

Safety Evaluation of a New Allergy Vaccine Containing the Adjuvant Monophosphoryl Lipid A (MPL[®]) for the Treatment of Grass Pollen Allergy

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A novel allergy vaccine (Pollinex[®] Quattro) has been developed for the prevention or relief of allergic symptoms caused by a variety of pollens. Within this range, the grass pollen allergy vaccine contains extracts of 12 grass pollens and rye cereal (all chemically modified by glutaraldehyde) that are adsorbed onto L-tyrosine with addition of the immunostimulatory adjuvant monophosphoryl lipid A (MPL[®]). A specific preclinical safety testing strategy was developed to support clinical use, comprising single-dose toxicity, repeat-dose toxicity and local tolerance studies. Dose levels of up to 0.5 ml per animal (mouse) and up to 1.0 ml per animal (rat and rabbit) were used with vaccines containing 2000 or 12 000 standardized units (SU) ml⁻¹ of grass pollen allergoids, 50 µg ml⁻¹ of MPL[®] adjuvant and 20 mg ml⁻¹ of tyrosine. Overall, the product showed no toxicological findings of significance at levels greatly in excess of those proposed for clinical use. A not unexpected, but relatively minor, immunostimulatory effect was seen following repeated dosing (once weekly for 3 weeks) at 1.0 ml per rat. Copyright © 2004 John Wiley & Sons, Ltd.

INTRODUCTION

Allergic asthma and allergy-related disorders such as hayfever are becoming increasingly prevalent in the developed world. Grass pollen allergy is of major concern and has been indicated in perennial allergic rhinitis and asthma (Varney, 1991; D'Amato *et al.*, 1998; Suphioglu, 1998). In recent years, allergy vaccination has been recognized as a safe and efficacious treatment strategy for patients suffering from these conditions (e.g. Bousquet *et al.*, 1998; Ebner, 1999; Malling, 2000; Rolland *et al.*, 2000). Such vaccination is believed to exert its beneficial effects on the immune system, at least in part, by modifying the T-lymphocyte response to subsequent natural allergen exposure. Studies of successful allergy vaccination have shown that the T-helper type 2 (Th2) cellular response influencing the expression of interleukin 4 (IL-4), IL-5 and immunoglobulin E (IgE) antibody is depressed in favour of a more T-helper type 1 (Th1)-like response, which is associated with increased interferon gamma (IFN-γ) and IL-10 or IL-12 (Jung *et al.*, 1995; Bellinghausen, 1997; Durham, 1998). Continuing development in this field has improved safety and efficacy, including optimal dosing, allergen modification (to reduce allergenicity while maintaining immunogenicity), adjuvant adsorption (to control release) and adjuvant

activity (to assist immunomodulatory action). A new range of vaccines (Pollinex[®] Quattro) embraces these properties for the prevention or relief of symptoms caused by pollens. Pollinex[®] Quattro Grass contains a mixture of 12 common grass pollen extracts and rye cereal pollen extract, which are all chemically modified by glutaraldehyde and adsorbed onto L-tyrosine with addition of the immunostimulatory adjuvant monophosphoryl lipid A (MPL[®]).

Vaccinations with allergen modification and improved adsorption are not new and include the modified pollen allergen tyrosine adsorbed (MATA) vaccines derived from extracts of one or more pollens that are chemically modified by glutaraldehyde and adsorbed onto L-tyrosine. Variants of the MATA range are currently marketed in various countries under various brand names (e.g. Pollinex[®], TA MIX top) and have been in use for at least 25 years. An array of data are available to show the safe and efficacious use of these materials (Palmer and Balacescu, 1977; Ko *et al.*, 1987; Muehlethaler *et al.*, 1990; Bonifazi *et al.*, 1991; Drachenberg *et al.*, 1999; 2003). Various publications show that L-tyrosine is a safe adjuvant for human use and has both an adsorptive role (as a short-term depot) and a favourable Th1 role (by stimulating production of IgG and not IgE) (Wheeler *et al.*, 1982; Newland *et al.*, 1999; Wheeler and Woroniecki, 2001; Baldrick *et al.*, 2002a). The addition of MPL[®], a detoxified form of lipid A derived from the lipopolysaccharide of *Salmonella minnesota* R595 as an immunostimulatory adjuvant to a range of vaccine materials, is the subject of ongoing clinical trials for many companies. Published non-clinical and clinical data indicate

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that MPL[®] is safe and effective with excellent immunological adjuvant activity (Alving, 1993; Ulrich and Myers, 1995; Thoelen *et al.*, 1998; Moore *et al.*, 1999; Lodmell *et al.*, 2000; Baldrick *et al.*, 2002b). Indeed, *in vitro* and *in vivo* studies show that non-toxic doses of MPL[®] have ideal adjuvant properties at dose levels at, or in the region of, 50 µg of MPL[®], a level that will be used in human allergy vaccine formulations (Baldrick *et al.*, 2002b). Preclinical immunogenicity studies have shown that the antigen absorbed to L-tyrosine/MPL[®] provides a Th1 skewed effect inducing IgG2a and IgG2b responses but no IgE response (Wheeler *et al.*, 2001). Finally, recent clinical work has been performed using a grass pollen MATA vaccine with MPL[®]. Using a four-injection course, the vaccine was found to be efficacious with no serious safety concerns (Drachenberg *et al.*, 2001).

Clinical trials of Pollinex[®] Quattro vaccines are progressing and this paper reports preclinical studies that were performed to show that the material has no toxicological safety issues. Relatively little 'regulatory' guidance for safety testing of these types of products exists. The World Health Organization (WHO) has designated standardized allergen extracts as 'vaccines' in a recent WHO Position Paper on allergen immunotherapy (Bousquet *et al.*, 1998). In Europe, guidance exists for non-clinical evaluation of vaccines (CPMP, 1995) and the WHO has recently issued draft guidance on the same topic (WHO, 2002). These aids have been followed but, owing to the nature of this allergoid material, a specific safety testing strategy was adopted. Studies comprised single-dose toxicity, repeat-dose toxicity and local tolerance studies. Multiple dosing by the subcutaneous route in the repeat-dose toxicity work reflected the clinical situation.

MATERIALS AND METHODS

All studies were performed at Covance Laboratories Ltd, Harrogate, UK in 2000–2001 and conformed to Good Laboratory Practice (GLP).

Test material and formulation

The Pollinex[®] Quattro vaccine for grass pollen allergy is a modified 12-grass/rye cereal pollen extract that is adsorbed by co-precipitation to L-tyrosine and then to MPL[®] adjuvant. The product is similar to some forms of the grass pollen vaccine marketed under the trade name Pollinex[®], which does not contain MPL[®] adjuvant. The pollen powder mix (see Table 1) is extracted into the aqueous phase and then chemically modified with glutaraldehyde, which covalently binds to primary amino groups on the extracted proteins. This procedure gives modified allergens (also known as allergoids) with an ability to reproduce the immunological response to the native allergen — particularly in terms of IgG induction — but with the safety benefit of reduced allergenicity or IgE reactivity (Moran and Wheeler, 1976; Wheeler *et al.*, 1976; Moran *et al.*, 1977). Although major allergens in the individual grass pollens are likely to be similar in terms of their structure and epitopes, some unique minor allergens may occur and the broad range of grass pollens and rye cereal addresses this situation. Indeed, a mixture of cross-reacting extracts is an acceptable approach to allergy vaccination (Bousquet

et al., 1998). The incorporation of L-tyrosine results in the MATA product to which MPL[®] adjuvant is added. A measured antigenic value is used for the starting pollen material, which forms the basis for the calculation of immunological potency in standardized units (SU) ml⁻¹ for the final product. The composition of the test material is given in Table 2.

The test material for the majority of the safety studies was formulated into vials containing 2000 SU ml⁻¹ of grass pollen allergoids (allergen extracts that have been chemically modified), 50 µg ml⁻¹ of MPL[®] adjuvant and 20 mg ml⁻¹ of tyrosine, administered as supplied. This material, referred to hereafter as Pollinex[®] Quattro, is the highest dose of vaccine currently in clinical use. For clinical administration, the vaccine is injected subcutaneously using four doses (300, 800 and 2 × 2000 SU) in weekly intervals.

In a second repeat-dose toxicity study, a high-strength vaccine containing 12 000 SU ml⁻¹ of grass pollen allergoids 50 µg ml⁻¹ of MPL[®] adjuvant and 20 mg ml⁻¹ of tyrosine was used. The control material for the repeat-dose toxicity studies was a 'tyrosine blank' formulation of 20 mg ml⁻¹ of L-tyrosine in 5 mg ml⁻¹ of phenol in neutral-buffered saline. In the single-dose toxicity studies, a further treatment arm of identical formulation to the latter control material but with the addition of 50 µg ml⁻¹ of MPL[®] adjuvant was included to confirm a lack of toxicity with the presence of this adjuvant in the new vaccine formulation. Owing to a lack of any adjuvant-induced study findings in the latter investigations and because other recent work showed a lack of toxicity with repeated oral administration of MPL[®] adjuvant in the rat at the levels used in the present work (Baldrick *et al.*, 2002b), it was considered unnecessary to have a stand-alone group with this material in the repeat-dose toxicity studies. For the local tolerance study, the control materials were 20 mg ml⁻¹ of L-tyrosine blank formulation + 50 µg ml⁻¹ of MPL[®] adjuvant and physiological saline; the latter treatment was included to allow the assessment of potential dose site effects due to adjuvant alone vs full formulation.

Single-dose toxicity studies

In these studies, groups of five male and five female CD-1 mice or Han Wistar rats were given a single subcutaneous or intraperitoneal dose of Pollinex[®] Quattro at dose volumes of 0.05 and 0.5 ml per mouse or 0.1 and 1.0 ml per

Table 1—Grass pollen/rye cereal identification

Common name	Latin name
Bent	<i>Agrostis tenuis</i>
Cocksfoot	<i>Dactylis glomerata</i>
Crested dogstail	<i>Cynosurus cristatus</i>
False oat	<i>Arrhenatherum elatius</i>
Meadow	<i>Poa pratensis</i>
Meadow fescue	<i>Festuca pratensis</i>
Meadow foxtail	<i>Alopecurus pratensis</i>
Rye	<i>Lolium perenne</i>
Soft brome	<i>Bromus spp.</i>
Sweet vernal	<i>Anthoxanthum odoratum</i>
Timothy	<i>Phleum pratense</i>
Yorkshire fog	<i>Holcus lanatus</i>
Cultivated rye (cereal)	<i>Secale cereale</i>

Table 2—Composition of test material^a

Ingredients	Function	Quantity per ml	Reference to standards
Active substances: Modified allergen extract (12 grasses/rye cereal)	Active ingredient	Normal strength: 2000 SU ml ⁻¹ High strength: 12 000 SU ml ⁻¹	HSE ^b
Other ingredients			
L-Tyrosine	Adsorbant base	20 mg (2%)	Ph.Eur.
Monophosphoryl lipid A/AF (MPL [®])	Adjuvant	0.05 mg	HSE ^b
Phenol	Preservative	5.0 mg (0.5%)	Ph.Eur.
Sodium chloride	Tonicity	9.0 mg	Ph.Eur.
Disodium phosphate dodecahydrate and sodium dihydrogen phosphate dihydrate	Buffering and tonicity	Not exceeding 4.0 mg	Ph.Eur.
Water for injection	Diluent	Up to 1.0 ml	Ph.Eur.

^a The control material was identical except that active substances and MPL[®] were not present.

^b HSE: in-house specifications are measured in SU (standardized units) ml⁻¹ (an amount of 2000 SU is approximately equivalent to 24 µg of grass pollen allergen).

rat. Similarly sized control groups were also included for each treatment regimen, using the higher dose volume. At dosing, mice were ca. 6–7 weeks old and weighed 27–36 g (males) and 23–31 g (females); rats were ca. 10–11 weeks old and weighed 257–338 g (males) and 183–212 g (females). Animal husbandry was similar to that described for the repeat-dose toxicity studies. All animals were examined for mortality and clinical signs, body weight was measured on days – 1, 1, 4, 8 and 15 and all animals were sacrificed after 2 weeks for full necropsy.

Repeat-dose toxicity studies

Two similar repeat-dose studies took place with either Pollinex[®] Quattro (top dose antigenic potency of 2000 SU) or at a higher strength not routinely used (12 000 SU). The material at 2000 SU ml⁻¹ was tested initially because it represented a form of allergy vaccine similar to that already marketed as Pollinex[®] but with the inclusion of MPL[®]. It is known that efficacy can be improved by having high antigen levels in allergy vaccines; to support the use of this high-strength vaccine in humans, a second toxicity study was performed. The studies took the form of a 3-week intermittent dose subcutaneous toxicity study in the rat, followed by a 2-week treatment-free period. Groups of up to 15 male and 15 female Han Wistar rats were given a single subcutaneous dose of low- or high-strength vaccine at dose volumes of 0.1 and 1.0 ml per rat once weekly for 3 weeks (a total of three doses). A control group of 15 males and 15 females was also dosed at a dose volume of 1 ml per rat. Rats were ca. 6 weeks old and had body weights of 124–161 g (males) and 112–144 g (females) on study initiation. They were allocated (males and females separately) to the various study groups by a randomisation procedure based on body weight and identified individually by subcutaneous electronic transponder. Animals were maintained under conditions of 22 ± 3 °C, 40–70% relative humidity and a 12-h light/dark cycle. Standard rodent diet and water were available *ad libitum* (other than during certain laboratory investigations) throughout the study. Rats were housed five to a cage.

All rats dosed with 0.1 ml and 10 animals of each sex per group from the control and from the 1.0-ml groups were sacrificed after 3 weeks; the remaining rats were retained for a 2-week recovery period. The following study parameters were measured: mortality and clinical observations, body weights, food consumption, ophthalmoscopy, clinical pathology, organ weights and macroscopic and microscopic examination. Cage-side observations were made daily. Body weights were recorded on day 1, twice weekly and prior to necropsy. Food consumption was measured on a weekly basis and expressed as g rat⁻¹ day⁻¹. An ophthalmoscopic examination was performed predose (all animals) and at week 3 (control and high dose); examination was carried out using an ophthalmoscope after induction of mydriasis. On one occasion during week 3, rats moved to metabolism cages and deprived of food and water overnight to allow the collection of urine samples. The following parameters were measured: volume, specific gravity, pH, glucose, blood, ketones, protein, bilirubin, urobilinogen, reducing substances and microscopy of the sediments. At the end of week 3, blood samples for haematology and clinical chemistry assessment were collected from the lateral caudal vein of all animals after an overnight period without food. Samples were collected in EDTA and lithium heparin tubes, respectively. A further sample was collected into trisodium citrate tubes for measurement of prothrombin time and activated partial thromboplastin time. Further samples were taken from the abdominal aorta of animals in the recovery groups during necropsy. The haematology parameters measured were: haemoglobin concentration, red blood cell count, packed cell volume, reticulocytes (high-strength vaccine study only), mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, platelets, white blood cell count, basophils, neutrophils, lymphocytes, monocytes, eosinophils and large unstained cells. The clinical chemistry parameters measured were: aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, sodium, potassium, chloride, calcium, inorganic phosphate, urea, total bilirubin, creatinine, total protein, albumin, globulin, albumin/globulin ratio, total cholesterol and glucose.

Rats were sacrificed after 3 weeks of treatment, or after the 2-week recovery period, by sodium pentobarbitone overdose prior to exsanguination and then examined grossly for pathological changes. Selected organs were weighed (paired organs together) and comprised: adrenals, kidneys, spleen, liver, heart, brain, pituitary, thyroids + parathyroids, prostate, testes + epididymides and ovaries. After processing to 5- μ m sections and staining with haematoxylin and eosin for light microscopy, the following organs/tissues were examined histopathologically for all animals of the control and high-dose groups (as well as injection sites from the low-dose group): adrenals, axillary lymph nodes, brain, caecum, colon, duodenum, eyes, femur with bone marrow and articular surface, gross lesions, heart, ileum, injection sites, jejunum, kidneys, liver, lungs with mainstem bronchi, mammary gland (female), mandibular and mesenteric lymph nodes, oesophagus, optic nerves, ovaries, pancreas, Peyer's patches, pituitary, prostate, salivary glands, sciatic nerves, skin, spinal cord (at three levels), spleen, sternum with bone marrow, stomach, testes + epididymides, thymus, thyroids + parathyroids, trachea, urinary bladder and uterus.

Statistical analysis involved comparison of the control group with those receiving the active vaccine. Body weight gains and clinical pathology parameters were analysed using a one-way analysis of variance (ANOVA) with Dunnett's test (for pairwise comparisons) and regression analysis (for a dose response), as well as Levene's test for equality of variances. For certain clinical chemistry parameters, re-evaluation with a Kruskal–Wallis non-parametric ANOVA followed by the Terpstra–Jonckheere test for a dose-related trend and the Wicoxon rank sum test for pairwise comparisons was necessary. Organ weights were analysed using analysis of covariance plus Dunnett's and Levene's tests. Probability values of $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered statistically significant.

Local tolerance study

In this study Pollinex[®] Quattro was injected subcutaneously, at a dose level of 1.0 ml per animal, into shaved left and right anterior sites of each of eight New Zealand White rabbits (four males and four females) on day 1. A control formulation was injected similarly into a left posterior side and 1.0 ml of physiological saline was injected into a right posterior side of each rabbit. Skin from an area posterior to the injected control site was identified as an untreated control. At dosing, rabbits were ca. 17 weeks old and weighed 2.7–2.9 kg. Animal husbandry was similar to that described for the repeat-dose toxicity studies. Groups of two males and two females were sacrificed on day 5 and the remaining animals were killed on day 10. Assessment of clinical signs, body weights (day –1, day 1 and prior to necropsy) and local dose site reaction was made throughout the study; dose sites were evaluated macroscopically and histologically following the day 5 and day 10 kills.

RESULTS

Single-dose toxicity studies

There were no study findings for mice given a single subcutaneous dose of Pollinex[®] Quattro at 2000 SU ml⁻¹ with

dose volumes of 0.05 and 0.5 ml per mouse. Mice dosed by the intraperitoneal route showed clinical signs of tremors and increased activity and/or unkempt appearance in controls and cases of lethargy or unkempt appearance with the 0.5-ml dose. Tremors continued for 1 or 2 h post-dose. These findings in both control and vaccine-treated mice were considered to be largely attributable to the phenolic content (0.5% w/v) of the dose formulation. For rats, there were no study findings following a single subcutaneous or intraperitoneal dose of Pollinex[®] Quattro at 2000 SU ml⁻¹ with dose volumes of 0.1 and 1.0 ml per rat. There were no differences between the control groups, which differed only by the presence of MPL[®].

Repeat-dose toxicity studies

A complete summary of study findings is given in Table 3. Single subcutaneous doses of both Pollinex[®] Quattro and high-strength (12 000 SU) vaccine at 0.1 and 1.0 ml per rat once weekly for 3 weeks were well tolerated, with no obvious signs of systemic toxicity. It is likely that the slight decrease in body weight gain (ca. 9%) seen for males treated with the high-strength vaccine at 1.0 ml per rat is coincidental. At 1.0 ml per rat, both strengths of vaccine showed effects associated with raised immunoactivity, which often reached statistical significance in comparison with the controls. In both studies and for both sexes, raised white blood cell count and associated increased neutrophil count, decreased albumin level, increased globulin level and an altered (reduced) albumin/globulin ratio were seen. Other findings comprised a slightly raised lymphocyte count for both sexes (high-strength vaccine) and an increased spleen weight (ca. 9%) for females (both vaccines). The only other notable findings were slightly increased platelets for both sexes (both vaccines) and an increase in liver weight (ca. 9%) for males receiving Pollinex[®] Quattro; because the latter finding was of minimal magnitude and seen only in one study and for one sex, it was considered to be a coincidental finding. None of these findings were seen after the 4-week recovery period. At the terminal kill for both studies there were macroscopic (red or dark discolouration and raised areas or thickening) and microscopic (cellulitis/fibrosis, abscess, haemorrhage and/or granulomas) findings suggestive of local damage at the injection site of both control and treated animals. However, the severity of the findings was generally greater in high-dose animals. Cellulitis was characterized by a mixed inflammatory cell infiltration of the subcutis, with varying levels of fibrosis, pigmented macrophages and foci of myositis of adjacent muscle. Abscesses took the form of an accumulation of necrotic, polymorph-rich exudate surrounded by a capsule of macrophages. Granulomas were characterized by aggregates of macrophages within the subcutis and sometimes in the surrounding cystic spaces. At the treatment-free recovery kill there was evidence of partial recovery from the microscopic observations. The main finding was cellulitis/fibrosis with a prominent fibrotic response and only a minor inflammatory cell component. Other than the dose site changes, there were no notable study findings at 0.1 ml per rat for both strength vaccines.

Local tolerance study

Single subcutaneous injections of 1.0 ml of Pollinex[®] Quattro at 2000 SU ml⁻¹ to the rabbit resulted in no clinical

Table 3—Key effect of repeat subcutaneous administration of Pollinex® Quattro on various toxicity study parameters after a 3-week treatment and a 2-week recovery period

Parameter		Normal strength study (2000 SU)			High strength study (12 000 SU)		
		Dose level (ml per animal) and findings ± SD					
		Control	0.1	1	Control	0.1	1
Body weight gain (g), weeks 1–3	M	72.3 ± 6.91	73.4 ± 17.8	76.0 ± 14.0	87.8 ± 4.85	91.9 ± 11.4	80.4 ± 7.68*
	F	38.3 ± 6.74	38.5 ± 4.58	41.5 ± 5.01	44.1 ± 5.96	41.5 ± 6.01	42.7 ± 6.26
Body weight gain (g), weeks 4–5	M	17.5 ± 2.67	–	18.0 ± 2.26	37.7 ± 3.73	–	49.1 ± 7.63**
	F	4.3 ± 1.36	–	5.7 ± 3.96	23.8 ± 2.91	–	27.0 ± 4.18
Platelets (1000 mm ⁻³), week 3	M	1016 ± 100	1095 ± 187	1185 ± 155**	1046 ± 209	1102 ± 150	1082 ± 166
	F	971 ± 110	951 ± 82	1061 ± 108**	1019 ± 246	1005 ± 124	1038 ± 242
Platelets (1000 mm ⁻³), week 5	M	950 ± 71	–	912 ± 43	Not measured	–	Not measured
	F	832 ± 168	–	940 ± 60	Not measured	–	Not measured
White blood cells (1000 mm ⁻³), week 3	M	7.0 ± 2.1	7.6 ± 1.2	7.5 ± 2.5	7.1 ± 1.7	7.4 ± 2.0	8.0 ± 1.7
	F	6.6 ± 1.8	5.0 ± 0.8*	7.3 ± 1.5	5.8 ± 1.2	5.4 ± 1.3	7.3 ± 1.4**
White blood cells (1000 mm ⁻³), week 5	M	2.7 ± 2.0	–	3.5 ± 0.8	5.3 ± 0.7	–	6.1 ± 1.4
	F	3.7 ± 0.6	–	2.4 ± 1.0	4.4 ± 0.4	–	6.0 ± 1.3*
Neutrophils (1000 mm ⁻³), week 3	M	1.1 ± 0.5	1.2 ± 0.3	1.5 ± 0.5	0.8 ± 0.2	0.8 ± 0.2	1.2 ± 0.4**
	F	0.9 ± 0.5	0.7 ± 0.3	1.4 ± 0.4**	1.1 ± 0.4	1.0 ± 0.5	2.0 ± 0.6***
Neutrophils (1000 mm ⁻³), week 5	M	0.5 ± 0.3	–	0.5 ± 0.2	0.9 ± 0.3	–	1.1 ± 0.4
	F	0.7 ± 0.2	–	0.5 ± 0.2	0.7 ± 0.2	–	0.9 ± 0.2
Lymphocytes (1000 mm ⁻³), week 3	M	5.7 ± 2.0	6.1 ± 0.9	5.7 ± 2.0	6.0 ± 1.5	6.2 ± 1.7	6.5 ± 1.4
	F	5.5 ± 1.4	4.0 ± 0.6*	5.5 ± 1.2	4.4 ± 1.0	4.1 ± 0.8	5.0 ± 1.0
Lymphocytes (1000 mm ⁻³), week 5	M	2.1 ± 1.7	–	2.9 ± 0.6	4.2 ± 0.5	–	4.8 ± 1.0
	F	2.9 ± 0.4	–	1.8 ± 0.8	3.5 ± 0.5	–	4.8 ± 1.1*
Albumin (g l ⁻¹), week 3	M	32 ± 1	32 ± 1	29 ± 1***	43 ± 2	43 ± 2	40 ± 2***
	F	32 ± 2	34 ± 2	29 ± 1***	41 ± 3	44 ± 2**	38 ± 2**
Albumin (g l ⁻¹), week 5	M	31 ± 1	–	31 ± 1	46 ± 2	–	44 ± 1
	F	32 ± 1	–	31 ± 1	48 ± 3	–	46 ± 3
Globulin (g l ⁻¹), week 3	M	33 ± 2	31 ± 1*	36 ± 2***	23 ± 2	22 ± 2*	27 ± 2***
	F	31 ± 2	32 ± 3	35 ± 2***	21 ± 2	20 ± 2	27 ± 2***
Globulin (g l ⁻¹), week 5	M	29 ± 1	–	29 ± 2	22 ± 2	–	22 ± 1
	F	28 ± 1	–	28 ± 2	19 ± 2	–	20 ± 2
Albumin/globulin ratio, week 3	M	1.0 ± 0.1	1.0 ± 0.0	0.8 ± 0.0***	1.9 ± 0.1	2.0 ± 0.2*	1.5 ± 0.1***
	F	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.0	1.9 ± 0.3	2.2 ± 0.3**	1.4 ± 0.1***
Albumin/globulin ratio, week 5	M	1.0 ± 0.1	–	1.1 ± 0.1	2.1 ± 0.3	–	2.0 ± 0.1
	F	1.1 ± 0.1	–	1.1 ± 0.1	2.5 ± 0.3	–	2.3 ± 0.2
Spleen weight (g) ^a , week 3	M	0.481	0.453	0.465	0.540	0.564	0.528
	F	0.384	0.379	0.424*	0.391	0.398	0.442
Spleen weight (g) ^a , week 5	M	0.580	–	0.543	0.536	–	0.516
	F	0.428	–	0.416	0.422	–	0.445
Liver weight (g) ^a , week 3	M	5.382	5.410	5.937*	5.957	5.918	5.901
	F	4.251	4.495	4.527	4.275	4.395	4.334
Liver weight (g) ^a , week 5	M	6.815	–	6.688	6.695	–	6.706
	F	5.051	–	4.840	4.851	–	4.867
Macroscopic findings ^b							
After 3 weeks		Yes ^c	Yes ^c	Yes ^c	Yes ^c	Yes ^c	Yes ^c
After 2 weeks' recovery		No	–	No	No	–	No
Microscopic findings: after 3 weeks ^b							
Cellulitis/fibrosis		16/20	18/20	20/20	19/20	18/19 ^e	20/20
Abscess		1/20	1/20	16/20	3/20	0/19 ^e	12/20
Haemorrhage		8/20	2/20	12/20	2/20	0/19 ^e	7/20
Granuloma		9/20	2/20	5/20	0/20	0/19 ^e	0/20
Microscopic findings after 2 weeks' recovery ^b							
Cellulitis/fibrosis		7/9 ^d	–	10/10	4/10	–	8/10
Granuloma		0/9 ^d	–	2/10	0/10	–	0/10

^a Adjusted for final necropsy body weight.^b Injection site only.^c Red or dark discoloration, and raised areas or thickening.^d One control group female died as a result of the blood sampling procedure during week 3.^e Due to accidental trauma (damage to the hind legs), one low-dose group was sacrificed on day 5.

M, male; F, female.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

signs, dermal reactions or macroscopic changes. Microscopic findings at vaccine injection sites revealed a local inflammatory response (primarily foreign body granulomas, characterized by aggregates of macrophages, together with minor levels of polymorphs and necrotic debris, sometimes surrounding cystic spaces, and cellulitis/fibrosis, characterized by a mixed inflammatory cell infiltration of the subcutis, with varying levels of fibrosis) in comparison with saline and untreated sites but were comparable with the degree of response seen at the control injection site. Findings at 10 days post-dosing were reduced in comparison with those at 5 days after administration, suggesting partial recovery. Cellulitis/fibrosis also tended to have reduced inflammation and a greater fibrotic component by day 10.

DISCUSSION

Modern allergy immunotherapy for the treatment of sensitivity to grass pollen can utilize allergoids to improve patient safety. The allergoids used in Pollinex® Quattro are pollen extracts chemically modified with glutaraldehyde, which are potentially useful, particularly in terms of reduced IgE reactivity while maintaining the ability of specific IgG induction (Moran and Wheeler, 1976; Wheeler *et al.*, 1976; Moran *et al.*, 1977). Adsorption of these allergoids to L-tyrosine to enhance both safety and efficacy has resulted in a range of allergen extract formulations with useful potential (Wheeler *et al.*, 1982; Newland *et al.*, 1999; Wheeler and Woroniecki, 2001; Baldrick *et al.*, 2002a). Clinical use of these vaccines (e.g. as Pollinex®) has shown a safe and efficacious profile over many years of human use (Palmer and Balacescu, 1977; Verstraeten and Wheeler, 1978; Metzger *et al.*, 1981; Ko *et al.*, 1987; Muehlethaler *et al.*, 1990; Bonifazi *et al.*, 1991; Drachenberg *et al.*, 1999; 2003). Recent animal antibody induction studies using ragweed pollen MATA materials with MPL® adjuvant have shown further evidence of a Th1 skewing effect (Wheeler *et al.*, 2001). A recent clinical study using Pollinex® Quattro for the treatment of grass pollen allergy has shown raised grass-pollen-specific IgG antibody but no rise in specific IgE as a result of immunotherapy (Drachenberg *et al.*, 2001).

The battery of non-clinical studies reported here support safe clinical use of this material by indicating a lack of toxicological findings. Single doses of Pollinex® Quattro (2000 SU ml⁻¹) by the subcutaneous route in the mouse and rat and by the intraperitoneal route in the rat showed no effects at levels greatly above a typical clinical dose volume of 1.0 ml for these types of materials. Thus, on a weight-to-weight basis, a safety margin of ca. 1200-fold (mouse) or ca. 240-fold (rat) was achieved for a 60-kg person. Limited clinical signs of central nervous system (CNS) origin were seen in all groups (including controls) following single intraperitoneal administration in the mouse and were considered to be attributable largely to the phenolic content (0.5% w/v) of the dose formulation. CNS toxicity has been reported in rodents for phenol (WHO, 1994).

Repeated clinical doses of Pollinex® Quattro will be used and potential adverse effects were examined following three weekly doses to the rat using Pollinex® Quattro (2000 SU ml⁻¹) and high-strength versions of the vaccine.

No obvious signs of systemic toxicity were found and study findings largely reflected the immunostimulating activity of the tested materials. Thus, findings of increased white cell parameters (notably neutrophils), altered albumin and globulin levels and/or increased spleen weight seen at 1.0 ml per rat are not unexpected considering that the modified pollen extract has activity *per se* and MPL® is an immunologically active adjuvant. Although a specific control group formulation with MPL® was not included in the present repeat-dose investigations, recent reviews of animal studies evaluating the potential toxicity of L-tyrosine and MPL® have highlighted consistent findings associated with stimulation of the immune system (notably increased white blood cell values and increased spleen weight) in the rat and dog (Baldrick *et al.*, 2002a,b). Furthermore, a recent 4-week repeat-dose toxicity study in the rat with MPL® adjuvant using the subcutaneous dose route showed remarkably similar changes to the present studies, namely limited study findings of raised neutrophil count and altered albumin and globulin levels at a dose level of 100 µg kg⁻¹ day⁻¹ (Baldrick *et al.*, 2002b). No findings were seen at a level to be used clinically in Pollinex® Quattro (namely, ca. 1.0 µg kg⁻¹ day⁻¹). A house-dust mite allergy vaccine (a 50:50 mixture of the house-dust mites *Dermatophagoides pteronyssinus* and *D. farinae* protein chemically modified by glutaraldehyde and adsorbed onto L-tyrosine with addition of MPL®), administered subcutaneously to the rat once weekly for 13 weeks, raised neutrophil (and total white cell) count and spleen weight (Baldrick *et al.*, 2002c). These findings again were related largely to the immunostimulatory components of the vaccine. The only other systemic finding in the present studies was slightly raised platelet levels for both strength vaccines. Results from the studies mentioned above show no similar change following the use of high levels of immunologically activity materials and it is considered that this finding is probably coincidental and of no toxicological relevance.

In the present studies, the limited signs of immunostimulation were not seen at the lower dose level of 0.1 ml per rat, nor following a 4-week non-dose period indicating a reversibility of the effects. On the basis of a 1-ml clinical dose volume, the dose levels used for Pollinex® Quattro at 2000 SU ml⁻¹ and the high-dose vaccine represent safety margins of ca. 24-fold and 240-fold, respectively, for a 60-kg person.

A local tolerance study in the rabbit using a subcutaneous dose volume of 1.0 ml of Pollinex® Quattro at 2000 SU ml⁻¹ showed notable microscopic findings indicative of a local inflammatory response at the dose site at 5 days post-injection. However, these findings were similar to those seen with a formulation lacking pollen extract (L-tyrosine plus MPL® adjuvant). Evidence of some recovery within 10 days of dose administration was noted. In the repeat-dose rat toxicity studies, findings at the injection site of control (L-tyrosine formulation) and Pollinex® Quattro-treated animals following once-weekly administration were consistent with those of local damage. Evidence of some recovery following a 2-week recovery period was noted. Subcutaneous administration of a glutaraldehyde-modified house-dust mite vaccine (containing L-tyrosine plus MPL® adjuvant) in a local tolerance study in the rabbit and a repeat-dose toxicity study in the rat also showed microscopic inflammatory changes at the dose site (Baldrick *et al.*, 2002c). These findings again showed evidence of recovery.

A potential for local effects with Pollinex® Quattro is not unexpected and dose site reactions are often seen following clinical vaccination and can be related to the presence of an adjuvant. Indeed, aluminium salts (which have been used as adjuvants for many years in human vaccines) are known to produce local reactions in animals and clinically (Gupta *et al.*, 1995; Vogel and Powell, 1995; Singh and O'Hagan, 1999). It is also well-known that granulomas can occur after administration of aluminium adsorbants. The findings reported here are likely, at least in part, to be related to the presence of L-tyrosine in the dose formulation. A recent review of animal study data using L-tyrosine has shown that injections may result in local swelling and an inflammatory response associated with deposited material (probably related to the low solubility of L-tyrosine) (Baldrick *et al.*, 2002a). However, these responses resolved rapidly, even after multiple injections, and showed no evidence of a progressive nature. An impressive human database using modified grass pollen L-tyrosine adsorbate vaccines has shown them to be well-tolerated and effective but with some evidence of local reactions (including swelling and/or erythema and itch) (Miller, 1976; Miller *et al.*, 1976; Palmer and Balacescu, 1977; Verstraeten and Wheeler, 1978; Drachenberg *et al.*, 1999; 2003). However, comparative studies have shown that local injection reactions are no worse than with conventional non-L-tyrosine-adsorbed materials such as aluminium-adsorbed extracts (Symington *et al.*, 1977; Muehlethaler *et al.*, 1990; Bonifazi *et al.*, 1991). Furthermore, no granulomas occur after administration of L-tyrosine adsorbates, which is due to complete solubilization of the natural amino acid. Incident rates from large clinical numbers are now available and it has been shown recently that local reactions (redness/swelling >5 cm diameter) following the use of nearly 13 000 injections of MATA vaccines in over 1800 patients have been <2% (Drachenberg *et al.*, 2003). A further recent study of MATA vaccines containing MPL® (with 2% L-tyrosine and 50 µg ml⁻¹ MPL®) in a similar number of patients showed

a higher injection reaction rate of 8.6% following 8500 injections (Drachenberg *et al.*, 2002). Possibly this higher rate could result from an immunostimulatory effect of MPL® adjuvant. However, these figures are not exactly comparable owing to the treatment of patients with several types of allergoids (modified pollens from trees, grasses, rye, herbs) in varying proportions for each study.

Animal studies have shown microscopic findings of an 'expected' local dose site effect from Pollinex® Quattro with evidence of reversibility. Although these effects may be related largely to the presence of tyrosine, it should be noted that there was evidence that the severity of the injection site findings in the rat (but not the rabbit) was greater when Pollinex® Quattro (notably at 1.0 ml per rat) was compared with the L-tyrosine control, indicating that the rest of the Pollinex® Quattro formulation may have a role in this enhanced response.

In conclusion, a specific preclinical safety testing strategy developed to support clinical use of Pollinex® Quattro and comprising single- and repeat-dose toxicity and local tolerance studies has shown that there were no toxicological findings of significance at levels greatly in excess to those proposed for clinical use. A not unexpected, but relatively minor, immunostimulatory effect was seen following repeated dosing (once weekly for 3 weeks) at 1.0 ml per rat. The Pollinex® Quattro formulation also resulted in an injection site reaction, which is probably due to the presence of L-tyrosine although it is possible that MPL® may contribute. It should be pointed out that Pollinex® Quattro vaccine has been evaluated recently in a multicentre, placebo-controlled, randomized, double-blind clinical study of over 80 grass-pollen-sensitive patients and was shown to be well-tolerated (Drachenberg *et al.*, 2001).

Acknowledgement

MPL® is a registered trademark of Corixa Corporation, Seattle, Washington, USA.

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