subjects react to a large number of different epitopes. Previously, 18 overlapping cDNA fragments covering the entire Der p 1 sequence was tested on sera from patients positive to recombinant Der p 1 and results showed that all fragments longer than 30 residues could bind IgE from all patients and shorter peptides reacted selectively [27]. Another study has also demonstrated that specific IgE binding epitopes of Der p 1 is discontinuous [28]. Mite allergy has also been shown to be associated with a specific profile of IgG epitopes such as those recognised on Der p 1 [29]. T-cell epitopes on Der p 1 has also been found to be discontinuous and the studies with T-cell clones demonstrated that not all individuals respond to the complete set of epitopes or the same sets of epitopes [30–32].

Hence, it is important to include sequences throughout the whole mature Der p 1 in the design of DNA vaccines.

In our previous studies, we have constructed Der p 1 constructs based on its predicted secondary structure to explore the DNA sequences required to raise Der p 1-specific protective immunity [33]. The proenzyme region was excluded, and the Der p 1 leader was replaced by the Der p 5 leader in all the three Der p 1 cDNA fragments: 1–116, 114–222, and 1–222 corresponding to amino acids 1–116, 117–222, and 1–222, respectively (Fig. 1). Each of the DNA constructs was inserted into the pCI-neo vector and i.m. immunisation studies were performed on mice. Results showed that the plasmids (p), pDer p 1 (1–116) and pDer p 1 (1–222) induced specific Der p 1 and rDer p 1 (1–116) antibodies in all mice. Here, we further show that pDer p 1 (114–222) did not induce Der p 1-specific antibodies. We further investigate the feasibility of oral administration using chitosan–DNA nanoparticles for raising specific antibodies to native Der p 1 and Der p 1-derived recombinant peptides in mice.

2. Materials and methods

2.1. Plasmid DNA

A plasmid, pCI-neo (Promega Corporation, Madison, WI) containing Der p 1 (1–222) cDNA fragment flanked upstream by the cytomegalovirus (CMV) immediate early region promoter, including the Der p 5 leader sequence for secretion and eukaryotic expression were constructed as described in [33]. Construction of Der p 1 (114–222) was carried out by PCR amplification of the Der p 1 cDNA using the following primers: 5'-CCT CCG GAT CAA ACT ATT GCC AAA TTT ACC CA-3' (the underlined sequence represents the restriction sequence of *BspEI*) and 5'-TTG GAT

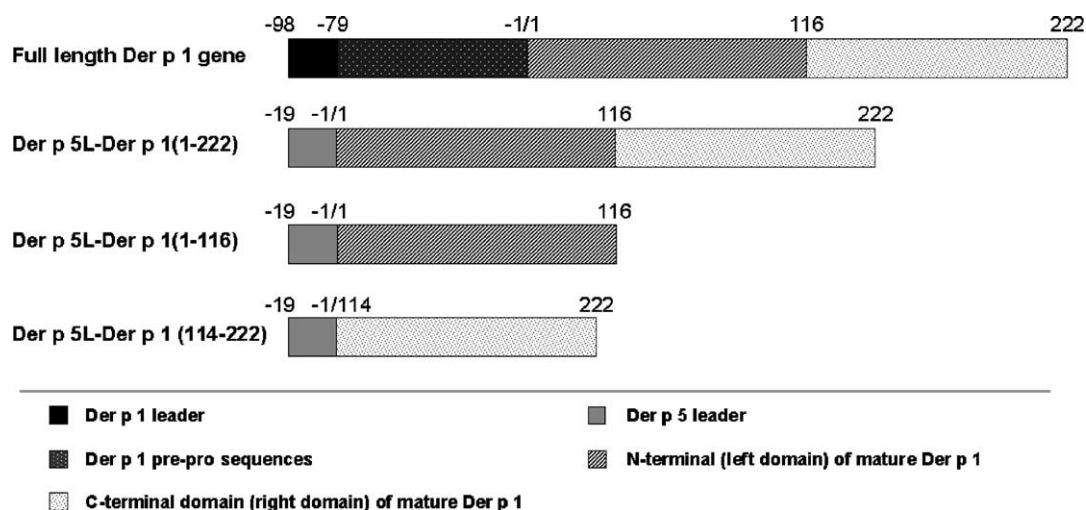


Fig. 1. Der p 1 cDNA constructs generated based on the predicted Der p 1 protein structure. The fragments derived from the full length *Der p 1* gene were subcloned into the pCI-neo vector for DNA immunisation studies.

CCG AGA ATG ACA ACA TAT GGA TAT TC-3'. The Der p 1 (114–222) fragment was then subcloned into the pCR2.1 (Invitrogen, CA) vector to generate pCR2.1-Der p 1 (114–222). Subsequently, the complementary DNA oligonucleotides corresponding to the Der p 5 leader sequence was inserted into the *Xho* I and *Bsp* EI sites of pCR2.1-Der p 1 (114–222) to generate pCR2.1-Der p 5L-Der p 1 (114–222). The expression construct pCI-neo-Der p 5L-Der p 1 (114–222) was produced by insertion of the Der p 5L-Der p 1 (114–222) released from pCR2.1-Der p 5L-Der p 1 (114–222) into the *Xho* I and *Not* I sites of the pCI-neo (Promega, Madison, WI). The plasmids, pCI-neo-Der p 5L-Der p 1 (1–222) and pCI-neo-Der p 5L-Der p 1 (114–222) are hereafter designated as pDer p 1 (1–222) and pDer p 1 (114–222), respectively. The plasmids were prepared according to the manufacturer's instructions using an endotoxin-free mega prep kit (Qiagen Corp., Chatsworth, CA).

2.2. Der p 1 proteins

Der p 1-derived (1–116, 117–222) peptides were expressed as GST-fusions from pGEX-T (Invitrogen, CA) plasmids containing inserts that encode for each of the Der p 1-derived peptides. The pGEX-T-Der p 1 (1–116, 117–222) were obtained from Thomas and coworkers [34,35] and protein expression was carried out as described in [36]. The peptides were purified using the glutathione–agarose affinity chromatography. The GST-rDer p 1 (1–116) and GST-rDer p 1 (117–222) are hereafter referred to as rDer p 1 (1–116) and rDer p 1 (117–222), respectively. Native Der p 1 protein was purified from spent mite media as previously described in [37].

2.3. Mice

Female BALB/cJ mice, 6–8 weeks of age, were purchased from the Laboratory Animals Centre (Sembawang, Singapore) and housed in the National University of Singapore (NUS) Animal Holding Unit.

2.4. Nanoparticles and size optimisation

Chitosan C390 (MW 390 kDa, deacetylation degree 83.5%) was a gift from Vanson Inc. (Redmond, WA). It was purified by reprecipitation from the filtered 1% acetic acid solution with ammonium hydroxide. The precipitate was washed with water and vacuum-dried. The purified C390 was dissolved in 1% acetic acid with gentle heating and the pH of the solution was adjusted to 5.5–5.7 with sodium hydroxide. The solution was diluted to a concentration ranging from 0.02 to 1.0% of chitosan (w/v) with 18 mM acetate buffer. The chitosan solution was readjusted to pH 5.5 and sterile-filtered through a 0.22 μ m filter. DNA solutions of concentrations ranging from 100 to 1000 μ g/ml in 25 mM sodium sulphate solution were used. The DNA and chitosan solutions were pre-heated to 55 °C separately. An equal

volume of both solutions were mixed and vortexed for 20 s to yield nanoparticles. The nanoparticles were observed under the ZEISS Axiovert 25 inverted microscope (Carl Zeiss, NY) to determine which pair of DNA and chitosan concentrations yielded uniform particles.

2.5. Nanoparticle characterisation

- Size distribution*: Sizing of the optimised nanoparticles was performed by photon correlation spectroscopy on the Zetasizer 3000 (Melfern Instruments Ltd., UK) to obtain average nanoparticle size measurements.
- Zeta potential determination*: Zeta potential of the nanoparticles was measured in demineralised water at neutral pH by laser Doppler anemometry using Zetasizer 3000 (Melfern Instruments Ltd., UK).
- Morphological examination*: Nanoparticles were observed under the transmission electron microscope. The freshly made nanoparticles were placed on a glow discharged copper carbon-coated grid (Agar Scientific Ltd., UK) and air-dried. The sample was negatively stained with uranyl acetate and viewed under the JEM1010 transmission electron microscope (JEOL, Japan). Digital micrographs were obtained from the DV 300W 1 camera using the Gatan Microscopy Suite™ Version 1.0 (Gatan Inc., CA).
- Determination of DNA complexed*: The amount of complexed DNA was determined by agarose gel electrophoresis of nanoparticles versus control naked DNA.

2.6. Immunisations

For intramuscular immunisation, 50 μ g of naked DNA in 50 μ l phosphate buffer saline were injected in the tibialis anterior muscle of each mouse under anaesthesia. For intramuscular immunisation with electroporation, DNA was injected as above followed by immediate in vivo electroporation using the ElectroSquare Porator (ECM 830) attached to a two-needle array (BTX Genetronix Inc., CA). Parameters were set at voltage: 82 V, pulse length: 20 ms, number of pulses: 4, and interval: 200 ms. Groups of mice were intramuscularly injected with or without electroporation with naked pDer p 1 (1–222), pDer p 1 (1–116) or pDer p 1 (114–222) at days 0 and 21.

For oral immunisation, mice in groups of six were fed with chitosan–DNA nanoparticles embedded in jelly. Mice were placed into separated cages before feeding and water was given ad libitum. Optimised freshly-made nanoparticles were then mixed in orange-flavoured Lady's Choice jelly crystals (CPC International Inc., Hong Kong) that were dissolved in double-distilled H₂O heated to 50 °C at 150% (w/v) (15 g jelly crystals in 10 ml H₂O). The mixture was then poured onto a weighing tray and left to solidify at 4 °C before being fed to mice. Chitosan nanoparticles containing 50 μ g of DNA were mixed in jelly and left for each mouse to eat once at day 0 or twice at days 0 and 3. After the

mouse had completely consumed the jelly, standard mouse food was replaced. At day 84, intramuscular immunisation with electroporation using 50 µg of DNA in 50 µl phosphate buffer saline was carried out. In order to test the reproducibility, these experiments were carried out twice with separately produced batches of nanoparticles, DNA and mice. Control groups include mice fed with chitosan–empty DNA vector nanoparticles in jelly and DNA in jelly without chitosan, followed by the same i.m. with electroporation immunisation protocol. Different combinations of plasmid DNAs (p): pDer p 1 (1–222), pDer p 1 (114–222) and pCI-neo were used in the oral and i.m. with electroporation immunisation regime (as described in caption of Fig. 4). All i.m. injections in orally primed mice were followed by electroporation. Mice experiments were performed in accordance with the Institutional Guidelines for Animal Care and Handling, NUS.

2.7. Sera, faeces and urine collection and ELISA

Mice were bled from the retro-orbital sinus and the sera were separated for the immunoassays. Urine samples were collected from each mouse and used undiluted. Mice were separately placed in cages without bedding for faeces collection. Lyophilised faecal pellets were homogenised in PBS containing protease inhibitors as described in [6] and centrifuged to collect the supernatant for immunoassays. Ninety-six-well plates (Costar 9018, Corning, NY) were incubated overnight at 4 °C with 5 µg/ml of purified GST-rDer p 1 (1–116 or 117–222) or native Der p 1 proteins in 0.1 M NaHCO₃, pH 8.3. Plates were washed and blocked by standard procedures. Diluted sera, faecal extracts or urine samples were added and incubated overnight at 4 °C. For quantification and internal control purposes, known serial dilutions of purified mouse IgG1, IgG2a and IgA κ-light chain isotype standard (Pharmingen, CA) was used on wells coated with anti-mouse κ-light chain (Pharmingen). Biotin-conjugated anti-mouse IgG1, IgG2a (Southern Biotech, AL) and IgA (Sigma, MO) were then added and incubated for 1 h at room temperature, followed by incubation with ExtrAvidin alkaline-phosphatase conjugate (Sigma, MO). The binding of specific antibodies was measured as absorbance at 405 nm after reaction of the immune complexes with *para*-nitrophenyl phosphate (*p*-NPP) substrate (Sigma, MO) in the dark. The antibody production units of antigen-specific antibodies were determined from the OD₄₀₅ using the plot of absorbance versus concentration of the standard. Antisera were also tested on plates coated with GST for specificity control.

2.8. Statistical analysis

Statistical differences were determined by the Student's *t*-test using SPSS, Version 11.0.1 (SPSS Inc., Chicago, IL, USA). A *P*-value of less than 0.05 was considered as significant.

3. Results

3.1. Preparation and characterisation of chitosan–DNA nanoparticles

The conditions for the nanoparticle preparations were optimised by light microscopic examination of the morphology of the particles. Relatively uniform particles were obtained using 100–200 µg/ml DNA and 0.2% chitosan. Particle size increased with the concentration of chitosan in the range of 0.02–1.0%. Aggregation occurred when 0.4–1.0% of chitosan were used. String-like and irregular shaped particles were observed when 500–1000 µg/ml DNA was mixed with 0.02–0.1% chitosan. A typical transmission electron microscopy (TEM) image of chitosan–DNA nanoparticles formed using 100–200 µg/ml DNA and 0.2% chitosan is shown in Fig. 2.

The size and zeta potential of chitosan–DNA nanoparticles were characterised by Zetasizer. The size of the optimised chitosan–pDer p 1 (1–222) nanoparticles was found to be 506.9 ± 9.7 nm (mean ± S.D.; *n* = 3) and the zeta potential was positive at 7.3 ± 0.2 mV. The size of optimised chitosan–pDer p 1 (114–222) nanoparticles was slightly smaller (422.8 ± 5.6) and its zeta potential was also lower (3.1 ± 0.3 mV) than chitosan–pDer p 1 (1–222) nanoparticles. The small differences in size and zeta potential between the different batches of nanoparticles demonstrate the good reproducibility of the preparation method.

3.2. DNA encapsulation efficiency

The optimised chitosan–DNA nanoparticles were analysed by agarose gel electrophoresis along with non-encapsulated DNA as controls (data not shown). DNA was completely retained in the loading well after complexation

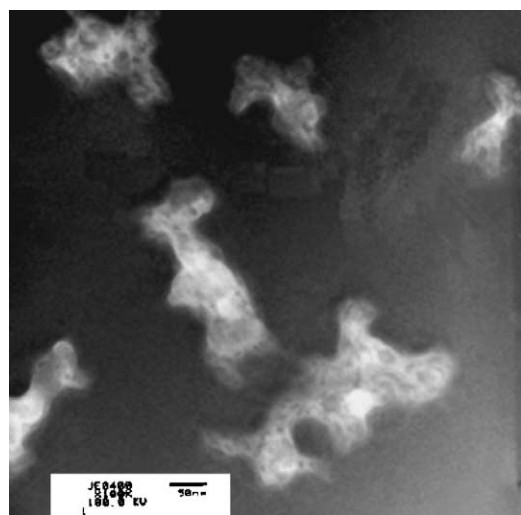


Fig. 2. Transmission electron micrographs of chitosan–pDer p 1 DNA nanoparticles ($\times 10^5$ magnification). The scale bar represents 50 nm.

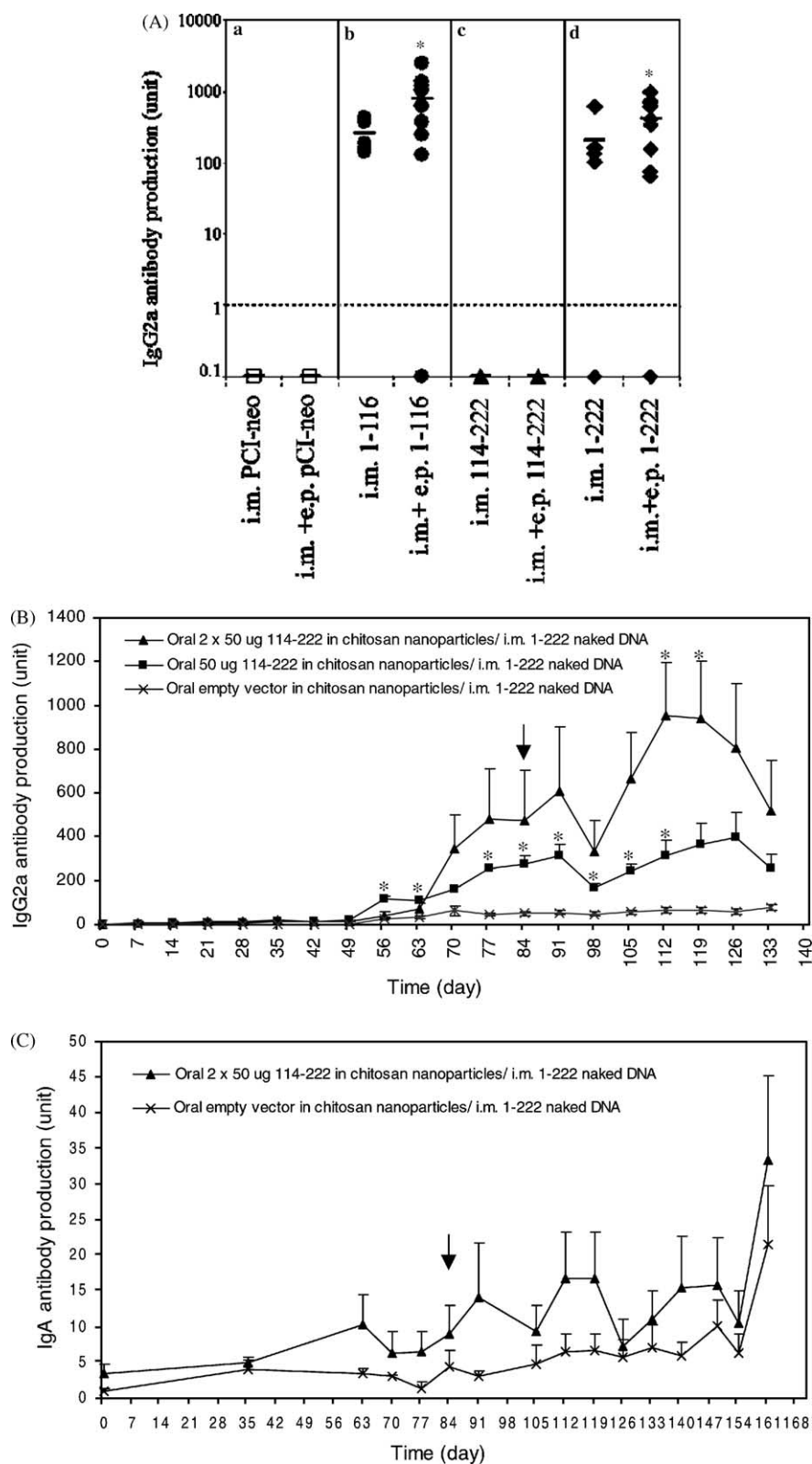


Fig. 3. Induction of antibody response by pDer p 1 (114-222). (A) Peak anti-Der p 1 IgG2a antibody responses in mice immunised intramuscularly with the different Der p 1 constructs. The mice were immunised on day 0 and boosted on day 21 using naked expression vector pCI-neo panel (a); pDer p 1 (1-116) panel (b); pDer p 1 (114-222) panel (c); and pDer p 1 (1-222) panel (d). The statistical significance of differences of means between respective groups with or without electroporation were $*P < 0.05$, as determined by the Student's *t*-test, e.p.: electroporation. (B) Average anti-rDer p 1 (117-222) IgG2a in mice fed with 50 μ g (solid square) and 2 $\times$ 50 μ g (solid triangle) of pDer p 1 (114-222) in chitosan nanoparticles in jelly. For the latter, oral administration was carried out on days 0 and 3. i.m. immunisation of 50 μ g pDer p 1 (1-222) was carried out on day 84 (as indicated by the arrow). Mice were immunised with empty vector as control (cross). The statistical significance of differences of means of experimental groups vs. the control group were $*P < 0.05$, as determined by the Student's *t*-test. (C) Average sera anti-rDer p 1 (117-222) IgA production kinetics in mice fed with 2 $\times$ 50 μ g of pDer p 1 (114-222). Significantly, there was no difference between the two groups, as determined by the Student's *t*-test. Results were mean $\pm$ S.E.M. per group.

with chitosan. This confirmed that the DNA was fully complexed into chitosan–DNA nanoparticles, suggesting a 100% encapsulation efficiency. The nanoparticles were also stained with propidium iodide (COULTER® DNA-Prep Stain, Coulter Corp., FL) and viewed under the FluoView™ 300 LSM (Olympus, Japan) confocal microscope. The images further confirmed that DNA was incorporated in the nanoparticles (data not shown).

3.3. Priming of humoral response to the right domain (peptide 117–222) of Der p 1

IgG2a antibodies against native Der p 1 were detected in mice intramuscularly immunised with pDer p 1 (1–222) and pDer p 1 (1–116) with and without electroporation. No anti-Der p 1 IgG2a were detected in mice intramuscularly injected with pDer p 1 (114–222) regardless of electroporation (Fig. 3A).

To investigate whether oral delivery of pDer p 1 (114–222) could prime for antibody production specific to the right domain (peptide 117–222), the kinetics of antibody production in mice fed orally with chitosan–pDer p 1 (114–222) nanoparticles followed by i.m. injection with pDer p 1 (1–222) were studied. IgG2a antibodies specific to rDer p 1 (117–222) were detected 56 days after oral administration and the antibody levels gradually increased to a peak and then began to decrease at day 98. A second peak of antibody response with higher magnitude was observed following the i.m. injection with full length *Der p 1* gene (Fig. 3B). Mice fed twice with 50 µg of DNA in chitosan nanoparticles produced higher antibody levels as compared to mice fed with a single dose. Control mice fed with vector DNA in chitosan nanoparticles did not produce any significant specific antibodies to rDer p 1 (117–222) before and after the i.m. injection of the full length gene. No significant IgG1 was detected in all mice. Mice fed with naked DNA without chitosan in jelly, did not prime any significant IgG2a or IgG1 antibody production in mice (data not shown). Furthermore, in the mice fed with a double dose of chitosan–DNA nanoparticles, low levels of specific IgA were detected in the sera (Fig. 3C) but not in the faeces and urine samples.

3.4. Oral priming of humoral responses to full length *Der p 1* and rDer p 1 (1–116) and (117–222)

Further in vivo studies with different combinations of the pDer p 1 constructs were performed to investigate whether oral immunisation with chitosan–DNA complexes can prime immune responses to full length *Der p 1* molecule. All mice were fed with 50 µg of chitosan encapsulated DNA nanoparticles in jelly at days 0 and 3, and boosted by i.m. injection with naked DNA at day 91. Kinetics of IgG2a and IgG1 production to full length native *Der p 1*, rDer p 1 (1–116) and rDer p 1 (117–222) were determined and shown in Fig. 4. Significant IgG2a antibodies against full length *Der p 1*, rDer p 1 (1–116) and (117–222) were elicited in mice orally fed

with the chitosan–DNA nanoparticles and i.m. boosted with naked DNA at day 91. All the experimental mice produced basal levels of specific IgG1 (data not shown). In comparison, control groups fed with chitosan encapsulated empty vector DNA nanoparticles in jelly followed by i.m. booster injection at day 91 with naked pDer p 1 (1–222), pDer p 1 (114–222) or empty vector did not show any significant antibody production (data not shown). Taken together, the results can be summarised as follows: first, pDer p 1 (1–222) gene delivered orally could prime IgG2a production to full length *Der p 1* and rDer p 1 (1–116) and (117–222); second, the antibody production against rDer p 1 (1–116) detected in mice fed with the unrelated molecule of pDer p 1 (114–222) in chitosan was due to the i.m. injection of pDer p 1 (1–222) (the results were consistent with our previous observation that i.m. injection with naked DNA preferentially induced higher levels of antibodies to rDer p 1 (1–116)); third, antibody response to rDer p 1 (117–222) was induced successfully by oral priming.

4. Discussion

Polymeric carriers are potentially advantageous over viral vectors, which have in vivo limitations including wild type reversion and immunogenicity. The use of cationic lipids in vivo is also hindered by its toxicity, complement activation and liver tropism. Chitosan is one of the few polycations of natural origin available, and may be superior to other vectors including liposomes due to its minimal toxicity [19]. Chitosan–DNA nanoparticles are spontaneously formed by the complex coacervation method using sodium sulphate as desolvating agent [21]. The morphology of nanoparticles in this study is slightly different from previous studies [6,21], owing to the higher concentration (0.2%) of chitosan used. We hypothesise that a higher concentration of chitosan may confer better protection of the DNA in the GI tract. Although particles can be prepared with high positive charge under the optimised conditions, their characteristics under the physiological conditions are difficult to control.

The data presented here show that oral feeding of chitosan–DNA nanoparticles could raise immune responses against the major dust mite allergen, *Der p 1* whereas intramuscular immunisation alone could not. In agreement with Wolfowicz et al. [33], pDer p 1 (114–222) failed to induce anti-native *Der p 1* antibodies in mice when immunised intramuscularly (Fig. 3A). The inability of the 114–222 construct to stimulate immune response was further confirmed by the lack of protein expression in the muscle cells after intramuscular immunisation with the pEGFP-*Der p 1* (114–222) both with and without electroporation as observed under the confocal microscope (data not shown). In Fig. 3B and C, mice orally primed with chitosan–pDer p 1 (114–222) nanoparticles produced specific IgG2a and IgA antibodies in the systemic circulation. No specific IgA antibodies were detected in the faecal extract and urine

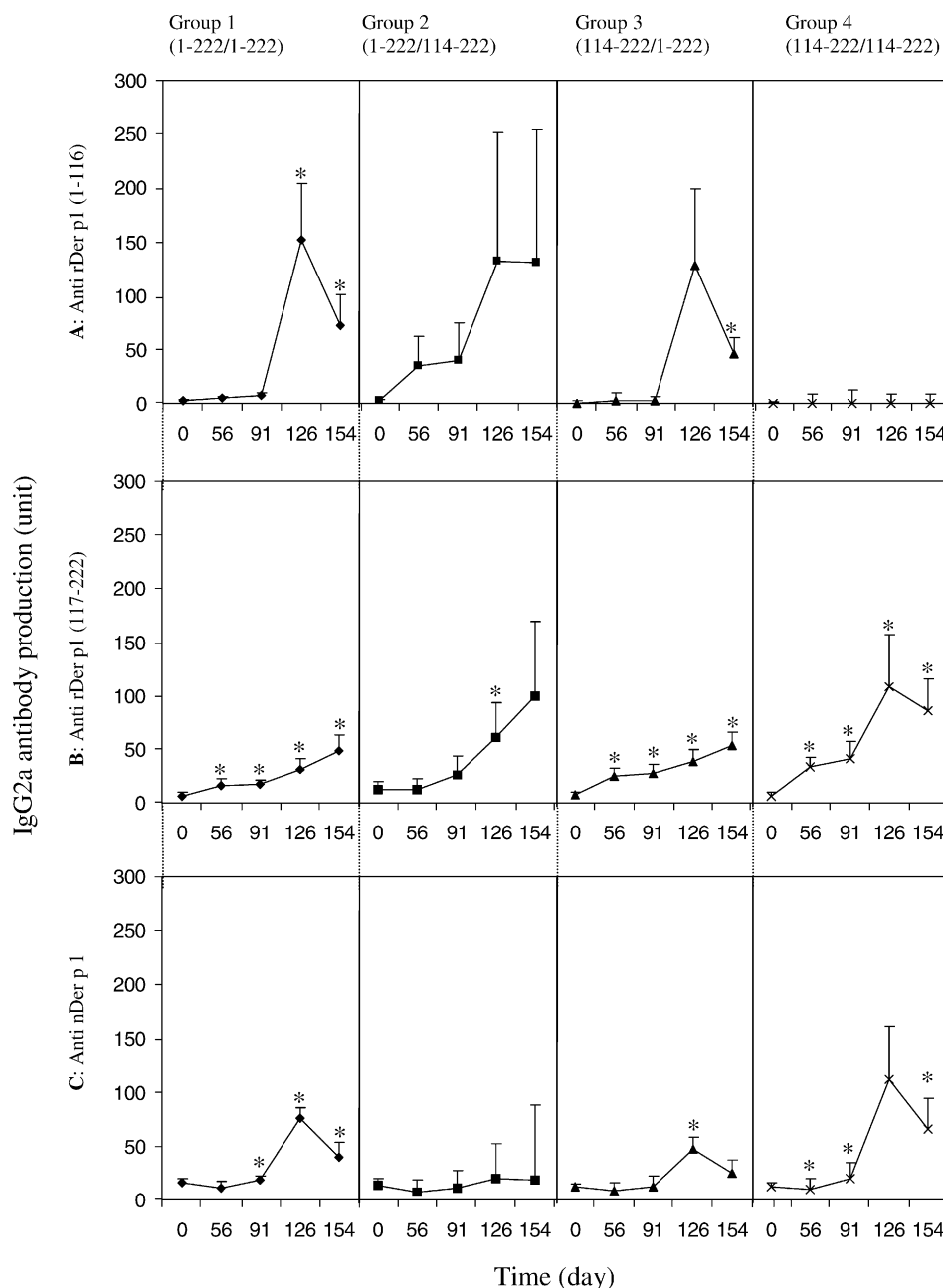


Fig. 4. Induction of IgG2a antibodies to Der p 1 and its derived peptides after oral immunisation with chitosan–DNA nanoparticles followed by i.m. immunisation with naked DNA. Panel (A) represents antibodies detected against rDer p 1 (1–116); panel (B) represents antibodies detected against rDer p 1 (117–222); panel (C) represents antibodies detected against native Der p 1 protein. Mice in group 1 were fed with chitosan encapsulated pDer p 1 (1–222) nanoparticles followed by i.m. injection of naked pDer p 1 (1–222); mice in group 2 were fed with chitosan encapsulated pDer p 1 (1–222) followed by i.m. injection of naked pDer p 1 (114–222); mice in group 3 were fed with chitosan encapsulated pDer p 1 (114–222) followed by i.m. injection of naked pDer p 1 (1–222); mice in group 4 were fed with chitosan encapsulated pDer p 1 (114–222) followed by i.m. injection of naked pDer p 1 (114–222). All mice were fed with 50 μ g of DNA at days 0 and 3, and i.m. injected with 50 μ g DNA followed by in vivo electroporation at day 91. Results were mean $\pm$ S.E.M. per group. The statistical significance of differences of means between experimental vs. control groups were $*P < 0.05$, as determined by the Student's *t*-test; n: native.

of these mice. This could be due to the fact that we have missed the peak of the kinetics for mucosal IgA production as the faecal and urine samples were collected from 42 days onwards post feeding.

Although Etchart et al. [38] has indicated that naked DNA is not significantly degraded in the stomach as observed

by the similar levels of measles virus-specific CTLs generated after oral (intra-gastric) and intrajejunal injection of naked DNA. However, our study showed that there was no significant humoral response in the control mice fed with naked DNA embedded in jelly, suggesting that DNA protection conferred by the chitosan nanoparticles was crucial.

The nanoparticles might also have facilitated DNA uptake by the host cells, leading to enhanced transfection efficiency as suggested by previous *in vitro* studies [21,23,24].

The initiation of immune response depends on the ability of particles to cross the mucosal epithelial barrier and to be captured and presented by antigen presenting cells. Since chitosan is mucoadhesive [17,39–41], the ingested chitosan–DNA nanoparticles may adhere to mucosa epithelia in the intestine and therefore facilitate its uptake by specialised M cells, located in the dome epithelium of the Peyer's patches, and translocate to the underlying lymphoid organs where antigen-presenting cells, including B cells and dendritic cells are present. Microparticles with a diameter of less than 10 μm have been shown to be taken up by M-cells and transported to the dome of the Peyer's patches of the small intestine [42,43]. Particles of less than 5 μm were transported to the spleen and lymph nodes where specific IgM and IgG were produced, while particles of 5–10 μm stayed in the Peyer's patches [43]. The chitosan–DNA particles (smaller than 1 μm) used in this study would be of suitable size for M cell uptake and translocation to Peyer's patches to initiate antigen-specific immune responses.

It is worth noting that although M cells have been the most extensively studied cell type in the uptake of nano and microparticles, studies have also shown that intestinal epithelial cells (IECs) in the villus epithelium may also play a role in the uptake and presentation of antigens [44]. IECs having intimate contact with intestinal intraepithelial lymphocytes (iIELs) and lamina propria lymphocytes (LPLs) may be able to regulate T-cell responses. Dendritic cells have also been found in different sites of the intestine and they may capture antigens directly from the lumen or from IECs. Since chitosan has been shown to be able to cross tight-junctions [45] between IECs, the chitosan–DNA nanoparticles may also be captured by dendritic cells through this route. Once the dendritic cells capture and process the antigen, they subsequently migrate to draining lymph nodes, where they acquire costimulatory signals necessary to prime naïve T cells.

Previously, our laboratory has shown that *i.m.* immunisations using pDer p 1 (114–222) and pDer p 1 (1–222) could not induce significant antibodies to the right domain, *i.e.* rDer p 1 (117–222) [33]. Data from this study showed that oral administration of chitosan–DNA nanoparticles containing pDer p 1 (114–222) and pDer p 1 (1–222) successfully primed antigen-specific immune responses to whole Der p 1 and Der p 1-derived peptides. The orally primed immune responses could be boosted 12–13 weeks post feeding by *i.m.* injection of naked DNA (Fig. 4). However, the magnitude and frequency of responders were lower compared to those that can be obtained through parenteral immunisation, as shown in [33]. Our data are in good accordance with other reported studies showing similar low levels of systemic antibody response after nano or microparticulate oral delivery [6,46]. It is interesting to note that IgG2a was preferentially induced in all mice and only basal levels of IgG1 were de-

tected in these mice; suggesting that oral priming may have induced Th1-skewed immune responses. Further studies on the cellular immune responses will be performed in order to gain a better understanding of the nature of the responses.

The kinetics of antibody production revealed that the serum antibodies were consistently induced at about 8 weeks after the initial oral gene administration. Antibody productions after parenteral immunisations are generally detected after 2–3 weeks. The delay in the antibody production kinetics was probably due to the higher percentage (0.2%) of chitosan used in our study. It has been reported that when 0.02% of chitosan was used for oral delivery of the peanut allergen gene, the immunised mice produced detectable levels of serum antibodies 3–4 weeks post feeding [6]. Furthermore, our preliminary studies showed that mice fed with allergen genes complexed in 0.02% chitosan showed similar antibody production kinetics reported by Roy et al. [6]. The higher chitosan concentration used in this study, 0.2%, would lead to more stable nanoparticles and a slower release of DNA. It may also account for the lower frequency of high responders in our study since in 0.2% chitosan–DNA complexes, the ratio of DNA to chitosan is comparatively low. Further optimisation of chitosan–DNA complexes preparation may help to improve the efficiency of gene delivery and magnitude of immune responses.

It has been shown by our previous study that *i.m.* immunisation with naked pDer p 1 (1–116) and pDer p 1 (1–222) DNA induced good humoral response to rDer p 1 (1–116) [33]. In contrast, oral delivery of chitosan encapsulated pDer p 1 (1–116) DNA induced poor humoral response to rDer p 1 (1–116) and full length Der p 1 (unpublished data). This differential immune response appears to have some specific correlation with the route of immunisation and DNA sequences administered.

Since the major challenge to successful oral immunisation is that most of the antigens are poorly immunogenic, it is important to develop good boosting methods to augment orally primed immune responses. To date most mucosal prime-parenteral boost studies have used viral vectors [47,48]. In our study, orally primed mice were boosted by an *i.m.* injection of 50 μg of naked DNA followed by electroporation. Although the immune response could still be boosted at 12–13 weeks post feeding, the magnitude of boosting effects was not satisfactory. Different boosting strategies are currently being explored in our laboratory. Since a single feeding of chitosan–DNA nanoparticles could successfully prime the murine immune response, we postulate that higher magnitude of immune responses can be achieved by multiple and optimised dosage.

In summary, our results demonstrate that by resorting to an oral route of DNA delivery, we could induce Der p 1-specific immune responses otherwise undetectable using parental route of immunisation. It may also be a promising gene delivery system for the induction of Th1-skewed immunity. Moreover, this study also shows that chitosan–DNA nanoparticles possess suitable characteristics for oral gene

vaccination and therefore could be a useful strategy for the development of vaccine regimes in general.

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