

Identification of adjuvants that enhance the therapeutic antibody response to host IgE

Jeannette Johansson^a, Anna Ledin^a, Molly Vernerström^a,
Karin Lövgren-Bengtsson^b, Lars Hellman^{a,*}

^a Department of Cell and Molecular Biology, Biomedical Center, Box 596, Uppsala University, S-751 24 Uppsala, Sweden

^b Isconova AB, Uppsala, Sweden

Received 15 July 2003; accepted 24 December 2003

Available online 25 February 2004

Abstract

In the development of a novel vaccine against atopic allergies, we have screened for adjuvants that enhance the therapeutic antibody response against self immunoglobulin E (IgE). The response against self IgE is induced by administration of a vaccine antigen, which contains both self and non-self IgE regions, together with an adjuvant. We evaluated five commonly used adjuvants; Freund's, aluminium hydroxide, ISCOMs, Montanide ISA 51 and Montanide ISA 720, and found that the mineral oil-based adjuvants; Montanide ISA 51 and Freund's induced at least 5–10-fold higher anti-self IgE titers than any of the other candidates. However, with one exception, Alum, the immune responses against the carrier, i.e. the non-self regions, were similar for all adjuvants, indicating that the ability to induce responses against self and non-self antigens differ among adjuvants. The responses against non-self IgE were more than 50-fold higher than antibody responses against self IgE in both the Freund's and Montanide 51-administered animals, indicating that the response against self molecules is markedly inhibited by tolerance-inducing mechanisms. Co-administration of Montanide ISA 51 with immuno-stimulatory substances from bacteria; muramyl dipeptide (MDP), monophosphoryl-lipid A (MPL) or a formyl-methionine-containing tripeptide (fMLP), did not elevate the anti-self IgE response. Hence, adjuvants based on pure mineral oil without additional immuno-stimulatory substances appear to be the best adjuvant candidates in therapeutic vaccines aimed at regulating the in vivo levels of self-proteins.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Therapeutic vaccines; IgE; Allergy

1. Introduction

Ever since Jenner's first attempt in 1798, vaccination has been the preferred and also the most successful method of controlling most viral and many bacterial diseases. Successful vaccination programs have resulted in a tight control or even in the eradication of many of the major human infectious diseases. This has encouraged researchers in other fields to develop vaccines against other clinical conditions.

During the past few decades several diseases caused by malfunctions of our own immune system, such as the allergic and autoimmune diseases, have become some of the major challenges of modern day medicine. Allergic diseases have increased to epidemic proportions in the past 20–30

years and estimates suggest that 20–30% of the total population is affected. The dominant form is atopic allergies, or IgE-mediated allergies, which include hay fever, fur allergies, dust mite allergies, insect venom allergies, extrinsic asthma and many types of food allergies. One interesting question is whether vaccines can also be developed against this type of diseases. One form of vaccination, so called hyposensitization therapy or immunotherapy has been used to treat allergies since the beginning of the 20th century [1,2]. This is an allergen dependent treatment strategy, which involves the use of allergen extracts to treat patients by injection. However, hyposensitization therapy has been questioned due to the often low efficacy and occasionally severe side effects, and this strategy is also limited by the fact that different extracts have to be used for each individual form of allergy. New strategies to treat allergies are therefore presently being evaluated. Vaccines aimed at modulating the levels of various self-antigens is an interesting area and IgE and IL-5 are two promising target molecules.

Abbreviations: IgE, immunoglobulin E; OVA, ovalbumin; BSA, bovine serum albumin; i.p., intraperitoneal; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant

* Corresponding author. Tel.: +46-18-4714532; fax: +46-18-4714434.

E-mail address: lars.hellman@icm.uu.se (L. Hellman).

New and powerful molecular tools facilitate the production of recombinant proteins to be used as vaccine antigens. However, most of these highly purified antigens lack some of the characteristics of classical vaccine antigens, e.g. heat-killed or attenuated viruses and bacteria. The recombinant antigens are usually less potent in inducing strong immune responses and they therefore need to be used together with strong immuno-potentiating agents, so called adjuvants. In an attempt to develop a vaccine against atopic allergies by targeting IgE, the central molecule in this type of disease, we have encountered some of these challenges [3–6]. In addition, the induction of immune responses against self-proteins poses additional challenges not normally seen with entirely foreign antigens, like bacterial or viral proteins. Due to tolerance induction self-proteins are usually much less immunogenic. In order to address these questions we have screened a panel of different adjuvants for their potency in inducing an antibody response against self IgE. We have also compared the response against self IgE to the response with the foreign carrier molecule to obtain an estimate of the difference in antigenicity between self and non-self epitopes.

Agents that enhance the immune response to an antigen beyond the level achieved by the antigen alone are referred to as immunological adjuvants, reviewed in [7]. Freund's complete and incomplete adjuvants (FCA and FIA, respectively) are the most widely used adjuvants for studies in laboratory animals. FCA is a water-in-mineral oil emulsion containing heat-killed *Mycobacterium tuberculosis* while FIA contains only mineral oil and the emulsifier, a lipophilic surfactant named mannide monooleate. Freund's adjuvant, which was used as a standard in this analysis is known to be a very potent adjuvant. However, due to the low purity of the oil and the emulsifier, FCA is considered too reactogenic for use in humans [8]. Aluminum compounds are the only commonly used adjuvants for clinical use. However, for most antigens, aluminum hydroxide is a relatively poor adjuvant (reviewed in [9]).

Due to the need for novel safe and potent adjuvants for human and veterinarian use many additional adjuvants are under active evaluation [8,10,11]. Highly purified mineral oils are one interesting set of candidates. Several companies are producing various combinations of well-defined mineral oils and emulsifiers [8]. In addition, combinations of mineral and biodegradable oils, primarily squalene or plant oils, are currently under evaluation. Several of these adjuvants are already in clinical trials [8]. One additional active area of adjuvant research is the use of immuno-stimulating complexes (ISCs) [12], especially the development of small well-defined particles made up of cholesterol and saponins from the bark of a tropical tree, *Quillaja saponaria*. ISCs are presently used in several veterinary applications, for example a commercial horse influenza vaccine [13]. A recent interesting development is the incorporation of specific affinity tags in the ISCOM, which allows more efficient binding of recombinant proteins to the ISCOM surface, e.g. incorporation of Cu^{2+} chelating agents on the

ISCOM that interacts with poly-histidine tags on the protein [14].

Many bacterial components have been shown to have strong immuno-potentiating effects. However, most of the crude extracts used to date are also rather toxic. To solve these problems, considerable effort has been devoted to isolating and purifying the specific components, and to identify regions within these bacterial components, that exert the immuno-stimulatory effect. Several such compounds have been identified and these are now available for a more detailed analysis of their potency as adjuvants. One such compound is muramyl dipeptide (MDP) a component of the cell wall of bacteria [15]. It has been shown not only to enhance the potency of weak antigens, but also to increase resistance to bacterial, fungal and viral infections. A second highly interesting molecule is lipid A, a structural component of the lipopolysaccharide (LPS) located in the outermost of the double lipid bilayers that constitute the cell membrane of Gram-negative bacteria. It has been shown that the toxic and adjuvant properties of LPS is due to the lipid A portion. By the removal of a phosphate group from a sugar moiety, Ribi et al. [16] managed to decrease the toxicity 100- to 1000-fold. However, the immune stimulatory properties of the molecule were maintained in the molecule. Baldrige and Crane [17] reduced the toxicity further by the removal of an ester-linked fatty acid group. A third class of molecules of interest is formyl-methionin-containing tripeptides (fMLP), a biproduct of bacterial protein synthesis that has also been shown to have immuno-stimulatory effects probably due mainly to its chemotactic activity on several immune cells.

As a further step in the development of novel strategies to treat atopic allergies in man, we present here an analysis of the capacity of these immuno-stimulatory substances to induce both an anti-self IgE and an anti-non-self IgE response in an animal model, the Wistar rat.

2. Materials and methods

2.1. The active vaccine component, ORO

The active component in the vaccine, ORO (1041 bp encoding the C3 domain of rat ϵ -heavy chain [18] flanked by the C2 and C4 domains of the opossum ϵ -heavy chain [19] (Fig. 1)) was expressed in 293-EBNA cells and purified on Ni-NTA Agarose (QIAGEN GmbH, Germany) as described previously [6]. The ORO component was prepared to a concentration of 1.5 mg/ml in PBS, pH 7.0.

2.2. Study 1

Twenty, 8–10-week-old female Wistar rats (Benton and Kingman, Sollentuna, Sweden) were sensitised to ovalbumin (OVA) as follows: the animals received an initial intraperitoneal (i.p.) injection of 10 μg OVA (A-5503 Sigma, MO, USA) in PBS, pH 7.0, followed by i.p. injections of 3 μg

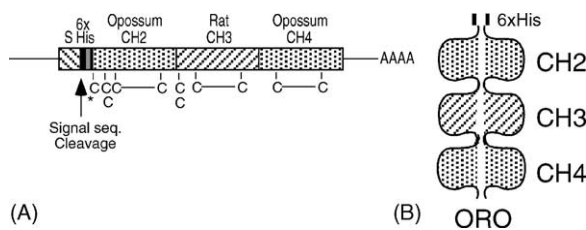


Fig. 1. (A) Schematic figure of the DNA construct encoding the ORO vaccine antigen. The signal sequence (derived from human BM40) is marked by an S. An asterisk denotes an unpaired cysteine residue that was changed to serine by point mutagenesis. (B) Structure of the recombinant ORO protein.

OVA in PBS, pH 7.0, weekly for 5 weeks prior to the initial vaccination and continuing throughout the vaccination program.

Groups of four animals received an initial i.p. vaccination of ORO mixed with one of the following adjuvants: Freund's complete adjuvant (Difco Laboratories, MI, USA), $\text{Al}(\text{OH})_3$ (Alhydrogel, Superfos Biosector A/S, Vedbeek, Denmark) or Cu^{2+} -conjugated ISCOM matrix [14]. Animals 1–4 received 100 μg ORO in 50 μl PBS, pH 7.0, mixed 50:50 with FCA. Animals 5–8 received 100 μg ORO in 100 μl PBS, pH 7.0 containing a 10 vol.% $\text{Al}(\text{OH})_3$ slurry by volume. Animals 9–12 received 100 μg ORO in 50 μl PBS, pH 7.0, mixed 50:50 with Cu^{2+} -conjugated ISCOM matrix. Animals 13–16 received 25 μg ORO in 50 μl PBS, pH 7.0, mixed 50:50 with Cu^{2+} -conjugated ISCOM matrix. Booster vaccinations were administered in week 3 of the treatment program. The booster vaccinations were identical to the initial vaccinations, with the exception that Freund's incomplete adjuvant (Difco Laboratories, MI, USA) was used instead of FCA in animals 1–4.

Blood samples of 1 ml were collected from the tail of the rats before initiating sensitisation, 3 days prior to vaccination and 2 weeks after the booster vaccination. The blood was allowed to coagulate overnight at 4 °C and spun for 10 min at 10,000 rpm (EBA12R, Hettich Zentrifugen, Germany). The sera were transferred to Eppendorf tubes and frozen until evaluation by ELISA.

The vaccine preparations containing $\text{Al}(\text{OH})_3$ were mixed with 100 μg ORO protein in PBS, pH 7.0 containing a 10 vol.% $\text{Al}(\text{OH})_3$ slurry by volume 1 day prior to vaccination and stored at 4 °C over night. The ISCOM matrix was prepared as follows: IDA matrix with Cu^{2+} had an estimated Quil A (QA) content of 1.7 mg/ml and an estimated cholesterol content of 0.5 mg/ml (Prep. 990823 B). According to analysis, matrix without Cu^{2+} had a QA content of 2.6 mg/ml, and the cholesterol concentration was estimated to be 0.8 mg/ml (Prep. 990320). To load the matrix with Cu^{2+} , a stock solution of 1 M $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in water was prepared. This solution was then added to the matrix preparation to a final concentration of 0.1 M Cu^{2+} . The mixture was incubated on a shaker at room temperature for 30 min followed by dialysis over-night at 4 °C against PBS. Protein

antigen was then added at a ratio of 1:1 to the cholesterol and incubated at 4 °C overnight.

2.3. Study 2

A total of 40, 8–10-week-old female Wistar rats (Benton and Kingman, Sollentuna, Sweden) divided into ten groups of four animals each received an initial 200 μl i.p. injection of 100 μg ORO in PBS, pH 7.0, together with an adjuvant, and in some groups, some additional immuno-stimulators. Animals 1–4 received FCA mixed 50:50 with antigen. Animals 5–8 received Montanide ISA 51 (MN51) (Seppic, Paris Cedex 07, France) mixed 50:50 with antigen. Animals 9–12 were injected with MN51:antigen (50:50) and 200 μg per animal of muramyl dipeptide (MDP also known as *N*-acetylmuramyl-L-alanyl-D-isoglutamine) (Sigma, St. Louis, MO, USA). Twenty-five milligrams of MDP was dissolved in sterile PBS to a final concentration of 10 mg/ml. Group 4, animals 13–16, received MN51:antigen (50:50) and 200 μg per animal of monophosphoryl-lipid A (MPL). A 10 mg/ml solution of MPL (RIBI ImmunoChem Research Inc., Hamilton, MT, USA) in methanol/chloroform (1:4) was aliquoted in volumes corresponding to doses of 0.2 and 0.1 mg MPL per animal and evaporated. Animals 17–20 were given MN51:antigen (50:50), 200 μg MDP and 100 μg MPL per animal. Animals 21–24 received MN51:antigen (50:50), 200 μg MDP, 100 μg MPL and 100 μg per animal of formyl-methionine (*N*-Formyl-Met-Leu-Phe) (Sigma, St. Louis, MO, USA). Ten milligrams of fMLP was dissolved in 1 ml sterile PBS and 1 ml 95% ethanol. Animals 25–28 received Montanide ISA 720 (MN720) (Seppic, Paris, France) in a 70:30 ratio with ORO. Animals 29–32 were injected with MN720:antigen (64:36) and 200 μg MDP per animal. Animals 33–36 received MN720:antigen (70:30) and 200 μg MPL per animal. Group 10, animals 37–40, were given MN720:antigen (64:36) with the addition of 200 μg MDP and 100 μg MPL per animal.

The booster dose comprised half the amount of ORO present in the primary injection, i.e. 50 μg , and FIA was used instead of FCA.

Blood was drawn from the tail of the rats 10 days prior to vaccination and 2 weeks after the booster. The blood was treated as described in Study 1 (Fig. 2).

2.4. Anti-rat IgE ELISA

The anti-rat IgE ELISA has been described previously [6]. The samples were assayed as single readings and horse sera served as the assay blank. In order to correlate the values, serial dilutions of two of the samples were assayed on every plate.

2.5. Anti-opossum C2-C3-C4 ELISA

The same procedure as described above was followed [6] with the exception that the coating antigen was opossum

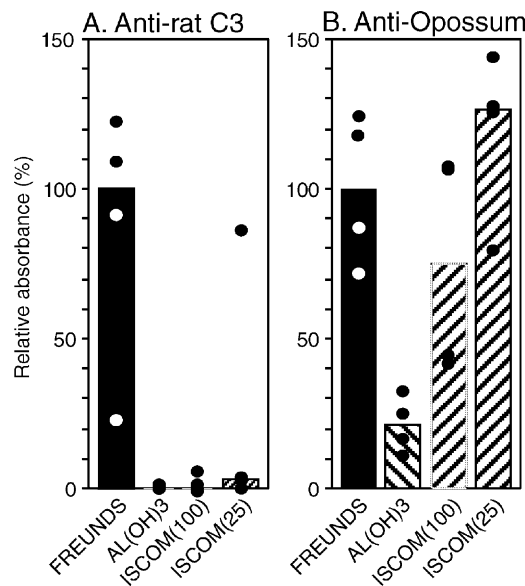


Fig. 2. Comparative analysis of sera from the blood drawn in week 5 of the treatment program of Wistar rats vaccinated with ORO mixed with either Freund's adjuvant, aluminium hydroxide or ISCOMs. (A) Relative anti-rat IgE antibody titers (anti-self IgE). (B) Relative anti-opossum antibody titers (anti-non-self). The mean value from rats vaccinated with Freund's was set to 100%.

C2C3C4 at a concentration of 5 µg/ml in carbonate buffer, pH 9.6.

3. Results

3.1. The vaccine component

To measure the immune response to self-components compared to that against non-self components, a hybrid molecule containing both self and non-self regions was designed and produced as recombinant protein. A vaccine containing the third constant domain from the ϵ -heavy chain of the rat, the target species, flanked by the second and fourth constant domains of the American opossum IgE-heavy chain (OpossumCH2-RatCH3-OpossumCH4 (ORO)) (Fig. 1), was expressed in 293-EBNA human embryonic kidney cells [6]. The opossum sequences differ by almost 60% from rat IgE and thereby serves as a non-self component. The opossum CH2 and CH4 domains have two main functions, acting both as a structural support for the self-component, the CH3 domain, and to break T cell tolerance to the self component by providing foreign T cell epitopes.

In order to measure the immune responses against opossum CH2 and CH4 by ELISA, a recombinant opossum C2C3C4 IgE was produced (OOO) by the same procedure as described above. Purified whole rat IgE was used for measurements of the immune responses against the rat CH3 domain [6].

3.2. Effects of three commonly used adjuvants on anti-self and anti-non-self IgE responses

As a first step in the screening process, three commonly used adjuvants were selected and their efficacy in inducing self and non-self responses was determined. The three adjuvants studied were Freund's adjuvant, Alum and a preparation of ISCOMs. The various adjuvants were administered by intraperitoneal injections together with the ORO vaccine component. The animals were divided into four groups; four animals were given 100 µg each of ORO in Freund's complete adjuvant, four received the same amount of protein absorbed to a commercially available preparation of Alum (a 10% Al(OH)₃ slurry by volume), four received 100 µg of ORO absorbed to the surface of 100 µg of ISCOMs and the last four animals were administered the same amount of ISCOMs but with only 25 µg of ORO. The rationale behind the reduced levels of antigen was to study the effect of a lower loading density on the ISCOMs, which may influence the accessibility to the immune system and therefore its ability to recognize the surface epitopes.

Booster vaccinations were administered in week 3 of the treatment program. The booster vaccinations were identical to the initial vaccinations, with the exception that FIA was used instead of FCA in animals 1–4. Blood samples were collected from the tail of the rats 3 days prior to vaccination and 2 weeks after the booster vaccination.

Comparative ELISA analysis was performed on sera from week 5 of the treatment program. The ELISA plates were either coated with whole rat IgE, to measure anti-rat C3 immune responses, the anti-self response, or with opossum C2C3C4 recombinant protein to measure anti-non-self responses. Substantial anti-self responses were detected only with Freund's adjuvant and in one of the four animals that received 25 µg of ORO absorbed on ISCOMs (Fig. 2A). No response was detected in any of the animals with Alum and not in any of the animals with 100 µg of ORO absorbed on ISCOMs (Fig. 2A). However, the responses against the non-self regions of the vaccine component, the opossum CH2 and CH4 domains, were quite different. The responses in ISCOM treated animals were comparable with Freund's treated animals and no significant difference in the magnitude of the response between these two adjuvants was detected. Alum was shown to be a less potent adjuvant giving a response of approximately only 20% of the levels seen in animals treated with Freund's and the ISCOMs (Fig. 2B).

The relative difference between the self and the non-self responses was also estimated by ELISA where different wells on the same plated were coated with either rat IgE or OOO. The ratio between the responses against non-self and self IgE in the Freund's-treated animals was found to be 150, 175, 150 and 750 for the four animals giving a mean value of an approximately 300-fold difference in titer. Although a substantial induction of antibodies against self IgE was seen, the titers of antibodies against self IgE were substantially lower than those against non-self IgE.

3.3. A comparative analysis of two mineral oil-based adjuvants and one adjuvant based on plant oils

Although the induction of immune responses against non-self IgE with the ISCOM preparation was comparable in magnitude to the levels obtained in the animals administered with Freund's adjuvant the titers against self IgE were undetectable or very low. In addition, no response against self IgE was detected when the Alum preparation was used. The only adjuvant that gave significant levels of antibodies against self IgE was Freund's adjuvant, an adjuvant based on mineral oil. However, due to its relatively severe side effects Freund's cannot be used as an adjuvant in humans. The negative effects of Freund's have been shown to originate primarily from impurities in the oil or the emulsifier preparation [8]. Other highly purified mineral oil preparations may therefore be the most promising alternative for future human applications. In order to address this possibility we decided to test one commercially available mineral oil adjuvant, Montanide ISA 51 and one adjuvant which is based on plant oil (Montanide ISA 720) but which has the same emulsifier (mannide monooleate) as Montanide ISA 51, to compare their effect with Freund's adjuvant [8].

Similarly to experiment 1, four animals in each group were tested for their induction of responses against self and non-self IgE. 100 μ l of adjuvant and 100 μ g of ORO were used. Comparative ELISA analysis were performed on sera from week 5 of the treatment program. A substantial response against self IgE was detected in all animals tested. However, the most prominent response was induced in Montanide ISA 51 treated animals, and this response was slightly higher than that response seen with Freund's adjuvant (160%). The response of the Montanide ISA 720 treated animals corresponded to only approximately 10% of that induced by Freund's. In contrast to the response against self IgE, all three adjuvants were shown to be almost equally potent in inducing an response against non-self IgE (Fig. 3).

The relative magnitude of the responses against self and non-self IgE was also determined. The ratio between the responses against non-self and self IgE in the Freund's-treated animals was 50, 65 and 75 for three of the four animals giving a mean 63-fold difference in titer (insufficient material was obtained from the fourth animal). For Montanide ISA 51 the ratio was 200, 30, 40 and 26 for the four animals giving a mean value of 74.

3.4. An analysis of the potential additive effect of MDP, lipid A and formyl-methionin peptides

Based on the results from the two experiments described above the mineral oil-based adjuvants are the most effective in inducing anti-self IgE responses.

However, the difference between the responses against non-self and self IgE is still quite substantial and frequently exceeds a 50-fold difference. The possibility was therefore tested that additive effects may be induced by bacte-

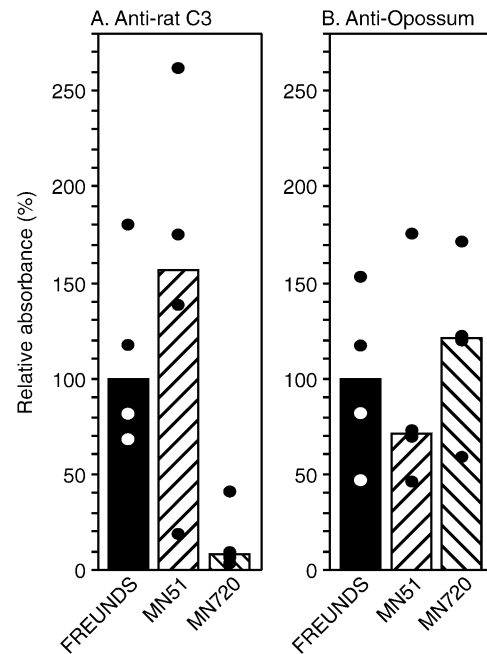


Fig. 3. Comparative analysis of sera from the blood drawn in week 5 of the treatment program of Wistar rats vaccinated with ORO mixed with either Freund's adjuvant, Montanide ISA 51 or Montanide ISA 720. (A) Anti-rat IgE antibody titers (anti-self IgE). (B) Anti-opossum antibody titers (anti-non-self). The mean value from rats vaccinated with Freund's was set to 100%.

rial immuno-stimulatory substances. A series of experiments was conducted in which rats were immunised in groups of four with 100 μ g ORO in Montanide ISA 51 or ISA 720 with additions of either 200 μ g of MDP, 200 μ g of monophosphoryl lipid A, 200 μ g of MDP and 100 μ g of MPL, or with three additions, 200 μ g of MDP, 100 μ g of MPL and 100 μ g of formyl-Met-Leu-Phe peptide (for details see Section 2).

Comparative ELISA analysis were performed on sera from week 5 of the treatment program. Absorbance was measured and the values were correlated to relative absorbance with the response induced by Freund's treatment set to 100%. The addition of MDP, MPL, fMLP or a combination of two or three of these had a slight negative effect (50% reduction in response) on the anti-self responses and no significant positive effect on the response against the non-self epitopes when administered together with Montanide ISA 51 (Fig. 4A and B).

In the experiment with Montanide ISA 720 a quite substantial enhancement of the response against self IgE was seen with MDP and MPL (Fig. 5A). An approximately four- and six-fold enhancement was seen with MDP and MPL, respectively (Fig. 5A). The addition of MPL resulted in an adjuvant with an activity of almost 60% of what was seen with a combination of Freund's complete and incomplete adjuvant (Fig. 5A). However, all additions had a weakly negative effect on the response against non-self IgE, the opossum CH2 and CH4 sequences (Fig. 5B).

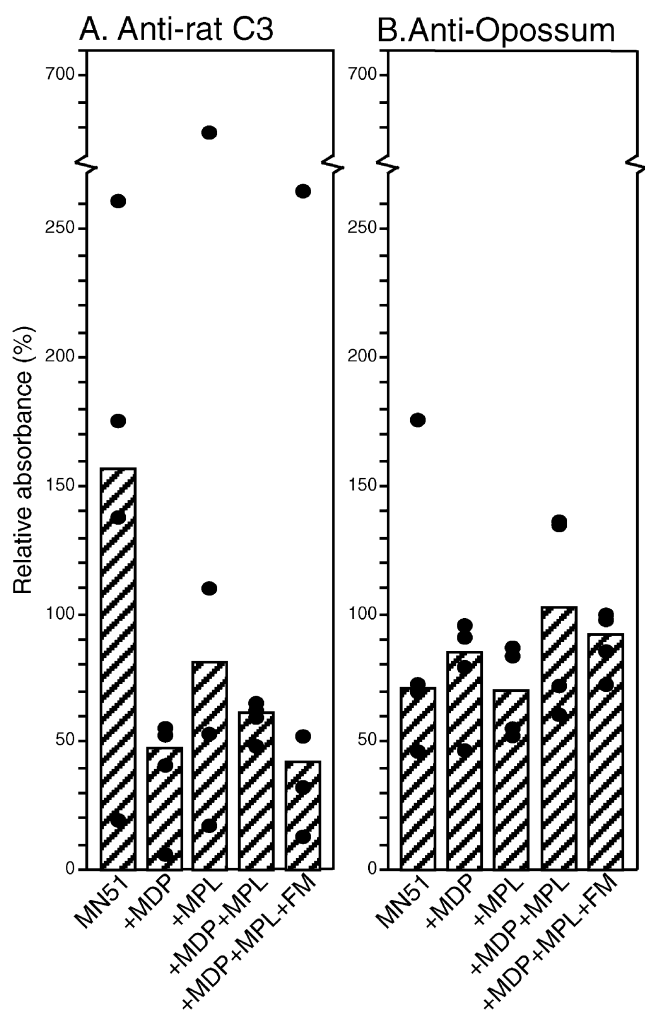


Fig. 4. Comparative analysis of sera from the blood drawn in week 5 of the treatment program of Wistar rats vaccinated with ORO mixed with Montanide ISA 51, plain or supplemented with 200 μ g muramyl dipeptide (MDP), 200 μ g monophosphoryl lipid A (MPL), both (200 μ g MDP + 100 μ g MPL) or with 200 μ g MDP, 100 μ g MPL and 100 μ g of a formyl-methionine-containing tripeptide (FM). (A) Relative anti-rat IgE antibody titers (anti-self IgE). (B) Relative anti-opossum antibody titers (anti-non-self). The mean value from rats vaccinated with Freund's was set to 100%.

4. Discussion

The present study was undertaken to identify a potential adjuvant that can be used in combination with a vaccine component and generate optimal therapeutic antibody responses against host IgE. Montanide ISA 51, an adjuvant based on mineral oil appears to be the most promising candidate. The response with this adjuvant was comparable to that seen using a combination of Freund's complete and incomplete adjuvants, which is known to be one of the most powerful adjuvants for immunisations in rodents and many other animal species. A substantially lower response was seen with Montanide ISA 720, an adjuvant primarily based on a bio degradable plant oil [8]. Compared to the plant oil

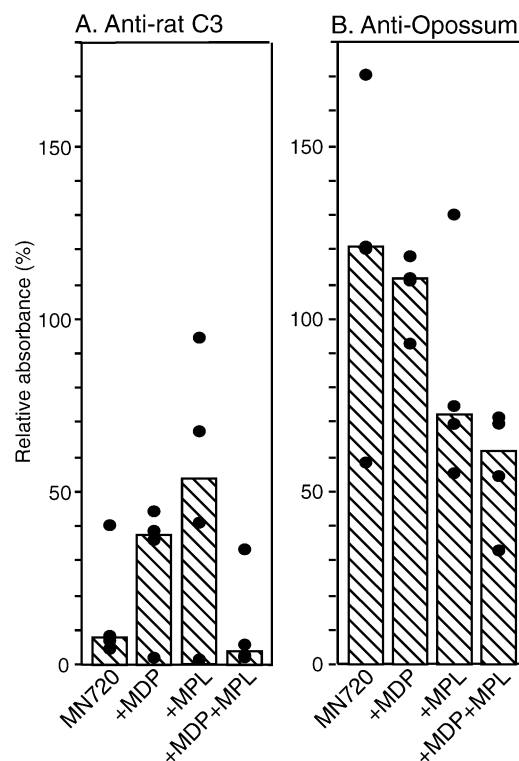


Fig. 5. Comparative analysis of sera from the blood drawn in week 5 of the treatment program of Wistar rats vaccinated with ORO mixed with Montanide ISA 720, plain or supplemented with 200 μ g MDP, 200 μ g MPL, or both (200 + 100 μ g). (A) Relative anti-rat IgE antibody titers (anti-self IgE). (B) Relative anti-opossum antibody titers (anti-non-self). The mean value from rats vaccinated with Freund's was set to 100%.

in Montanide ISA 720, the mineral oil in Montanide ISA 51 probably has a longer half-life in the tissue and can thereby function more efficiently as an antigen depot. This extends the time the vaccine antigen is available for antigen presenting cells, which may significantly effect the type and magnitude of the response.

Surprisingly, we do not observe any significant difference between Freund's adjuvant and Montanide ISA 51 in inducing a response against self IgE. Freund's complete adjuvant contains heat-killed mycobacteria, and bacterial compounds have frequently been shown to have strong immunopotentiating effects. In line with the finding we did not see any enhancement in response when Montanide ISA 51 was administered together with any of the three bacterial immuno-stimulatory compounds, MDP, MPL or fMLP, neither alone nor in various combinations (Fig. 4A and B). However, in contrast, the response against self IgE was increased almost 400% when MDP was added to Montanide ISA 720, and yet another 40% when MPL was the additive component (Fig. 5A). One possible explanation to this phenomenon can be that pure Montanide ISA 51 has reached a maximum in possible adjuvant activity without the additives. Actually, we see a decrease in the response against self IgE following the addition of immuno-stimulatory

compounds to Montanide ISA 51 (Fig. 4A). The bacterial immuno-stimulatory compounds may lead to a faster inflow of phagocytic cells to the area of antigen depot, which in turn may result in a faster clearance of the antigen and thereby to a shorter time the antigen is available for more professional antigen presenting cells, such as Langerhans cells and other dendritic cells. In contrast to Montanide ISA 720, which seems to be a milder adjuvant, the adjuvant effect can possibly still be improved by MDP and MPL.

Interestingly, all adjuvants used in this study, except Alum, did induce comparable antibody titers against the non-self part of the vaccine antigen, the opossum C2 and C4 domains. This suggests that the difference between the adjuvants primarily lies in their strength to break the tolerance against self-proteins, but that they are almost equally good at inducing response against a non-self protein.

Today, the only commonly used adjuvant for human applications is Alum. However, with Alum we did not see any detectable response against self IgE. This is in contrast to the promising results seen with Alum-based anti-fertility vaccines for two other self-proteins [20,21]. Preliminary animal experiments with the β -subunit of ovine luteinizing hormone and the human chorionic gonadotropin linked to tetanus toxoid as carrier and adsorbed onto $Al(OH)_3$, has indicated that Alum may be a sufficiently strong adjuvant to induce a response that prevents monkeys and humans from getting pregnant [20,21]. This indicates that there is either a species-specific difference in the activity of the adjuvant or that important differences exist between different self-antigens. Additional experiments are therefore needed before the possibility of using Alum-based adjuvants can be ruled out. However, the most promising adjuvant candidate for the allergy vaccine is presently Montanide ISA 51.

The use of mineral oil-based adjuvants in human has been questioned, primarily because of the toxic effects seen with Freund's adjuvant. However, Montanide ISA 51, which is based on highly purified components, is presently used in several veterinary applications and seems to be safe [8]. This adjuvant is also being used in several clinical trials, indicating that it may be approved for a more general use in humans in the near future [8]. If mineral oil will turn out not to be safe for long term treatment, non-mineral oil-based adjuvants such as Montanide ISA 720, with addition of one or several highly purified immuno-stimulatory compounds may serve as an almost equally potent alternative. Based on the results shown in Fig. 5A, we can see that Montanide ISA 720 with MPL showed an activity of almost 60% of the response seen with the mineral oil-based adjuvants.

In this study, Montanide ISA 51 was shown to be the most potent adjuvant in inducing a response against self IgE. However, the response against self IgE is weak in comparison with the antibody response against non-self regions of the fusion protein, the ORO protein. The response against opossum IgE was shown to be at least 50 times higher than the response against rat IgE indicating that the tolerogenic effect of the self-regions is very strong. Although the levels

of antibodies against self IgE are relatively low, they have been shown to be capable of reducing the circulating IgE levels to a clinically significant extent in low and medium IgE responder rats [3,6]. However, the response may not be sufficiently strong to reduce IgE levels significantly in animals and human patients with higher IgE levels. Additional improvements in the presentation of the antigen may have positive effects on the response. Both the use of multimeric forms but also cytokine doping of the vaccine may increase the magnitude of the response, which presently is under investigation (Johansson, et al., work in progress). However, on the basis of the data presented in this study Montanide ISA 51 is the most promising candidate for the allergy vaccine.

Acknowledgements

We should like to thank Katarina Ramlund at Isconova AB for preparing the ISCOMs.

References

- [1] Noon L, Cantab BC, Eng FRCS. Prophylactic inoculation against hay fever. *Lancet* 1911;i:1572–3.
- [2] Freeman J. Vaccination against hay fever: report of results during the first three years. *Lancet* 1914;i:1178–80.
- [3] Hellman L. Profound reduction in allergen sensitivity following treatment with a novel allergy vaccine. *Eur J Immunol* 1994;24(2): 415–20.
- [4] Hellman L, Carlsson M. Allergy vaccines: a review of developments. *Clin Immunother* 1996;6(2):130–42.
- [5] Hellman L. Vaccines against allergies. In: Perlmann P, Wigzell H, editors. *Handbook of experimental pharmacology (vaccines)*, vol. 133. Berlin: Springer-Verlag; 1999. p. 499–526.
- [6] Vernersson M, Ledin A, Johansson J, Hellman L. Generation of therapeutic antibody responses against IgE through vaccination. *FASEB J* 2002;16:875–7.
- [7] Lövgren-Bengtsson K. Preparation and use of adjuvants. In: *Methods in microbiology*, vol. 25. New York: Academic Press; 1998. p. 471.
- [8] Aucouturier J, Dupuis L, Deville S, Ascarateil S, Ganne V. Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines. *Expert Rev Vaccines* 2002;1(1):111–8.
- [9] Lindblad EB. Aluminium adjuvants. In: Stewart-Tull DES, editor. *The theory and practical application of adjuvants*. Chichester: Wiley; 1995. p. 21–35.
- [10] Cox JC, Coulter AR. Adjuvants: a classification and review of their modes of action. *Vaccine* 1997;15(3):248–56.
- [11] Singh M, O'Hagan D. Advances in vaccine adjuvants. *Nat Biotechnol* 1999;17(11):1075–81.
- [12] Barr IG, Sjolander A, Cox JC. ISCOMs and other saponin based adjuvants. *Adv Drug Deliv Rev* 1998;32(3):247–71.
- [13] Mumford JA, Jessett D, Dunleavy U, Wood J, Hannant D, Sundquist B, et al. Antigenicity and immunogenicity of experimental equine influenza ISCOM vaccines. *Vaccine* 1994;12(9):857–63.
- [14] Andersson C, Wikman M, Lövgren-Bengtsson K, Lundén A, Ståhl S. In vivo and in vitro lipidation of recombinant immunogens for direct iscom incorporation. *J Immunol Methods* 2001;255(1–2):135–48.
- [15] Takada H, Kotani S. Muramyl dipeptide and derivatives. In: Stewart DES, editor. *The theory and practical application of adjuvants*. Chichester: Wiley; 1995. p. 171–202.

- [16] Ribi EE, Cantrell JL, Von Eschen KB, Schwartzman SM. Enhancement of endotoxic shock by *N*-acetylmuramyl-L-alanyl-(L-seryl)-D-isoglutamine (muramyl dipeptide). *Cancer Res* 1979;39(11):4756–9.
- [17] Baldrige JR, Crane RT. Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines. *Methods* 1999;19(1):103–7.
- [18] Hellman L, Pettersson U, Engstrom A, Karlsson T, Bennich H. Structure and evolution of the heavy chain from rat immunoglobulin E. *Nucleic Acids Res* 1982;10(19):6041–9.
- [19] Aveskogh M, Hellman L. Evidence for an early appearance of modern post-switch isotypes in mammalian evolution cloning of IgE. *Eur J Immunol* 1998;28(9):2738–50.
- [20] Talwar GP. Vaccines for control of fertility and hormone-dependent cancers. *Immunol Cell Biol* 1997;75(2):184–9.
- [21] Talwar GP, Singh OM, Gupta SK, Hasnain SE, Pal R, Majumbar SS, et al. The HSD-hCG vaccine prevents pregnancy in women: feasibility study of a reversible safe contraceptive vaccine. *Am J Reprod Immunol* 1997;37(2):153–60.