

Original article

Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults

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Background: In mice, highly repetitive antigens, such as those present on bacterial or viral surfaces, efficiently cross-link B-cell receptors and therefore induce strong IgG responses. In this study we covalently coupled a synthetic 16-amino-acid sequence of the allergen Der p 1 to a virus-like particle derived from the bacteriophage Q β (Q β -Der p 1).

Objective: We evaluated the safety and immunogenicity of Q β -Der p 1 in human subjects and compared different doses and routes of immunization.

Methods: In a phase I trial 24 healthy volunteers were randomly assigned to one of 4 treatment groups. Group 1 received 50 μ g of Q β -Der p 1 intramuscularly, group 2 received 50 μ g of Q β -Der p 1 subcutaneously, group 3 received 10 μ g of Q β -Der p 1 intramuscularly, and group 4 received 10 μ g of Q β -Der p 1 subcutaneously. Boosting immunizations with 10 μ g were given after 1 and 3 months. Antibody titers were measured after 1, 3, 4, 6, 12, and 18 months.

Results: The vaccine Q β -Der p 1 was well tolerated. Significant IgG responses were observed 4 weeks after a single injection. Individuals receiving 50 μ g of the vaccine had significantly higher IgG titers than those vaccinated with 10 μ g. However, the route of immunization (subcutaneous vs intramuscular) had no effect. In the 50- μ g dose group, strong antibody responses against Der p 1 with average titers of 1:2000 were obtained. **Conclusion:** Vaccination with a peptide antigen covalently coupled to highly repetitive virus-like particles represents an adjuvant-free means of rapidly inducing high antibody titers in human subjects.

Clinical implications: Allergens coupled to virus-like particles can be used to enhance the efficiency of allergen-specific immunotherapy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Der p 1, virus-like particle, immunotherapy, clinical trial

Vaccination is one of the most effective means for the prevention of infectious disease. The majority of vaccines that have been used in the past are attenuated or inactivated forms of the original pathogen, such as the Sabin¹ and Salk² vaccines, respectively. Most classical vaccines, in particular the attenuated ones, induce strong B- and cytotoxic T-cell responses. Although inactivated or attenuated vaccines are relatively safe, there is nevertheless a certain probability that pathogens might revert to a virulent phenotype *in vivo* and cause disease, as is sometimes observed after vaccination against polio virus. In fact, poliomyelitis has essentially been eradicated from the Western world, and now the only reported cases are vaccine induced.^{3,4} In light of the decreasing probability of infection, the acceptance of vaccine-induced side effects is continuously decreasing. Consequently, modern vaccines are usually produced recombinantly and do not replicate in the host. Unfortunately, isolated soluble components of viruses and other pathogens are often poorly immunogenic in the absence of nonspecific inflammatory stimuli.⁵⁻⁸ Recombinant vaccines therefore need to be administered together with an adjuvant to enhance immunogenicity. However, effective adjuvants are not well tolerated, and only a few of them have the potential to be used in human subjects. Hence there is a great need for safe and effective recombinant vaccines that do not require adjuvants.

The presentation of an antigen in a highly ordered and repetitive array normally provokes strong antibody responses in mice, whereas the same antigen presented as a monomer is nonimmunogenic.⁹⁻¹³ Furthermore, repetitive antigens induce strong antibody responses in the absence of adjuvant. Examples of repetitive antigenic arrays inducing potent B-cell responses are viral and bacterial surfaces^{13,14} and virus-like particles (VLPs) that are formed by certain viral proteins.^{12,15-18} The correlation between immunogenicity for B cells and antigen repetitiveness has been established in mice,^{9,10,12,13,19-21} and there is also epidemiologic evidence that similar rules might govern B-cell activation in human subjects. Specifically, viruses that exhibit a highly organized, quasicrystalline surface structure are primarily eliminated by antibodies, whereas viruses with less repetitive surfaces are often

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Disclosure of potential conflict of interest: A. Fulurija, F. Hennecke, K. Sladko, G. T. Jennings, and M. F. Bachmann have stock in and are employed by Cytos Biotechnology. The rest of the authors have declared that they have no conflict of interest.

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Abbreviations used

GMP: Good manufacturing practice
 Q β -Der p 1: Der p 1 coupled to a virus-like particle derived from the bacteriophage Q β
 VLP: Virus-like particle

cleared by T cells.¹³ In addition, viruses and VLPs are known to be potent inducers of B-cell responses in human subjects.^{2,22-25}

However, proteins that spontaneously assemble to highly repetitive structures, such as VLPs, are rare exceptions. In this study we chose a model protein (Der p 1) that by itself does not spontaneously assemble and attempted to enhance its immunogenicity by covalently linking it to another protein that forms a VLP (Q β). We have previously shown that the bacteriophage-derived protein Q β , as well as antigens coupled to Q β , induce potent B-cell responses in mice.¹² The present study extends these findings to human subjects. Twenty-four volunteers were vaccinated with Der p 1 coupled to Q β by using 2 different doses (10 μ g and 50 μ g of total protein) and 2 different routes of immunization (subcutaneous and intramuscular). No adjuvant was used. After only one single injection, we found that all volunteers mounted a significant response against both Der p 1 and Q β . The response was dose dependent, whereas the route of immunization appeared to be of minor importance.

These results shed new light on B-cell responsiveness to repetitive antigens in human subjects, as well as demonstrating that antigens coupled to highly repetitive VLPs are a highly efficient means of rapidly inducing high titers of antibodies in human subjects without the need for adjuvants.

METHODS

Participants

We assessed healthy volunteers aged 18 to 50 years from November 2002 through November 2003 at the Zürich University Hospital, Switzerland. Exclusion criteria were blood donations or surgery within the previous 30 days, use of investigational drugs within the previous 90 days, mastocytosis defined by a serum tryptase level of greater than 13.5 μ g/mL, significant cardiovascular disease, hypertension, active infectious disease (white blood cell count, >12,000 cells/ μ L), seropositivity for HIV, hepatitis B or hepatitis C, significant other hepatic disease with alanine aminotransferase and aspartate aminotransferase or bilirubin levels greater than 2 $\times$ normal values, significant renal disease with a creatinine level of greater than 105 μ mol/L, significant hematologic disorders with a hematocrit value of less than 30% or platelet counts of less than 100,000/ μ L, significant pulmonary disease, autoimmune disease, history of malignancy, obesity with a body mass index of greater than 30 kg/m², and moderate or severe asthma or allergy defined by daily use of β -agonists or topical steroids. IgE against *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* had to be lower than CAP class 2. Participants were not taking immunosuppressive medications, β -blockers, angiotensin-converting enzyme inhibitors, tricyclic antidepressants, or angiotensin II receptor antagonists. The ethics

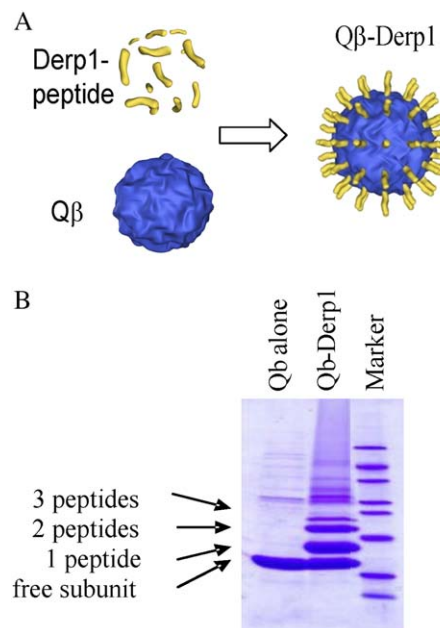


FIG 1. Characterization of vaccine. **A**, Der p 1 chemically coupled to Q β particles, consisting of 180 Q β monomers, adopts a highly repetitive format. **B**, Up to 3 Der p 1 peptides are coupled to Q β monomer, as determined by means of SDS-PAGE.

committee approved the study, and Swissmedic gave regulatory approval. Volunteers provided signed informed consent.

Procedures

Production of Q β -Der p 1. All syntheses, production, and coupling of the investigational new drug were performed by Cytos Biotechnology AG under conditions stipulated by Annex 18 of the EU good manufacturing practice (GMP) guidelines. The monomeric coat protein of Q β was cloned and expressed in *Escherichia coli*. The spontaneously assembled VLPs were purified, involving precipitation and chromatography. The peptide (Der p 1) used in this study was derived from a cysteine protease, the major fecal allergen of the house dust mite *D pteronyssinus*. Der p 1 was chemically synthesized under GMP conditions and comprised the amino acid sequence CGIYPPNANKIREALAQTHSA, amino acids 117 through 133, which are known to include B-cell epitopes.²⁶ The amino acid residue CG were introduced at the amino terminus of the synthetic peptide to facilitate chemical cross-linking. The Der p 1 peptide was coupled to purified Q β VLPs by using a heterobifunctional chemical cross-linker, resulting in a highly organized array of peptides on the VLP surface (see schematic Fig 1, A). Numerous quality control criteria, including epitope density and particle size, were used to analyze Q β -Der p 1. SDS-PAGE and densitometry indicated that approximately 70% of the Q β subunits had 1, 2, or 3 covalently coupled peptides (Fig 1, B). Size exclusion chromatography showed homogeneity in the size of the particles (data not shown), a finding that was confirmed by means of dynamic light scattering, where particles with a diameter of approximately 30 nm diameter were observed (data not shown).

Assessment of safety, tolerability, and immunogenicity. Safety and tolerability of the vaccine were determined by measuring vital signs, clinical laboratory analyses (hematology, blood chemistry, and urinalysis), and assessment of any reported symptoms.

Immunogenicity of the vaccine was assessed by measuring the humoral immune responses (ie, specific antibodies against Q β and

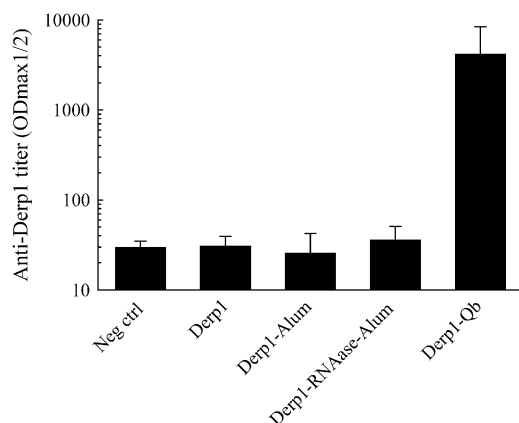


FIG 2. Immune responses in mice. Mice (5 per group) immunized once with 50 μ g of Der p 1 in saline or alum or coupled to RNase in alum or coupled to Q β . *Neg ctrl*, Preimmune sera. Serum IgG titers after 3 weeks by means of ELISA with Der p 1 coupled to an independent carrier (hepatitis B core antigen). Titers are expressed as serum dilution leading to half maximal OD_{450nm} values.

Der p 1 by means of ELISA). A total of 10 μ g/mL peptide coupled onto RNase or 4 μ g/mL Q β protein in coating buffer (0.1 M NaHCO₃ pH 9.6) was coated onto ELISA plates (Nunc MaxiSorp; Nalge Nunc International, Rochester, NY), and ELISAs were performed according to standard protocols by using horseradish peroxidase-conjugated secondary antibodies (goat-anti-human IgG, Jackson ImmunoResearch Europe [Cambridgeshire, UK]; goat anti-human IgM, Zymed Laboratories Inc [San Francisco, Calif]; mouse anti-human IgE, Southern Biotechnology Associates Inc [Birmingham, Ala]; IgG subtype-specific antibodies were obtained from Southern Biotechnology). Plates were developed with orthophenylenediamine substrate buffer (0.4 mg/mL orthophenylenediamine, 0.01% H₂O₂, 0.066 M Na₂HPO₄, and 0.035 M citric acid, pH 5.0; 100 μ L in each well), the reaction was stopped with 50 μ L of 5% H₂SO₄ per well, and optical density was read in an ELISA reader at 450 nm (OD_{450nm}). A standard antiserum (pooled from 3 donors) was run on each plate as an internal standard and positive control. For detection of specific IgE antibodies, IgG was preabsorbed from sera by using protein G sepharose (Amersham Bioscience, Uppsala, Sweden), according to the manufacturer's instructions.

End-point titers were calculated in the following way. Curves of OD_{450nm} versus serial dilution of immune sera were plotted. The average optical density of all preimmune sera at all dilutions was calculated. The value of the end-point titer was determined from the curve as the dilution of immune sera corresponding to the calculated average OD_{450nm} of all preimmune sera. Titers of half-maximum absorbance were determined as described.¹²

Subjects responding to the vaccine were defined as those with anti-Der p 1 antibody titers measured at 1:50 dilution of serum with an OD_{450nm} value greater than 0.1 above the corresponding preimmune serum in 2 independent measurements.

Statistical analysis

Sample-size considerations were based on expected ELISA titer values. It was assumed that 5 subjects per group would have a power of 97% to detect a difference in means of 0.900 log₁₀ (0.00 vs 0.900) with a common SD of 0.32 by using a 2-group *t* test with a 5% 2-sided significance level. Six subjects per group were recruited to account for possible subject withdrawals. Under the same assumptions, a power of 79% should be obtained with a 2-group *t* test with a 0.8% 2-sided significance level.

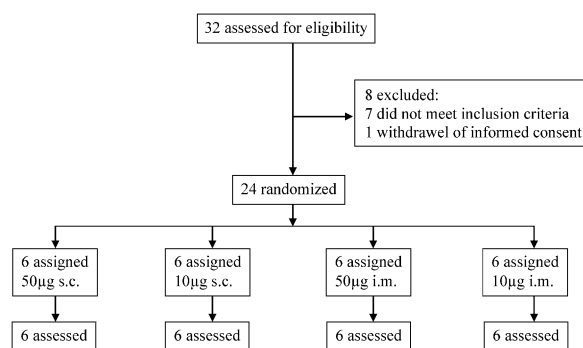


FIG 3. Trial profile. Thirty-two subjects were assessed for eligibility. Eight subjects were excluded because 7 did not meet the inclusion criteria, and 1 subject withdrew informed consent. Twenty-four subjects were included in the study and randomized. None of these 24 subjects dropped out of the study. *s.c.*, Subcutaneous; *i.m.*, intramuscular.

Role of the funding source

The sponsor Cytos Biotechnology AG approved the study design, determined the ELISA titers, and helped with the preparation of the manuscript. The sponsor had no part in clinical data collection, data quality management, data analysis, or data interpretation.

RESULTS

Key preclinical data

To compare the immune response to Der p 1 when presented as a highly ordered array on Q β with standard conjugation technologies, Der p 1 was also coupled to a protein carrier (RNase) and used to immunize mice. Animals were immunized with either Der p 1 alone or Der p 1 in alum, Der p 1 coupled to RNase in alum, or Der p 1 coupled to Q β without adjuvant. IgG titers against Der p 1 were determined 3 weeks later by means of ELISA (Fig 2). Although the Q β -Der p 1 preparation induced strong IgG responses in the absence of adjuvants, titers obtained against Der p 1 alone or coupled to RNase were weak, even when administered in alum. Thus, as previously observed, antigens conjugated to VLPs are much more immunogenic than antigens conjugated to carrier proteins.^{11,12,27}

Participants

Thirty-two volunteers were assessed for eligibility. Seven volunteers did not meet the inclusion criteria, and 1 volunteer withdrew informed consent (Fig 3). Twenty-four healthy volunteers were included and randomized: 14 were female subjects and 10 were male subjects, and most of the participants were students. Their average age was 26.9 $\pm$ 5.5 years.

Adverse events

The vaccine was well tolerated. No serious adverse events were reported, and no subject had to interrupt the study or change the dose of the study drug. Of the other 55 adverse events reported, only 3 were considered possibly

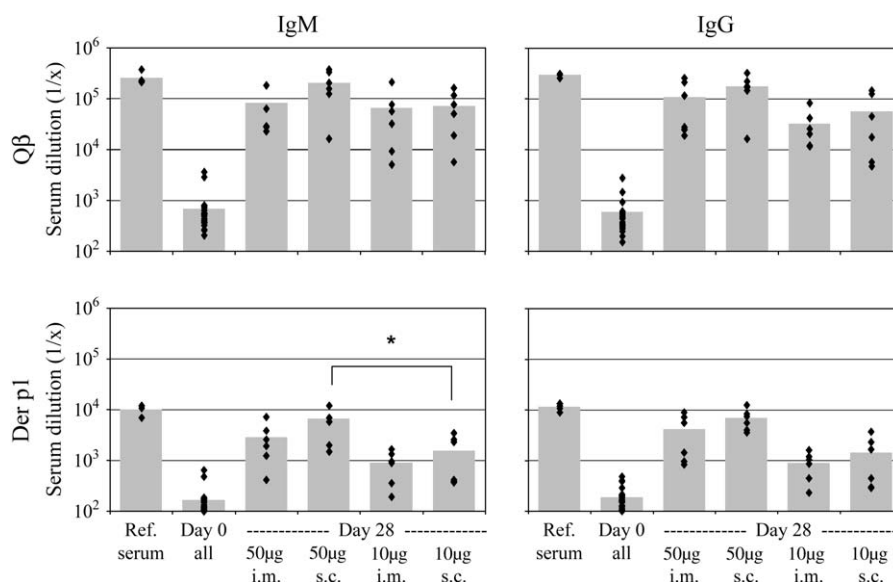


FIG 4. ELISA determination of IgM and IgG titers against Q β and Der p 1: individual end-point titers. Each filled symbol represents the average of 2 measurements. The gray bar represents the average end-point titer of each treatment group. As reference (Ref.) serum, a pool of sera from high responders was used. Routes of administration: *i.m.*, intramuscular; *s.c.*, subcutaneous.

related to the study drug. These adverse events included a mild vagovasal reaction, more probably because of blood sampling; a mild abdominal skin rash after jogging; and a mild subclavicular lymph node swelling. All 3 events resolved spontaneously. None of the events required termination of the study.

Local reactions

Injection site examinations were performed 10 minutes, 30 minutes, and 1 day after injection, as well as 1 month (day 120), 5 months (day 240), and 9 months (day 360) after the last injection. The highest number of abnormal findings was observed on the day after the injection, where after the 72 injections, slight pain was observed in 26 subjects, erythema was observed in 16 subjects, and swelling was observed in 9 subjects. Local reactions were mild and transient, recovering spontaneously mostly within 2 days. There was a trend toward a higher incidence of erythema, swelling, and/or pain on palpation for the subcutaneous application route.

Systemic reactions

None of the 24 participants showed signs of a systemic reaction. Statistical analysis of all recorded clinical or laboratory parameters revealed no consistent between-group or time-dependent variations. The following parameters were recorded at the screening visit and at days 0, 1, 30, 31, 90, 91, 120, 240, and 360: medical history and physical examination, including electrocardiography and spirometry, clinical chemistry panel, hematology panel, and urinalysis. Vital signs, including weight, blood pressure, heart rate, body temperature, and respiration rate, remained stable throughout the study without major changes.

Antibody responses against Q β

Antibody titers were determined on days 0, 30, 90, 120, 240, 360, and 540. Already at day 30 (ie, after one single administration of Q β -Der p 1), all volunteers responded with specific antibodies against Q β . Not only high titers of specific IgM (average of groups ranging from 1:80,000 to 1:200,000; Fig 4) but also specific IgG titers were measurable (average of groups ranging from 1:30,000 to 1:170,000; Fig 5). A dose-response effect was evident, with the 50- μ g doses inducing significantly higher titers than the 10- μ g doses ($P < .05$ for both IgM and IgG titers).

Antibody responses against Der p 1

Antibody titers against the covalently coupled antigen Der p 1 were also analyzed by means of ELISA. At day 30 (ie, after a single injection of Q β -Der p 1), all subjects in the 50- μ g groups showed increased IgM specific for Der p 1 (Fig 4). Also, within 30 days after a single injection, all subjects generated Der p 1-specific IgG. Again, a dose-response effect was observed. Volunteers receiving 50 μ g subcutaneously had average IgG titers of 1:6900 (± 1333 SEM), and those receiving 50 μ g intramuscularly had IgG titers of 1:4172 (± 1452). Volunteers receiving 10 μ g subcutaneously generated IgG titers of 1:1468 (± 564), and they generated IgG titers of 1:897 (± 205) if injected intramuscularly. These IgG differences were statistically significant (50 μ g intramuscularly vs 10 μ g intramuscularly, $P = .05$; 50 μ g subcutaneously vs 10 μ g subcutaneously, $P = .004$; Student *t* test). Two volunteers in the low-dose groups responded with titers only marginally greater than those measured in preimmune sera. As already observed with antibody titers against Q β , the route of immunization had no significant effect on the antibody response.

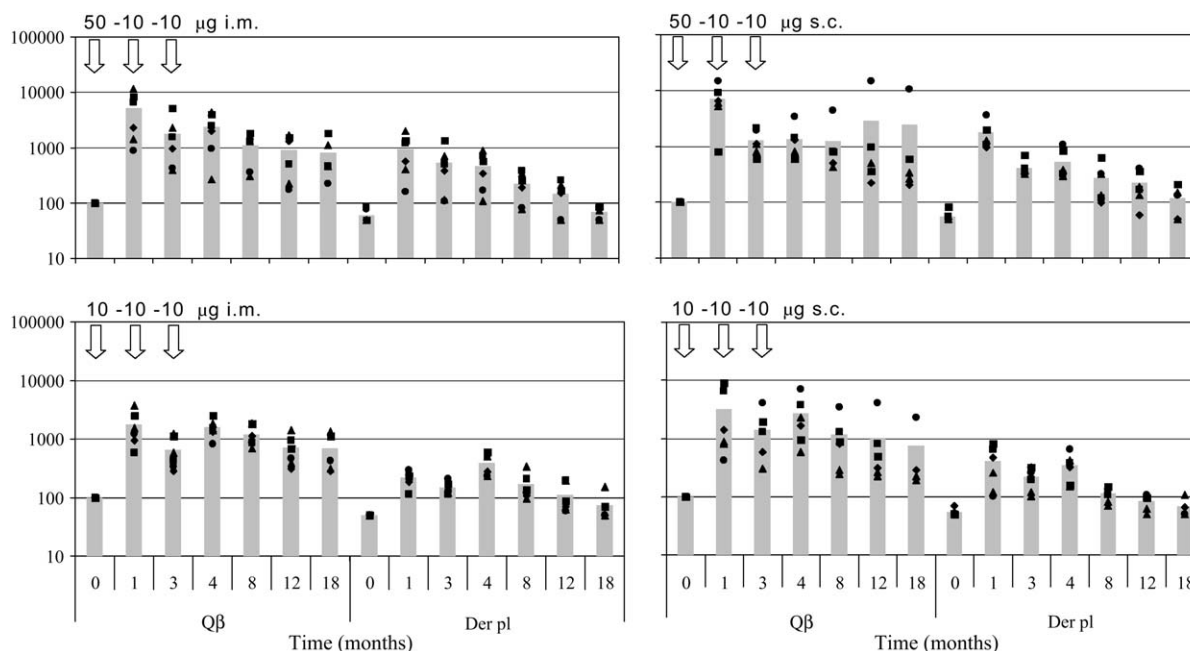


FIG 5. Duration of IgG titers against Q β and Der p 1: individual titers of half-maximal absorbance. Each *filled symbol* represents the average of 2 measurements. The *gray bar* represents the average end-point titer of each treatment group. Each panel represents 1 of the 4 treatment groups. Routes of administration: *i.m.*, intramuscular; *s.c.*, subcutaneous.

Subclass distribution of antibody response

The subclasses of antibodies specific for both Q β -Der p 1 (not shown) and Der p 1 (Fig 6) were determined on day 30. The dominant anti-Q β and anti-Der p 1 subclasses were IgG1 and IgG3, whereas IgG2 and IgG4 values were low or negligible. Moreover, no specific IgE was detectable in any of the samples (not shown).

Longevity of antibody response

Longitudinal analysis of the IgG antibody titers against Q β and Der p 1 showed a similar kinetics of the response (Fig 5). The highest IgG titers were found on day 30 in all groups. The boosting immunizations on days 30 and 90 apparently did not further enhance the IgG titers. When looking at the decrease in antibody titers between day 120 and day 360, titers decreased by 75%.

DISCUSSION

It has been found in mice that the presentation of an antigen in a highly ordered and repetitive array normally provokes strong antibody responses, whereas the same antigen presented as a monomer is nonimmunogenic.^{9-12,14} In support of these data, clinical trials in human subjects have demonstrated that viral proteins that self-assemble to VLPs (ie, capsid-like structures) potentially induce antibody responses in the absence of adjuvants.²²⁻²⁵

However, the vast majority of proteins do not spontaneously assemble and are poorly immunogenic if not administered with an adjuvant. In this study we chose

Der p 1 as a model protein that does not assemble spontaneously. However, we conjugated a peptide sequence of the allergen Der p 1 to a highly repetitive structure by covalently linking it to a carrier protein that forms a VLP. We previously demonstrated that such repetitive alignment of an antigen on a VLP enhances immunogenicity in mice.²⁷ This is the first human use of this novel class of vaccines. With this vaccine, Q β -Der p 1, administered in the absence of adjuvant, we observed rapid induction of IgM and IgG responses to both Der p 1 and Q β in all volunteers. These results demonstrate that antigen repetitiveness drives B-cell responses in human subjects and mice in a similar way. Furthermore, we demonstrate that VLPs have the potential to serve as carriers for the antigen of choice to induce strong antibody responses in human subjects.

The key parameters for any successful vaccine are as follows: rapid induction of high antibody titers in the majority of subjects and in the absence of adjuvants and major side effects. All parameters seem to be fulfilled by the Q β -Der p 1 vaccine. First, the vaccine was administered without adjuvant and was well tolerated with only mild local reactions in a few subjects. This is in line with the previous clinical studies with VLP vaccines. All these studies have thus far reported good safety and tolerability.²²⁻²⁵

Second, the response to the vaccine was rapid. Within 4 weeks, all subjects responded with significant antibody titers. Surprisingly, marked IgG titers were already present in the first serum samples drawn after 4 weeks. Because IgM responses peak considerably earlier than IgG

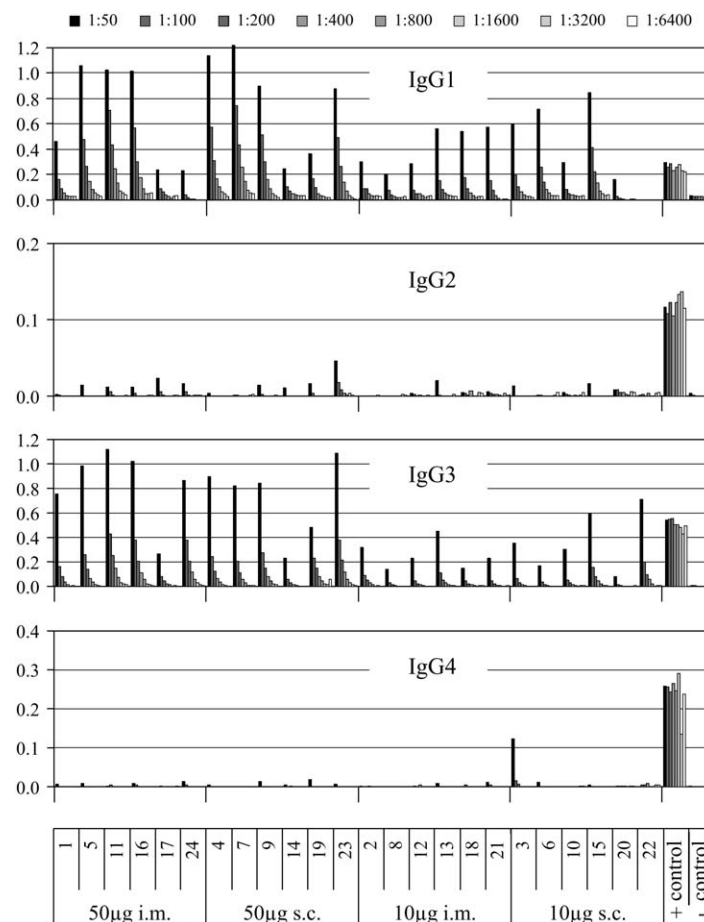


FIG 6. IgG subtype composition of the anti-Der p 1 immune responses. Individual sera from each treatment group have been tested in ELISA for reactivity with the peptide Der p 1 and developed with IgG subtype-specific secondary antibodies. The titers are given as optical density at the indicated serum dilution. Routes of administration: *i.m.*, intramuscular; *s.c.*, subcutaneous.

responses, it must be assumed that the maximum IgM response was achieved before this first measurement, probably within the first 2 weeks. Such rapid induction of IgM against antigens in a highly repetitive form is also observed in mice, in which IgM production against many repetitive antigens is independent of T help.¹⁴

Third, high titers of specific anti-Der p 1 and anti-Q β antibodies were induced. Already after 4 weeks, the IgG titers induced by Q β -Der p 1 conjugate were higher than those published in the literature. Although subcutaneous immunization with 50 μ g (containing approximately 8 μ g of Der p 1 peptide) induced an approximately 30-fold increase in IgG within 4 weeks, the published increases in IgG titers range from 2- to 10-fold after 14 weeks to 18 months of SIT with house dust mite allergen preparations containing Der p 1 within a similar dose range.²⁸⁻³¹ Finally, a high responder rate was achieved. Indeed, all 24 individuals produced antibody responses to the target antigen Der p 1 and the VLP carrier.

In this study the antibody response against the carrier Q β is consistently higher than the response against Der p 1. We have previously found in mice that the

density of peptide antigens on VLPs has an effect on the ratio of the anti-Q β versus the anti-peptide response. The denser the peptide, the more of the Q β surface is covered and the lower the Q β -specific response. Correspondingly, the anti-peptide response increased (unpublished data).¹² Furthermore, the VLP Q β is highly stable and likely more protease resistant *in vivo* than the coupled Der p 1 peptide. Thus Q β -specific B cells might be stimulated for longer time periods than Der p 1 peptide-specific cells, explaining the higher immunogenicity of the former antigen.

Der p 1-specific antibody titers reach background values earlier than Q β -specific antibody titers. Whether this is due to an intrinsically more short-lived anti-Der p 1 response or might simply be explained by the fact that anti-Der p 1 responses were lower to begin with is subject to further investigation.

Successful immunotherapy is associated with a significant IgG4 response.^{32,33} The subclass distribution after vaccination with Q β -Der p 1, however, was mainly IgG1 and IgG3, which are the prevailing subclasses induced by bacterial and viral proteins. IgG4 production is

thought to reflect repeated long-term antigenic stimulation with T cell-dependent antigens.³⁴ Also, in the antibody response to conventional immunotherapy, initially mainly IgG1 is formed, whereas IgG4 becomes more prominent after 1 to 2 years. It might therefore not be surprising that 3 immunizations with VLPs did not induce IgG4. However, the fact that there is a correlation of long-term conventional immunotherapy with IgG4 production does not rule out that IgG1 and IgG3 could also ameliorate allergic symptoms. This remains to be seen in a phase II study. Most importantly, however, Q β -Der p 1 did not induce any measurable IgE.

The presented method therefore appears ideally suited to further expand the growing repertoire of allergen immunotherapy.³⁵⁻³⁷ The observed extremely rapid induction of the antibody response could shorten the duration of immunotherapy. Also, this class of vaccines is ideally suited for postexposure vaccinations or for other vaccinations in which a rapid response is key (eg, in case of threat by biologic weapons, such as anthrax and ricin). The vaccination technology presented here might therefore be ideal for the development of such vaccines.

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