

A Th1-Inducing Adjuvant, MPL[®], Enhances Antibody Profiles in Experimental Animals Suggesting It Has the Potential to Improve the Efficacy of Allergy Vaccines

Alan W. Wheeler^a Jean S. Marshall^b J. Terry Ulrich^c

^aAllergy Therapeutics Ltd, Worthing, UK, ^bDalhousie University, Halifax, Canada and ^cCorixa Corporation, Hamilton, Mont., USA

Key Words

Th1 · Adjuvant · Monophosphoryl lipid A · MPL[®] · Mice · Rats · IgG1 · IgG2a · IgE · Allergy vaccines

Abstract

Background: Monophosphoryl lipid A (MPL[®]) is a detoxified derivative of the lipopolysaccharide (LPS) moiety of *Salmonella minnesota* R595, which has retained immunostimulatory activities. MPL[®] has been administered to many subjects in clinical trials as an adjuvant component of infectious disease vaccines and is currently a component of a licensed cancer vaccine, Melacine[®] (Corixa Inc., Schering Plough). MPL[®] has, in particular, been shown to promote Th1-type antigen specific responses. L-tyrosine is a depot adjuvant which is fully metabolisable and has been successfully employed in allergy vaccines for a number of years. **Methods:** Mice were immunised with MPL[®] adjuvant in conjunction with separate preparations of either ovalbumin or glutaraldehyde-modified ragweed pollen extract both coprecipitated with L-tyrosine. The specific antibody isotypes IgG1, IgG2a, IgG2b and also IgE were measured. Rats received booster injections of keyhole limpet haemocyanin (KLH) in con-

junction with MPL[®] adjuvant following priming with KLH in alum alone. KLH-specific antibody responses were measured. **Results:** It was shown that a combination of L-tyrosine and MPL[®] were synergistic in enhancing murine antigen specific IgG antibody responses without enhancing antigen specific IgE responses. Furthermore, this adjuvant combination promoted strong IgG2 antigen specific responses indicative of a Th1 directed response. In KLH sensitised rats, treatment with MPL[®] was shown to prevent a secondary IgE antibody response when injected with booster injections of antigen. **Conclusions:** Immunisation of mice with two different antigens adsorbed to L-tyrosine induced a Th1-skewed primary response when in conjunction with MPL[®] adjuvant which also generally enhances a specific IgG response. Incorporation of MPL[®] adjuvant in the immunisation of rats prevented a secondary specific IgE response. These results suggest that the employment of this new adjuvant in clinical allergy vaccination formulations may result in an improved efficacy which could be utilised in various ways to improve compliance.

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Introduction

Allergen immunotherapy (AI) is currently used in a somewhat restrictive manner for the treatment of type I hypersensitivity. Very few patients suffering from respira-

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Fax +41 61 306 12 34
E-Mail karger@karger.ch
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1018–2438/01/1262–0135\$17.50/0

Accessible online at:
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Correspondence to: Dr. Alan W. Wheeler
Allergy Therapeutics Ltd.
Dominion Way
Worthing, West Sussex BN14 8SA (UK)
Tel. +44 1903 844 724, Fax +44 1903 844 744, E-Mail alan.wheeler@allergytherapeutics.com

tory symptoms due to inhaled materials such as pollens or house dust mites currently receive AI. Yet, AI has the capacity to modify the underlying disease process [1] and a long-term cure can often be achieved following several years of treatment [2]. It is surprising therefore, that the treatment is not used more widely. Reasons for this are a perceived inconsistent efficacy, the potential for allergic adverse events during treatment and poor compliance due to the large number of injections required.

The likelihood of serious adverse events occurring during AI has been reduced by the introduction of measures such as optimal dosing and allergen modification to reduce allergenicity while maintaining immunogenicity [3] and by adsorption to alum [4] or coprecipitation with L-tyrosine [5] in order to control allergen release. Although AI has been shown in placebo-controlled studies to be effective, there is a need to identify strategies that can enhance effectiveness and thus make AI acceptable as a first-line treatment of appropriately diagnosed allergy. For example, aluminum salts are often used in allergy vaccines but have been shown to promote a Th2 response which leads to undesirable IgE production at least in experimental animals [6]. Although there is some evidence that alum can induce IgE antibodies in a clinical study, the evidence is relatively weak [7]. The depot adjuvant L-tyrosine, on the other hand, has been shown not to have this strong Th2 bias in animals [8] and as it is also fully metabolisable it should be considered for AI.

Some studies have suggested that one mechanism involved in successful AI is a reorientation of the allergen specific Th response to a stronger more favourable Th1 profile [9]. Other possible mechanisms that may not necessarily be mutually exclusive are a deviation in the Th2 response from an IgE to IgG4 focus or enhanced IL-10 induction, possibly from T-regulatory cells bringing about an allergen specific anergic state [10]. An ideal adjuvant, in addition to enhancing the immune stimulus, would favour a Th1 response, which should result in a significant reduction of clinical symptoms. Treatment should ideally induce an eventual reduction in specific IgE antibody and the late-phase reaction of type 1 allergy should be modified. Th1-like immunomodulation has been demonstrated in laboratory animals using a new adjuvant, 3-deacylated monophosphoryl lipid A (MPL®), derived from the lipopolysaccharide (LPS) of *Salmonella minnesota* [11]. Hence this adjuvant was used in these studies to extend and confirm these findings.

The following animal studies endorse the useful potential of MPL® as an adjuvant for AI.

Materials and Methods

Animals

Female BALB/c mice 10–12 weeks old were purchased from the Jackson Laboratory (Bar Harbor, Me., USA). Female Brown Norway rats, 150–200 g, were purchased from Harlan Sprague Dawley (Indianapolis, Ind., USA). On arrival, the animals were housed in isolator cages, provided with autoclaved food and water ad libitum and quarantined for 7 days. On release from quarantine, the animals were entered into the studies following protocols on file with the US institutional Animal Care and Use Committee and in compliance with the Animal Welfare Regulations (9CFR).

Preparation of MPL® Adjuvant Formulation

MPL® is a product derived from the LPS of *S. minnesota* R595, purified by a series of organic solvent extractions followed by mild acid and alkaline treatments, as described by Ulrich and Myers [11]. The final product has suitably attenuated toxicity but has retained immunostimulatory activity. The detailed structures of the forms of the species present in the MPL® product have been reported [11]. For adjuvant use MPL® was prepared as 1.0 mg/ml aqueous formulation containing MPL® at a 4:1 molar ratio with dipalmitoylphosphatidylcholine in water. This formulation is termed MPL®-AF and was used as an admixture with the tyrosine co-precipitates.

Preparation of Ovalbumin-Tyrosine Co-Precipitates

Ovalbumin (OA)-tyrosine co-precipitates were prepared as follows: OA (16.0 mg) was solubilized in 20 ml of Dulbecco's phosphate buffered saline (DPBS), pH 7.2 (Sigma Chem. Co., St. Louis, Mo., USA) in a 40 ml beaker containing a magnetic stir bar. While stirring, 6.65 ml of a 3.2 N NaOH solution and 6.65 ml of a 3.8 N HCl solution containing 24% w/v tyrosine were added dropwise over a 10 min period to the OA solution. After the tyrosine precipitate formed it was allowed to stir for an additional 5 min. The precipitate was decanted into a 50 ml conical centrifuge tube, DPBS added to bring the volume to 40 ml, and the tube centrifuged at 2,000 rpm for 10 min. The supernatant was decanted and assayed for protein, i.e. OA, by the bicinchoninic acid method [12]. The results (data not shown) indicated undetectable levels of protein in the supernatant suggesting that virtually all of the OA was bound to the tyrosine precipitate. The pelleted tyrosine precipitate was washed an additional 3 times using centrifugation and resuspension with DPBS. After the final wash the precipitate was resuspended in 80 ml of DPBS containing 0.4% w/v glycerol and 0.01% w/v thimerosal as a preservative. Analysis of the preparation by a gravimetric method indicated the tyrosine precipitate to be at 40 mg/ml wet weight. Assuming 100% adsorption of the OA to the tyrosine precipitate, the stock preparation contained 200 µg/ml OA and 40 mg/ml tyrosine. For use the OA-tyrosine co-precipitate was diluted with an equal volume of DPBS or MPL®-AF at 500 µg/ml. BALB/c mice were injected with 0.2 ml subcutaneously (s.c.), with these diluted preparations. For booster injections all materials were freshly prepared.

Use of Commercial Ragweed Pollen Extract Vaccine

Pollinex-R® (Lot No. EX14432), a commercial tyrosine co-precipitate of glutaraldehyde-treated ragweed pollen extract (GRWP) was obtained from Allergy Therapeutics Ltd. (Worthing, UK). This product was diluted with an equal volume of DPBS or MPL®-AF at 500 µg/ml. BALB/c mice, 5 per group, were injected, s.c., with 0.2 ml of these preparations.

Table 1. OA antibody titres in pooled sera from mice immunised with combinations of OA, L-tyrosine and MPL[®] adjuvant

Formulation	IgG1 titre	IgG2a titre	IgG2b titre	IgE OD at 1/10
OA/tyrosine	102,400	100	200	0.213
OA/tyrosine + MPL [®]	409,600	102,400	102,400	0.104
OA + MPL [®]	102,400	200	400	0.218
OA alone	6,400	<100	<100	0.235
Pre-immune	<100	<100	<100	0.095

Results as endpoint titres defined as the reciprocal of the last serum dilution giving an adsorbance value greater than or equal to 0.1 OD of the enzyme substrate chromophore.

Immunizations, Sample Collections and Immunoassays

Mice (4 per group) were lightly anaesthetised with CO₂ and bled from the retro-orbital plexus to provide sera on day 10 following primary and booster (day 14 or day 21) injections of the immunising preparations. Serum samples were bulked from each group and frozen at -70°C. The bulking was to ensure sufficient serum volume to carry out the required antibody assays. Following thawing, the samples were tested in a standard ELISA using Nunc Maxisorb plates. For analysis of IgG isotype specific anti-OA or anti-GRPE responses, plates were coated overnight at 4°C with 2 µg/ml OA or 5 µg/ml ragweed pollen extract in 0.05 M carbonate buffer, pH 9.6. Antigen-specific IgG antibody isotypes were measured using goat anti-mouse horseradish peroxidase (HRP) labelled antibodies (Southern Biotechnology Assoc., Inc., Birmingham, Ala., USA). Endpoint titres were defined as the reciprocal of the last serum dilution giving an adsorbance value greater than or equal to 0.1 OD of the enzyme substrate chromophore. Mouse serum IgE antibody to OA was measured as follows: The wells of 96-well Nunc Maxisorb plates were coated overnight at 4°C with rat antimouse IgE monoclonal antibody (Pharmingen, San Diego, Calif., USA) at 1.0 µg/ml in borate-buffered saline, pH 8.3. Serum samples diluted in PBS containing 0.3% w/v bovine serum albumin and 0.002% v/v Tween 20 (PBS/BSA/T20) were added at 100 µl per well. The plates were incubated overnight at 4°C. Then a 0.1 µg/ml solution of biotinylated OA in PBS/BSA/Tween 20 was added to the wells and the plates were incubated for 2 h at room temperature. Next a 1/4,000 dilution of HRP-labelled avidin (Neutralite™, Southern Biotechnology) was added to the wells and the plates incubated for 2 h at room temperature. Finally, the OD of the enzyme substrate chromophore was read following colour development.

Rat Model to Study the Effect of MPL[®] on a Boosted IgE Response

Ten female Brown Norway (high IgE responding) rats received a subcutaneous (s.c.) primary injection, containing 10 µg of keyhole limpet hemocyanin (KLH) (Sigma)- alum adsorbate (Alhydrogel, Superfos, Denmark) mixed with 1×10^9 heat-killed *Bordetella pertussis* organisms (Connaught Lab., Swiftwater, Pa., USA) in order to induce a strong IgE response to KLH. On day 14, the rats were randomised into two groups of 5 each and all boosted, s.c., with 30 µg KLH in saline. The rats were either injected, s.c., with 200 µg of MPL[®] in 0.2 ml saline (group A) or 0.2 ml saline (group B) at a site in very close proximity to the booster injection. A schedule of further antigen booster injections of the same concentration followed by MPL[®] or saline control treatments was carried out on days 21, 28, and 35. All animals were bled (tail vein) prior to treatment on days 14, 28, and 42. Serum IgE-specific KLH responses were measured by

a paper radio allergosorbent assay technique (PRAST) assay with KLH-coated paper discs and bound IgE detected by an ¹²⁵I-labelled mouse anti-rat IgE (MARE-1) monoclonal antibody.

Results

The Addition of MPL[®] to an OA-Tyrosine Co-Precipitate Induces a Strong IgG2 Isotype Antibody Response

Previous studies have shown that the addition of MPL[®] adjuvant to an alum adsorbed vaccine significantly enhances the overall immunogenicity of the vaccine antigens, with a significant enhancement of the IgG2 isotype antibody responses when compared to alum alone [13, 14]. The preparation of a model antigen, OA/L-tyrosine co-precipitate allowed us to evaluate the adjuvant activity of MPL[®] in this system. Several parameters of the system were established such as: dose of MPL[®], pH of MPL[®] solution, and adsorption kinetics of MPL[®] to the tyrosine adsorbate. Regarding the adsorption kinetics, MPL[®] at concentrations up to 500 µg/ml at pH 7.2, was essentially 100% bound to an equal volume of tyrosine precipitate at 40 mg/ml within 15 min of mixing. Quantification of L-tyrosine-bound MPL[®] was carried out by solubilizing a pelleted sample of the L-tyrosine precipitate in 0.2 N glacial acetic acid, and analysing the MPL[®] content by high pressure liquid chromatography [15]. The results of a representative immunogenicity experiment are shown on table 1. Although, as expected OA-tyrosine-MPL[®] appeared to induce less anti-OA IgE-specific antibody than the other combinations studied, the data was of limited robustness as the assay was carried out only at one concentration of serum. However, a strong anti-OA IgG2 isotype response was induced only in this group. These data support the concept that the addition of MPL[®] to the OA-tyrosine co-precipitate is serving to promote the Th1-type of antibody response in these animals.

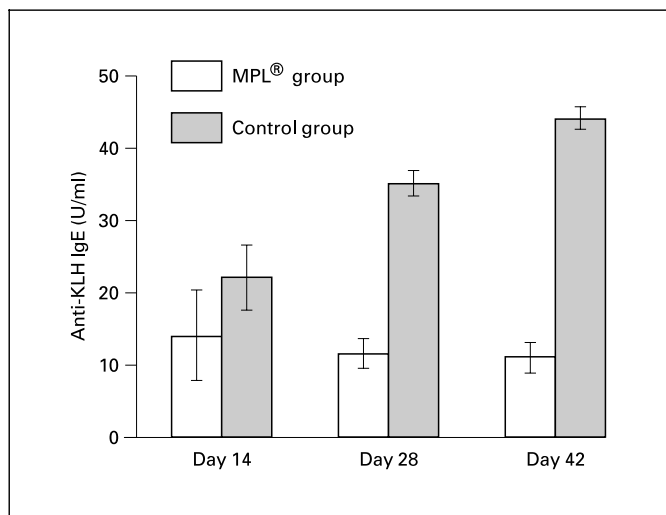


Fig. 1. KLH-specific IgE antibody levels in sera from KLH/alum immunised rats injected with MPL® and KLH or diluent (saline) and KLH. Five animals in each group. Error bars indicate standard deviation.

Table 2. Ragweed extract-specific antibodies in pooled sera from mice immunised with Pollinex R or Pollinex R plus MPL® adjuvant

Formulation	IgG1 titre	IgG2b titre	Ratio IgG1:IgG2b
Pollinex R alone	3,200	<200	16:<1
Pollinex R + MPL®	12,800	6,400	2:1

Results as endpoint titres defined as the reciprocal of the last serum dilution giving an adsorbance value greater than or equal to 0.1 OD of the enzyme substrate chromophore.

MPL® Promotes an IgG2b Response to Pollinex R

The effect of the addition of MPL® adjuvant to a commercially available L-tyrosine adsorbate co-precipitate of glutaraldehyde-modified ragweed pollen extract, Pollinex R, is shown in table 2. Unfortunately no IgG2a assays were carried out, as the developing antiserum required was temporarily unavailable and only enough mouse serum was collected to measure one of the IgG2 subclasses. However, IgG2b is also believed to be very much under the control of Th2 modulation. Clearly, it can be seen that the ratio of IgG2b:IgG1 antibody was much higher in those animals given MPL®. Once again these data suggest that MPL® is preferentially enhancing the Th1 component of the immune response to ragweed pollen extract.

MPL® Treatment Down-Regulates the Rat Anti-KLH IgE Antibody Response

The results shown in figure 1 demonstrate that the administration of MPL® serves to blunt further increases in anti-KLH IgE induced by booster injections in these hypersensitized animals. The induction of high serum concentrations of interferon (IFN γ) in vivo by MPL® in a mouse system has been reported [16]. Although not measured in this rat model, the induction of a rapid IFN γ response by MPL® could serve to inhibit further activation of IgE-promoting Th2 cells by booster administrations of KLH.

Discussion

Experiments described here confirm that MPL® can act as an immunostimulant for the induction of antibody in animals. The profile of the particular subclasses of IgG antibody induced in mice is believed to reflect the Th1:Th2 response stimulated, though a measurement of cytokines not carried out in these studies would provide more solid evidence. It is generally accepted, however, that an IgG1 response is induced by help from Th2 cells whereas Th1 activity leads additionally to IgG2a and IgG2b antibody production [17]. It was necessary to pool the mouse sera to reach sufficient volumes required for the antibody assays. This procedure has a potential drawback in that a high- or low-responding animal may skew the antibody titres. However, skewing should be minimal since the mice were inbred and all of the same sex and age.

The combination of MPL® adjuvant and insoluble L-tyrosine was synergistic at inducing, in particular, IgG2a and IgG2b responses to the co-precipitated antigen. The reason for this synergy is not clear although it is known that tyrosine can act as a depot adjuvant in its own right and in some experiments has tended to produce a more Th1 rather than Th2 like response. IgG1 antibody levels were also raised by immunisation with the combination of tyrosine and MPL® but the differences between the immunising potentials of this and the other preparations in terms of this IgG subclass were not as marked. These results suggested that a clinical study of the immunostimulant as an additive to existing L-tyrosine-based formulations of allergens or allergoids would be worthwhile. The IgG2b response to the Allergy Vaccine Pollinex R was enhanced substantially by the addition of MPL® compared with the enhancement of the IgG1 isotype which again suggests that MPL® has a strong Th1-inducing bias as an adjuvant for promoting ragweed-specific antibodies.

The experiments described above have shown that the adjuvant MPL® has the propensity to induce a Th1-skewed primary response. Prolonged immunisation though was not studied and could possibly have resulted in a different outcome.

In the case of the allergic patient we are dealing with a well established ongoing Th2 biased allergen-specific response. Theoretically we need to switch the existing response to a more pronounced Th1 profile [18] or stimulate a de novo Th1 cell population. Existing allergy immunotherapy may act by striking a new balance between Th1 and Th2. In addition, it may change the nature of the Th2 response, diverting the focus away from IgE towards IgG4 antibodies which may be as important a feature of AI as a change in the Th1:Th2 ratio. A strong, high-dose immunogenic stimulus possibly including many injections is probably required to achieve this. MPL® is a general adjuvant for the induction of IgG antibodies as well as having a Th1-directing activity, and therefore its inclusion in vaccines may very well obviate the need for the long schedule and high doses of allergen required for successful allergy vaccination.

It was observed that MPL® was capable of preventing the further increase in IgE seen when booster injections of antigen were given to rats previously immunised to induce a primary IgE response. This might be expected of a known Th1 adjuvant such as MPL® but these results also give encouragement that this adjuvant could provide benefit in an allergy vaccine.

The antibody profiles induced in mice when MPL® was added to the immunising formulations suggest that the new adjuvant can stimulate an enhanced general specific IgG antibody response but also has a greater Th1 than Th2 stimulating potential compared with the effects seen when the formulations were injected alone. T cell modulation of antibody responses in man and rodents may well be different. However, these results and the known properties of MPL® have led us to consider the use of MPL® in allergy immunotherapy. The general as well as selective immunoenhancing activity of MPL® adjuvant may augment the effectiveness of allergy vaccines to which it is added if the effects in rodents are reproduced in man. Benefits gained from this strategy could potentially include enhanced efficacy of AI, which could possibly be exploited by a reduction in the number of injections required to achieve a therapeutic effect.

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