



Modifications increasing the efficacy of recombinant vaccines; marked increase in antibody titers with moderately repetitive variants of a therapeutic allergy vaccine

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Abstract

Development of vaccines targeting important self-molecules like tumor antigens, IgE, cytokines or other regulatory molecules, brings about challenges that are not met in classical vaccine development. Tolerance inducing mechanisms reduce the levels of therapeutic antibodies in the vaccinated subject, and anti-self antibody titers are frequently more than 50-fold lower than the anti-non-self response to the carrier. In order to overcome this limitation in efficacy we have explored various methods to enhance the immunogenicity of the vaccine antigen. Vaccination with a molecule containing two IgE C ϵ 3 domains and thereby a low level of repetitiveness markedly increased the efficacy. The anti-IgE antibody titers in the animals treated with the dimeric vaccine antigen were 4.5, 5 and 8 times higher than in the animals treated with the monomer, in three independent experiments. In addition, this increase in efficacy was not masked by the use of potent adjuvants. The effect persisted even in the presence of Freund's or Montanide ISA 51, two mineral oil based adjuvants. This in contrast to most Toll-like receptor (TLR) agonists, which appear to enhance the immune response only when administered together with weak adjuvants. This clearly shows that the introduction of a moderately repetitive structure is enough to substantially increase the efficacy of a therapeutic vaccine.

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1. Introduction

Vaccines are classically based on crude preparations of attenuated or killed viruses and bacteria. However, safety considerations have made it difficult to launch new vaccines based on such uncharacterized and complex antigens. Many new vaccines are instead based on subunits or modified recombinant antigens of very high purity. These antigens are however, usually much less potent in inducing strong immune responses. In new areas of vaccine development, like therapeutic vaccines this phenomenon is even more pronounced. Most presently studied therapeutic vaccines target various self-molecules, and immune responses against self-antigens

are normally heavily suppressed by tolerance inducing mechanisms. We have previously observed more than 50-fold lower antibody titers to the self-region of the antigen, the IgE C ϵ 3 domain, compared to the response against the foreign carrier epitopes [1–3]. In order to achieve good therapeutic effects with vaccines against self-molecules it is therefore of major interest to enhance the immunogenicity of these vaccines. One possibility is to use characteristic features of viral and bacterial antigens.

What are the reasons for the superior efficacy of crude virus or bacterial vaccine antigens? One important factor is probably that these preparations often contain one or several molecules interacting with pattern recognition receptors of the Toll-like receptor family (TLR) [4]. Activation of the TLRs then provides the danger signals needed to trigger innate immune mechanisms. This is in turn a prerequisite for efficient induction of adaptive immunity. Examples

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of molecules that bind and activate TLRs are, bacterial lipopolysaccharides, different fungal or bacterial carbohydrates and lipids, double stranded or single stranded RNA and DNA fragments containing unmethylated CpG motifs [4,5]. Such molecules can be used as additives to the vaccine formulation to activate the immune system. Large efforts are presently being made in this area to find formulations that are safe and still have good effect [4,5].

The use of potent adjuvants may in itself, however, not be sufficient to obtain good clinical effect for therapeutic vaccines. We have therefore studied additional differences between recombinant proteins and whole bacteria or viruses to see if they can be used as immuno stimulators in future therapeutic vaccines. One example of such a difference is the fact that, viral and bacterial antigens frequently are present in a highly repetitive manner. Repetitive epitopes on the surface of both bacteria and viruses have been observed to effectively stimulate B cells [6]. The immune response to many antigens has also been shown to increase markedly by covalent coupling to virus coat protein [6,7]. However, fusion proteins between a target antigen and a coat protein of a virus or phage impose certain very important restrictions. For example, only small peptides or small stable domains can normally be coupled to the surface of a virus due to folding constraints [8]. However, more complex structures and larger antigenic regions are required for many applications.

We have encountered this problem during the development of a therapeutic vaccine against one of major challenges of modern medicine, IgE-mediated allergies [2,9]. The target region of the allergy vaccine antigen is the Cε3 domain of IgE, and this domain is particularly difficult to produce as a recombinant protein. Due to its size and complexity, this protein cannot be produced as a fusion with a viral coat protein. We therefore tested the possibility of using a low level of repetitiveness as a way to enhance immunogenicity. The results from a study of the stimulatory effects of this dimeric variant of the hybrid IgE like molecule are here presented. An increase in the anti-IgE titer by 4–8 times compared to a monomeric antigen was observed in three independent experiments. This clearly shows that the introduction of a moderately repetitive structure is enough to substantially increase the efficacy of a therapeutic vaccine.

2. Materials and methods

2.1. DNA constructs

The following DNA constructs were made from cDNA clones by using PCR amplification and subsequent subcloning (Fig. 1A). *ORO*: a hybrid IgE like molecule containing the C2, C3 and C4 domains of IgE. The C2 and C4 domains were derived from opossum IgE ε-chain [10] and the C3 domain of rat ε-heavy chain [11]. For a more detailed description of the construct and primers see Ref. [2]. *ORORO*: a construct encoding a duplicated C2C3 region from the *ORO*

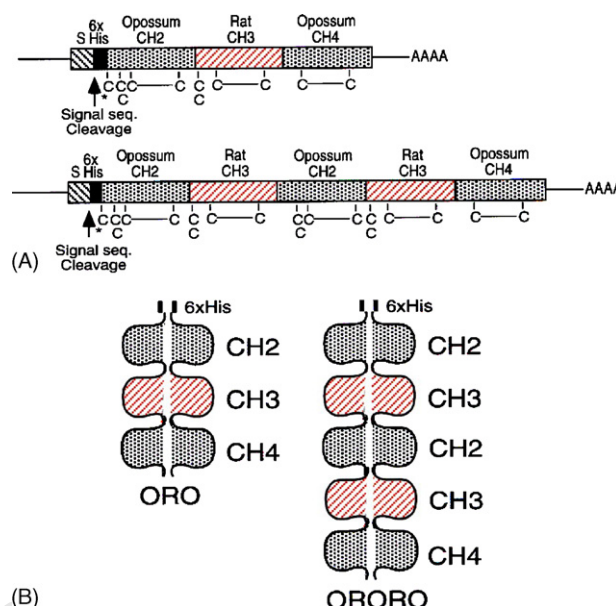


Fig. 1. Schematic drawings of vector constructs and the dimerized fusion proteins *ORO* and *ORORO*. Panel A shows the vector constructs of the two fusion proteins. Individual domains, cysteine bridges, the N-terminal signal sequence and the six histidine region are indicated. The signal sequence cleavage site is also indicated. Panel B shows a schematic drawing of the resulting fusion proteins in their dimeric cystine-bridged form.

vaccine antigen followed by the opossum C4 domain. The original cDNA clones encoding the ε-heavy chain of opossum (*Monodelphis domestica*) and rat (*Rattus norvegicus*) IgE were derived from total spleen cDNA libraries [10,11].

For purification purposes, the 5' primers directed against the N-terminal region of the opossum C2 domains (oligo 394-5' OpC2) both contained an 18 nucleotide (nt) region encoding a histidine hexapeptide downstream of an *EcoRI* site. In addition, the native sequence was altered at one base (*A) in both 5' C2 primers in order to avoid an unpaired cysteine residue and the formation of unwanted interchain cysteine bridges [2].

All PCR amplifications were carried out under the following conditions: 0.1 μg template DNA, 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 0.25 mM of each of the four nucleotide triphosphates, approximately 1.5 μg of each of the two oligonucleotides used to obtain the various fragments, and 1 unit of Taq DNA polymerase (Promega, WI, USA). The reactions were initiated with a 2 min incubation at 90 °C followed by 35 cycles of 30 s at 94 °C, 60 s at 60 °C and 90 s at 72 °C in a MJ Research mini cycler (MJ Research, Inc., MC, USA).

2.2. Cloning and expression

PCR products were purified from agarose gels and cloned into the expression vector pCEP-Pu2. Nucleotide sequences were confirmed using the dideoxy chain-termination method.

The pCEP-Pu2 vector is a modified version of the pCEP4 vector (10.4 kb, Invitrogen, Netherlands). The major modifi-

cations, including replacement of the hygromycin resistance gene with the gene for puromycin resistance to allow for puromycin selection in mammalian cells, and insertion of a 105 bp region containing some of the 5'UTR and the signal peptide sequence region of human BM40, were done by Kohfeldt and coworkers and resulted in the vector pCEP-Pu/BM40s [2,12,13]. We modified this vector further. An *EcoRI* site positioned upstream of the ampicillin-resistance gene was removed by fill-in treatment and religation. In addition, the polylinker was extended to include an *EcoRI* and a *NotI* site between the *NheI* and *XhoI* sites. The resulting expression vector was named pCEP-Pu2.

Human Embryonic Kidney Cells, 293-EBNA (Invitrogen, Netherlands), were cultured to about 70% confluency in 9 cm² wells, washed with neutral PBS and serum-free Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Life Technologies), respectively, and transfected as follows. About 15 µg DNA in 50 µl sterile TE (10 mM Tris, pH 7.5, 1 mM EDTA) and 40 µl Lipofectamine (Gibco BRL) were each diluted in serum-free DMEM to 800 µl. Subsequently, the DNA and Lipofectamine preparations were combined, mixed by vigorous vortexing and incubated for 45 min at room temperature. The DNA/Lipofectamine mixtures were then diluted to 6 ml with serum-free DMEM and added to the washed cells. After 12–16 h, the medium was supplemented with FBS (Gibco BRL) to a final concentration of 10%. The cultures were split 1:4 the following day and 1–2 days later selection was initiated by the addition of 0.5 µg/ml puromycin (P8833) (Sigma Chemical Co., MO, USA) to the culture medium: DMEM supplemented with 2 mM L-glutamine (Gibco BRL), 50 mg/ml gentamicin (Gibco BRL), 5% FBS and 0.25 mg/ml G418 Sulphate (Geneticin) (Calbiochem, CA, USA). Growing cells were expanded and conditioned media were collected every 4–5 days. Cells and debris were removed by centrifugation for 10 min at about 1200 × g.

2.3. Protein purification

Ni-NTA Agarose slurry (QIAGEN GmbH, Germany) containing about 0.5 ml beads in 20% ethanol (EtOH) was added per 500 ml of conditioned media. Following overnight incubation at 4 °C on a shaker, the Ni-NTA Agarose was pelleted by centrifugation for 5 min at about 500 × g and transferred to columns. The beds were washed with PBS, pH 7.0, in the presence of 1 M NaCl and 0.1% Tween-20 and eluted with 100 mM imidazole (Merck, Germany) in 20 mM Tris, pH 8.0, with 0.1 M NaCl, according to the manufacturer's protocol. Protein-containing fractions were pooled and dialyzed against PBS, pH 7.0, using a membrane with a molecular weight cut-off of 12,000–14,000 Da (Spectra/Por® Membranes, Spectrum Medical Industries, Inc., CA, USA). In order to reduce the volume required for immunization, some of the samples were concentrated using Minicon-B15 microconcentrators (Amicon, Inc., MA, USA). Final protein concentrations were estimated both by comparison with a

BSA standard curve on SDS-PAGE and by Bradford (BIO-RAD Protein Assay, Bio-Rad Laboratories, CA, USA). A Rainbow protein molecular weight marker (Amersham International plc, England) was used for size estimation.

2.4. Immunization of animals

Separate groups of animals were vaccinated with ORO or ORORO. An initial s.c. injection of 100 µg ORO or ORORO in 100 µl neutral PBS mixed 50:50 with FCA (Difco Laboratories, MI, USA) or with Montanide ISA 51 (Seppic, Paris, France) was administered to Wistar rats. The week of the initial vaccination was designated week 0. The rats received booster vaccinations with 100 µg ORO or ORORO in 100 µl PBS, mixed 50:50 with IFA or Montanide ISA 51 in week 3 of the experiment. Blood samples were taken at weeks 0 and 5. The blood was allowed to coagulate over night and the serum was collected. The resulting serum was stored frozen at –20 °C until further processing.

2.5. Anti-rat IgE ELISA

Plates, Nunc-Immuno™ Plate MaxiSorp™ Surface (NUNC Brand Products, Denmark), were coated with 5 µg/ml of rat IgE derived from the IR 2 immunocytoma (a kind gift from Prof. H. Bennich, Tucson, Arizona and Dr. T. Karlsson, Pharmacia Diagnostics, Uppsala, Sweden) in carbonate buffer, pH 9.6. The 1:5 serial dilutions (1:5, 1:25, 1:125, 1:625, 1:3125, 1:15625) of the samples were prepared in horse sera (SVANOCONE, SVA, Håttanaholm, Sweden) and horse serum was used as assay blank. In order to correlate the values, serial dilutions of two of the samples were assayed on every plate. Equal amounts of biotinylated mouse anti-rat IgG₁/IgG_{2a} and anti-rat IgG_{2b} (G28-5, RG7/11.1, PharMingen, CA, USA) served as the secondary antibody. Alkaline-phosphatase-labeled streptavidin (DAKO, Denmark) was added and *p*-nitrophenyl phosphate disodium tablets (Sigma 104 phosphatase substrate tablets, Sigma diagnostics, Inc., St. Louis, USA) in diethanolamine buffer, pH 9.8, served as substrate. The absorbance at 405 nm (*A*₄₀₅) was read in a multiscan photometer (Labsystems Multiskan® MCC/340).

3. Results

3.1. Design of new vector constructs and the production of vaccine antigens

Many mammalian proteins or protein domains are very difficult to produce in soluble and properly folded form in other expression systems than in mammalian cells. We have experienced a particularly difficult example of this phenomenon during the development of an allergy vaccine targeting IgE [2,9]. The early prototype vaccine antigens consisted of a fusion protein between the C2C3 domains of rat or human IgE and a parasite or bacterial protein produced in a bacterial

expression system. The majority of these fusion proteins were found to form insoluble inclusion bodies or not to fold properly. *Pichia pastoris* expression systems were also tested but no recombinant protein could be detected in the conditioned media. The lack of secreted protein indicated aberrant folding and lysosomal degradation, a phenomenon commonly observed when expressing immunoglobulin-like domains in fungal expression systems.

The difficulties in expressing the IgE Cε3 domain in bacterial expression systems is most likely caused by the lack of a carbohydrate structure, which apparently is needed for proper folding. This carbohydrate addition site is conserved among all presently characterized IgE and IgG molecules, from the egg-laying monotremes to placental mammals [14]. The folding of this domain probably also needs the help from several chaperons. The lack of certain such chaperones is probably the primary reason why these proteins are difficult to produce in fungal expression systems.

In order to solve the problem with solubility and folding, we looked into alternative expression systems and other potential fusion partners. We decided to try to produce the target region, the IgE Cε3 domain, as a fusion protein with the Cε2 and Cε4 domains in the form of a chimeric IgE molecule. The Cε3 domain was derived from the IgE gene of the species to be vaccinated, here rat, dog or human and the carrier domains originate from a non-placental mammal. The flanking domains were aimed to function both as structural support for the Cε3 domain and as a carrier providing T cell help. For initial tests in an animal model we produced a fusion protein consisting of the Cε2 and Cε4 domains of the American Opossum (*M. domestica*), a marsupial, and the Cε3 domain of the domestic rat [2]. When being expressed in a mammalian expression system this molecule, which was designated ORO, folded properly and was exported from the producer cell line as a disulphide-linked dimer (Figs. 1 and 2) [2].

To study the immuno stimulatory effect of repeated epitopes, a chimeric IgE molecule with two target domains was produced. This new vaccine antigen contains two rat C3 domains and two C2 domains from opossum IgE. This recombinant protein, named ORORO thus holds five Ig domains (C2-C3-C2-C3-C4) (Fig. 1A). The ORO and the ORORO vaccine antigens were expressed in 293-EBNA human embryonic kidney cells in an average yield of about 2 and 0.5 mg protein per liter of conditioned media, respectively. SDS-PAGE analysis under reducing conditions revealed that ORO and ORORO migrate as proteins with molecular weights of ~48 and 85 kDa, respectively (Fig. 2). However, under non-reducing conditions, most of the protein migrates at ~100 and 230 kDa, respectively, suggesting that recombinant ORO and ORORO are both produced as a disulphide-linked homodimers (Fig. 2).

We also produced vector constructs for two additional variants of the vaccine antigens with three and four C3 domains, respectively (ORRORO and ORRRORO). However, it was difficult to obtain sufficient protein quantities

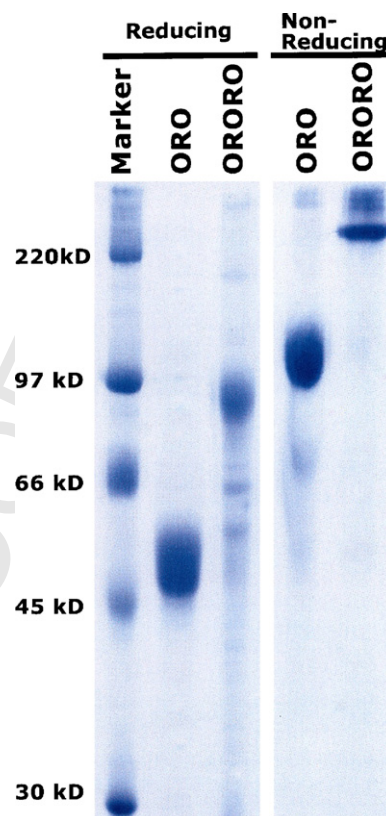


Fig. 2. SDS-PAGE analysis of Ni column purified ORO and ORORO under reducing and non-reducing conditions.

in sufficiently high purity to analyze the effect of these multimeric variants in an animal model. These proteins were therefore not further analyzed.

3.2. Effects of vaccination with ORO and ORORO

Groups of Wistar rats were injected with ORO or ORORO in Freund's complete adjuvant or Montanide 51 (Montanide 51 is a pure mineral oil based adjuvant). Three weeks after the first injection the animals were administered a booster dose in Freund's incomplete adjuvant or Montanide 51. Five weeks after initiation of the experiment serum samples were taken and analyzed for anti-IgE titers. Antibodies against native rat IgE were detected in the sera of all ORO- and ORORO vaccinated animals, and the most prominent anti-IgE antibody response was observed in the ORORO treated rats (Fig. 3). In three independent experiments with two different adjuvants (Freund's and Montanide ISA 51) the anti-IgE antibody titers, were 4.5, 5 and 8 times higher in the ORORO treated animals than in the ORO treated animals (Fig. 3). In each of the three independent experiments four rats were included in each group, ORO and ORORO, which in total was 12 animals per antigen variant. A statistical analysis using the Student's *T*-test of all 24 animals showed a highly statistical significant difference between the two groups of animals with a *p*-value of 0.00018 (***). When comparing all 24 animals the differ-

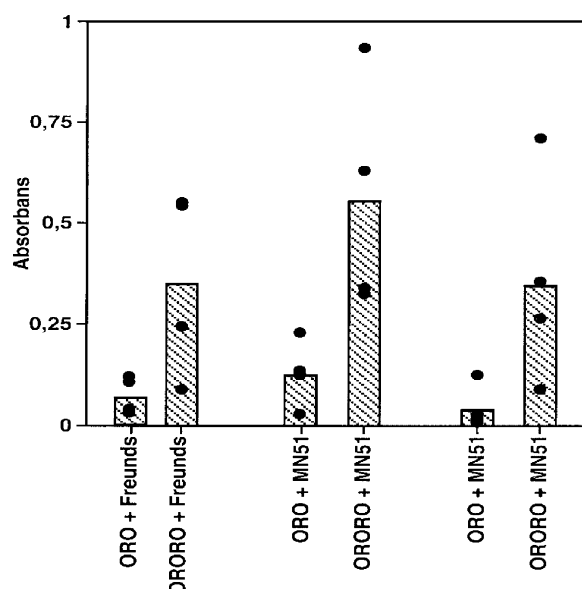


Fig. 3. Analysis of anti-IgE antibody levels in rats vaccinated with ORO or ORORO. Absorbance values of individual animals are indicated by black dots, and the mean value in each group of animals is shown by hatched bars. Three independent experiments are depicted separately, one using Freund's as adjuvant and two with Montanide ISA 51.

ence in titer of was found to be 5.3 between the two groups. The reason why we chose to do divide the study into three independent experiments, which were separated in time by several months and using three independent shipments of animals was to avoid errors coming from an individual shipment or a potential unrecognized infection during an experiment.

4. Discussion

Vaccines have one very important advantage over treatment strategies using pre-produced proteins directly targeting the specific organ or the target molecule in that a therapeutic effect can usually be achieved by the use of very low amounts of protein. For example, the amounts of recombinant protein required for the treatment with a therapeutic vaccine is estimated to 10,000–20,000 times lower than for the treatment with monoclonal antibodies [2]. This is one important reason why therapeutic vaccines most likely will become an interesting alternative to conventional therapies. The concept of therapeutic vaccines has also been boosted by the enormous number of potential patients in both human and veterinary medicine, and is being studied extensively in such important areas as birth control, cancer, allergy and autoimmunity [15]. Initial tests in various animal models have been very promising [2,9,16–19]. However, recent clinical trials have not met the high expectations from the field. A recent clinical trial with an anti-TNF- α vaccine induced no detectable antibody titers against native TNF- α [20]. A recent study with the tumor antigen HER-2 has also been rather discouraging. Only 6 out of 10 patients showed detectable

anti-HER-2 antibody titers. In addition, the anti-HER-2 antibody levels in two of these six patients were barely detectable (Pharmexa, www.pharmexa.com). In both of these studies, the antigen was administered together with Alum, a relatively weak adjuvant. These studies therefore implicate that more potent adjuvants and other modifications increasing the efficacy of a vaccine antigen are needed in order to obtain good clinical affect.

The development of therapeutic vaccines is a demanding task where numerous important questions that have to be addressed. The vaccine antigen has to be produced in a soluble and properly folded form to elicit an immune response that is also directed against the native target protein. Purification schemes with sufficient yield have to be developed for the product to be within reasonable price range. Animal models that can give information concerning the potential efficacy of the different vaccines have to be available. Although these steps in the development process are often both cost and time consuming, most of these issues can normally be solved. However, an additional critical issue in the development of vaccines against self-antigens is the strong tolerizing effect of the self-antigen on the B cell response, which may result in poor vaccine efficacy.

Many factors can influence the development of tolerance. However, for therapeutic vaccines the level of the particular self-protein appears to be the critical factor. High levels of the self-protein appear to suppress the antibody response very efficiently at the B cell level [2,9,21,22]. In contrast, the T cell tolerance is to a large extent circumvented by the covalent coupling of the self-antigen to a foreign carrier protein, as exemplified by bacterial proteins or the opossum C2 and C4 domains of the hybrid IgE molecule used in this study [2,9]. These carrier regions contain multiple non-tolerized T cell epitopes, which leads to an efficient recruitment and activation of T cells directed against the vaccine antigen. However, the B cell responses are down regulated by binding of self-antigen in the absence of costimulatory signals like CD40–CD40 ligand interactions and cytokines [22,23]. In order to overcome the limited B responses strong adjuvants or structural modifications of the antigen that increase the antigenicity are probably necessary. We may even have to use both strategies simultaneously, in particular with target self-proteins that are present in relatively high concentrations in vivo [3].

In our previous work, we have studied the effect of different adjuvants on anti-IgE levels and anti-carrier antibody levels to optimize the adjuvant for therapeutic vaccines. Almost all tested adjuvants were shown to induce high antibody titers against the non-self regions, the carrier. Of the five adjuvants tested, i.e. Freund's, Montanide 51, Montanide 720, Alum and Iscoms, only one, Alum, yielded significantly lower titers than the other adjuvants [1]. Alum is the most commonly used adjuvant in humans. However, this adjuvant is known to have relatively weak immuno stimulatory properties and to show a clear TH2 profile [1,24]. Alum has recently also been shown to induce contact hypersensitivity to aluminum in a

high percent of the patients (77%) and this adjuvant remains at the injection site for several months to years, making it a less favorable alternative in therapeutic vaccines [25].

We also analyzed the antibody titers elicited against the self-component. In contrast to the anti-carrier responses the responses against the self-component varied markedly between adjuvants. The only adjuvants that did induce a strong anti-self response were the mineral oil based adjuvants, Freund's and Montanide 51. This clearly indicates that there is a large difference in the effect of adjuvants in inducing anti-self and anti-non-self responses.

Thus, mineral oil-based adjuvants appeared to be the most promising alternative. However, mineral oils also confer a substantial risk of adverse side effects and there are serious doubts concerning their future use in vaccine development. One very interesting alternative was identified in an analysis of biodegradable adjuvants in combination with various Toll-like receptor agonists. Montanide 720 is a squalene-based adjuvant with relatively low activity when administered without additional immuno stimulatory substances. However, when administered together with the lipid A portion of bacterial lipopolysaccharides this adjuvant turned out to induce anti-IgE levels almost at 60% of the titers induced by Freund's and Montanide 51 [1]. Surprisingly, the Toll-like receptor agonists did not augment anti-IgE levels when added to the strong mineral oil-based adjuvants [1]. Thus, the effect of the TLR agonists was primarily seen with weak or moderately potent adjuvants.

It was an extremely encouraging finding that a combination of TLR agonists and biodegradable adjuvants like Montanide 720 gave almost as good effect as with the strongest known adjuvants, the mineral oil based adjuvants. However, for many therapeutic vaccines this may not be sufficient, due to high in vivo levels of the target antigen. In order to obtain a vaccine that have a good therapeutic effect we may need to enhance the immunogenicity even further. A very promising complement is here repetitive epitopes. For example, the use of empty virus particles is a powerful way to increase the immunogenicity of small peptides or small protein domains. However, for many therapeutic vaccines this is not an alternative due to folding constraints. The study presented here introduces another promising alternative. The results are very encouraging as an only moderately repetitive variant, the dimer, increased the efficacy of the vaccine by 4–8-fold. It should be noted that the same amount of antigen was used for both groups and the experiment was not performed under equimolar conditions. An even larger difference between the two groups of animals may have been observed if equimolar conditions had been used. One additional important finding was that this enhancing effect persisted with strong adjuvants. The effect was seen in the presence of two mineral oil based adjuvants, Freund's and Montanide 51. This indicates that the effect most certainly also will remain after optimizing the adjuvant formulation. The enhancing effect of multimeric variants of vaccine antigens has previously also been observed in experiments with recombinant aller-

gens [26,27]. Interestingly, hybrid allergens with epitopes from two or several allergens seems also to have a similar effect [28–30].

In summary, the use of detoxified TLR agonists as immuno stimulators in vaccine formulations will most likely substantially increase the chances of obtaining good clinical effects in future clinical trials. In addition moderately or highly repetitive variants of vaccine antigens can also further increase vaccination efficacies. With these two new alternatives to enhance immunogenicity at hand chances have increased dramatically that many important therapeutic vaccines not only remain in the development stage but also reach the clinic.

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