

A battery of genotoxicity studies with an allergy vaccine adjuvanted with monophosphoryl lipid A (MPL[®]) for the treatment of grass pollen allergy

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ABSTRACT: The allergy vaccine Pollinex[®] Quattro Grass, developed for the prevention/relief of allergic symptoms caused by grass pollen in adults and children, contains extracts of 12 grass pollens and rye cereal (all chemically modified by glutaraldehyde), adsorbed onto L-tyrosine with addition of the immunostimulatory adjuvant monophosphoryl lipid A (MPL[®]). This paper represents the first publication on genetic toxicology data for such a vaccine. An Ames test using five strains of *Salmonella typhimurium* at concentrations up to 2000 standardized units (SU) per plate in the absence (–) and presence (+) of metabolic activation (rat liver S9) showed negative results, as did treatment – S9 in the mouse lymphoma assay (MLA). However, a reproducible positive response was noted in the MLA + S9, which could not be attributed to extreme culture conditions, but may have been an artefact of the exogenous metabolizing system. Up to 60% false positive results are reported for the MLA with noncarcinogens. Hence, *in vivo* genotoxicity studies were conducted. These further assessments comprised a rat bone marrow micronucleus test following subcutaneous (s.c.) doses of 10 000, 20 000 or 40 000 SU kg^{–1} per day for 2 days, and a comet assay in liver and kidney cells from rats treated with Pollinex[®] Quattro Grass at 20 000 or 40 000 SU kg^{–1} per day s.c. for 2 days. Plasma analysis showed evidence of systemic antibodies to Pollinex[®] Quattro Grass immunogens, but the exposure levels could not be quantified. No evidence of genotoxicity was observed in either of the *in vivo* studies. Overall, the genotoxicity test results supported safe clinical use of Pollinex[®] Quattro Grass. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: grass pollen; allergy vaccines; Pollinex[®] Quattro Grass; MPL[®] adjuvant; Ames test; mouse lymphoma assay; micronucleus test; comet assay

INTRODUCTION

Grass pollen has been identified as a common causative allergen in perennial allergic rhinitis and asthma, in both adults and children (Bufe *et al.*, 2009; D'Amato *et al.*, 1998; Suphioglu, 1998). For patients suffering from pollen allergy, a form of vaccination known as specific immunotherapy (SIT) has been established. SIT involves subcutaneous administration of increasing doses of specific allergen extracts in an attempt to prevent the allergic symptoms caused by binding of immunoglobulin E (IgE) antibodies to grass pollen, with subsequent binding to mast cells and basophils, resulting in degranulation and release of histamine (Brozek *et al.*, 2010; Ebner, 1999; Larche, 2006; Malling, 2000; Niederberger, 2009; Rolland *et al.*, 2000). The immunological mechanism of SIT in reducing inhalant allergies is not fully understood, but may involve induction of an allergen-specific T cell tolerance; modification of a T-helper 2 (Th2) lymphocyte-dominated allergen-specific response to a Th1 response; or shifting the response to induce allergen-specific IgG antibodies which antagonize the effect of IgE (Akdis and Akdis, 2007; James and Durham, 2008; Larche, 2006; Niederberger, 2009).

The need to produce safer and more effective SIT therapy products has led to the development of the Pollinex[®] Quattro Grass vaccine, a 12 grass/rye cereal pollen extract that is chemically modified by glutaraldehyde and adsorbed onto L-tyrosine with the addition of the immunostimulatory adjuvant, monophosphoryl

lipid A (MPL[®]). Previous work (Baldrick *et al.*, 2002a) has shown that L-tyrosine is a safe adjuvant for human use and has both an adsorptive role (as a short-term depot) and a favourable immunological effect. The safe and immunostimulatory nature of the adjuvant material MPL[®] – a detoxified form of lipid A from the lipopolysaccharide of *Salmonella minnesota* R595 – has also been reported and reviewed elsewhere (Baldrick *et al.*, 2002b, 2004). MPL[®] is also present as an adjuvant in Cervarix[™] and Fendrix[™], vaccines used for the prevention of cervical cancer and against hepatitis B virus, respectively (Segal *et al.*, 2011; EMA, 2011a, b).

To support product licence application for marketing and safe clinical use of Pollinex[®] Quattro Grass, a package of nonclinical studies has been performed based on scientific judgement, as no specific guidelines on such studies exist for allergen preparations. The results of single and repeat doses as well as local tolerance studies have been published and showed no

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toxicological safety concerns (Baldrick *et al.*, 2004). Although unusual for vaccines in general, genotoxicity testing was considered useful to confirm the lack of potential findings, and this paper presents the results of genotoxicity studies performed *in vitro* and *in vivo*, on Pollinex[®] Quattro Grass.

MATERIALS AND METHODS

The battery of genotoxicity studies were performed at Covance Laboratories Ltd, Harrogate, UK in 2007 (bacterial reverse mutation assay; Ames test), 2008 (mouse lymphoma assay, MLA) and 2008–2009 (rat bone marrow micronucleus test and comet assay) and conformed to Good Laboratory Practice. Animal care and use was compliant with the Association for Assessment and Accreditation of Laboratory Animal Care International recommendations and study designs underwent internal ethical review.

Test Material and Formulation

The Pollinex[®] Quattro Grass vaccine is a modified 12 grass/rye cereal pollen extract, derived from natural source material, which is adsorbed by co-precipitation to L-tyrosine and then to MPL[®] adjuvant. The product is similar to some forms of grass pollen vaccine marketed under the trade name Pollinex[®], which do not contain MPL[®] adjuvant. The pollen powder mix (see Table 1) is extracted into the aqueous phase, then chemically modified with glutaraldehyde, which covalently binds to primary amino groups on the extracted proteins. This procedure produces 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; Moran *et al.*, 1977; Wheeler *et al.*, 1976). The incorporation of L-tyrosine results in the modified pollen allergens tyrosine adsorbed (MATA) product (Wheeler *et al.*, 1982), to which MPL[®] adjuvant is added. A measured antigenic value is used for the starting pollen material and appropriate subsequent stages that forms the basis for the calculation of immunological potency in standardized units per millilitre (SU ml⁻¹) for the final product. The composition of test material is given in Table 2.

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>

The test material, referred to hereafter as Pollinex[®] Quattro Grass, used for the genotoxicity studies was formulated into vials containing 4000 SU ml⁻¹ of grass pollen allergoids along with 100 µg ml⁻¹ MPL[®] adjuvant and 2% w/v tyrosine. The maximum feasible test concentrations and doses of Pollinex[®] Quattro Grass in the battery of genotoxicity tests were 2000 SU per plate (Ames test), 200 SU ml⁻¹ (MLA) and 40 000 SU kg⁻¹ per day in the micronucleus test and in the comet assay, both conducted in the rat. In comparison, weekly ascending 0.5 ml subcutaneous doses of 300, 800 and 2000 SU per 0.5 ml Pollinex[®] Quattro Grass represent the clinical dose situation.

Animals and their Maintenance

For the *in vivo* genotoxicity studies, male and female rats of the strain Han Wistar Crl:WI (Han) were obtained from Charles River (UK) Ltd. Animals were randomized to groups of six using a system of random numbers and were maintained under conditions of 22 ± 3 °C, 40–70% relative humidity and a 12 h light/dark cycle. Standard rodent diet (SQC Rat and Mouse Maintenance Diet no. 1, expanded, obtained from Special Diets Services Ltd, Witham, UK) and water were available *ad libitum* throughout the study. All animals were observed daily for clinical signs of ill health or overt toxicity.

Dose range-finding work showed no differences in toxicity between the sexes, hence only male animals were used for the main experiments. After an acclimatization period, treatment proceeded with administration of subcutaneous doses of Pollinex[®] Quattro Grass or control at a dose volume of 10 ml kg⁻¹. At initiation of treatment, the animals were 6–7 weeks old and weighed 209–260 g.

Ames Test

Pollinex[®] Quattro Grass was evaluated for the induction of reverse mutations and cytotoxicity in bacterial cells using five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) using a design in compliance with the published recommendations (Maron and Ames, 1983; Gatehouse *et al.*, 1990; ICH, 2009; OECD, 1997a). All the tester strains, with the exception of TA102, were originally obtained from UK NCTC. Strain TA102 was derived from a culture obtained from Glaxo Group Research Limited. Following an initial range-finding experiment, two independent experiments were performed using treatments in the absence and presence of exogenous metabolic activation (S9) with three replicate plates per test concentration and six concentrations in each experiment (from 0.64 to 2000 SU per plate in experiment 1 and a narrowed dose range of 6.325–2000 SU per plate in experiment 2). The S9 was obtained from Molecular Toxicology Incorporated, USA, where it was prepared from male Sprague–Dawley rats induced with Aroclor 1254. Negative controls (purified water) were included in quintuplicate; positive control chemicals were obtained from Sigma Aldrich Chemical Co. (Poole, UK) and were included as triplicate plates as follows: 5 µg ml⁻¹ 2-nitrofluorene (TA98 –S9), 2 µg ml⁻¹ sodium azide (TA100, TA1535 –S9), 50 µg ml⁻¹ 9-aminoacridine (TA1537 –S9), 0.2 µg ml⁻¹ Mitomycin C (TA102 –S9), 10 µg ml⁻¹ benzo[a]pyrene (TA98 + S9) and 2-aminoanthracene at 5 µg ml⁻¹ (TA100, TA1535 and TA1537 + S9) and 20 µg ml⁻¹ (TA102 + S9). The highest feasible test concentration was 2000 SU ml⁻¹, based on the supplied stock concentration of 4000 SU ml⁻¹ of Pollinex[®] Quattro Grass and a maximum

Table 2. Composition of test material

Ingredient	Function	Content per 0.5 ml	Reference to Standard
<i>Active substance</i>			
Allergen extract (12 grasses/rye cereal)	Active ingredient	Strengths of 300, 800 and 2000 SU	IHRP ^a
<i>Other ingredients</i>			
L-Tyrosine	Adsorbant base	10 mg (2%)	Ph.Eur.
MPL [®]	Adjuvant	0.05 mg	In house specification ^b
Phenol	Preservative	2.5 mg (0.5%)	Ph.Eur.
Sodium chloride	Tonicity	4.5 mg	Ph.Eur.
Disodium phosphate dodecahydrate	Buffering and tonicity	0.25 mg	Ph.Eur.
Sodium dihydrogen phosphate dihydrate	Buffering and tonicity	0.75 mg	Ph.Eur.
Water for injection	Diluent	to 0.5 ml	Ph.Eur.

^aIHRP, in-house reference preparation: SU, standardized units (an amount of 2000 SU per 0.5 ml is approximately equivalent to 8.42 µg of Timothy grass major allergen)/

^bAdded as MPL-AF, an aqueous formulation of MPL at a concentration of 200 µg ml⁻¹. Ph.Eur. *European Pharmacopoeia*.

volume of 0.5 µg ml⁻¹ of test article solution to allow the agar to set sufficiently. The standard plate incorporation method was used for all treatments in experiment 1 and for treatments in the absence of S9 in experiment 2. Treatments in the presence of S9 in the second experiment included a pre-incubation step (60 min at 37 °C). Colonies were counted electronically using a Seescan colony counter (Seescan plc.) or manually where confounding factors such as splitting or bubbles in the agar affected the accuracy of the automated counter. The background lawn was inspected for signs of toxicity.

Standard statistical tests (De Serres and Shelby, 1979; Mahon *et al.*, 1989) were used to analyse the data for the Pollinex[®] Quattro Grass and the positive controls including calculation of the *m*-statistic to check that the data were Poisson-distributed; use of Dunnett's test to compare the counts at each concentration with those of the vehicle control; and linear regression analysis to determine the presence or otherwise of a concentration response.

Mouse Lymphoma Assay

Pollinex[®] Quattro Grass was evaluated for its potential to induce tk gene mutations in mouse lymphoma L5178Y cells. The assay was carried out using the microwell method. L5178Y TK^{+/−} mouse lymphoma cells were originally obtained from Dr D. Clive, Burroughs Wellcome Co. and stored as frozen stocks in liquid nitrogen. The protocol employed was consistent with current guidelines (Cole *et al.*, 1983; Cole *et al.*, 1990; OECD, 1997b) and consensus agreements discussed during successive Mouse Lymphoma Workshops (Clive *et al.*, 1995; Moore *et al.*, 2000, 2003, 2006).

Following initial range-finding experiments, two independent experiments were performed in the absence and presence of S9 using a 3 h treatment period in the presence of S9 and 3 or 24 h treatment in the absence of S9. Nine concentrations were tested in each experiment using duplicate cultures (ranging from 12.5 to 200 SU ml⁻¹ in experiment 1 and from 20 to 200 SU ml⁻¹ in experiment 2). Appropriate negative control (vehicle, purified water) and positive control treatments [4-nitroquinoline 1-oxide in the absence of S9 and benzo(a)pyrene in the presence of S9, from Sigma Aldrich Chemical Co. Poole, UK] were included. Precipitation of the test material occurred at concentrations of

100 SU ml⁻¹ and above. Hence, the highest test concentrations selected for determination of viability and 5-trifluorothymidine (TFT)-resistance were 100 or 125 SU ml⁻¹. Cultures were maintained for an expression period of 2 days prior to plating out in microtitre plates for viability assessment (192 wells averaging 1.6 cells per well) and TFT-resistance (384 wells at 2 × 10³ cells per well). Following incubation, wells containing clones were identified by eye using background illumination. Standard evaluation methods were used to analyse study parameters for the Pollinex[®] Quattro Grass comprising the global evaluation factor (GEF, Moore *et al.*, 2006) and one-sided linear regression analysis (chi-squared test).

Micronucleus Test

Pollinex[®] Quattro Grass has been evaluated for the induction of micronuclei (MN) *in vivo* following subcutaneous (s.c.) administration in the rat [Han Wistar Crl:WI (Han)]. A preliminary range-finding experiment (*n* = 3/sex) showed no clinical signs of toxicity in the 48 h observation period following 2 daily s.c. doses at the maximum feasible dose of 40 000 SU kg⁻¹ per day (based on a supplied concentration of 4000 SU ml⁻¹ and a dose volume of 10 ml kg⁻¹). No gender differences were seen; therefore male animals alone (*n* = 6/dose group) were chosen for the main study and administered 10 000, 20 000 or 40 000 SU kg⁻¹ per day s.c. Dosing occurred on two successive days at a 24 h interval. Treatments using negative control (vehicle, sterile saline for injection) and positive control (cyclophosphamide, a single s.c. dose of 20 mg kg⁻¹, 24 h before sampling) were included. Animals were killed approximately 24 h after the second and final dose and bone marrow was sampled and smears prepared for the estimation of MN frequency following staining with acridine orange. Micronuclei (MN) were scored in polychromatic erythrocytes (PCE, 2000 per rat) and the ratio of polychromatic to normochromatic erythrocytes (expressed as %PCE) was determined from a sample of 1000 total erythrocytes of the bone marrow. All scoring was conducted following 'blinding' of the data. This study was designed to comply with the recommendations in the published literature (Richold *et al.*, 1990; Hayashi *et al.*, 1994, 2000) and relevant regulatory guidelines (OECD, 1997c; ICH, 2009).

Standard statistical tests were used to analyse study parameters for the Pollinex[®] Quattro Grass-treated groups vs control: The number of micronucleated polychromatic erythrocytes (MN PCE) in vehicle control animals was compared with the laboratory's historical control data to determine if the assay was acceptable. For each group, inter-individual variation in numbers of MN PCE was estimated using a heterogeneity chi-squared test and the numbers of MN PCE in each treated group were compared with the numbers in the vehicle control group using a 2 × 2 contingency table to determine chi-squared (Lovell *et al.*, 1989). A test for linear trend was applied to evaluate possible dose-response relationships.

Comet Assay

Pollinex[®] Quattro Grass has also been evaluated for its ability to induce DNA damage in rat liver and kidney *in vivo* using the comet assay. The liver and kidney were selected as it has been reported that MPL has the potential to accumulate in these tissues (Reddy and Kishore, 1996). The maximum dose and the use of only male animals was based on the preliminary toxicity evaluation for the micronucleus test (see above).

Groups of six male rats [Han Wistar Crl:WI (Han)] were dosed s.c. with vehicle (sterile saline for injection) or Pollinex[®] Quattro Grass at 20 000 or 40 000 SU kg⁻¹ per day, on two successive days at a 24 h intervals and animals were killed 3 h after the second dose. The highest dose was the maximum achievable with the preparation of Pollinex[®] Quattro Grass provided. Positive control animals received a single oral (gavage) dose of ethyl methanesulfonate (250 mg kg⁻¹), 3 h before sacrifice. This study design allows assessment of cells that have been exposed to the test agent over a 3–27 h period prior to sampling in accordance with published recommendations (Hartmann *et al.*, 2003, 2004). Samples of liver and kidney cells were taken and processed for assessment of DNA damage (comets) according to the recommendations of Tice *et al.* (2000). Four slides were prepared per single cell suspension, per tissue: two to three for assessment of comets and one (diffusion slide) for assessment of cell damage. Comet induction was assessed using 100 cells per animal. DNA migration was assessed using the tail moment, although tail intensity (%DNA in tail) was also measured. Cytotoxicity was assessed in terms of the proportion (%) of cells with morphology indicative of severe toxicity, necrosis and/or apoptosis (termed 'clouds') and the percentage 'diffused cells' based upon a sample size of 100 cells per slide. All scoring was conducted following 'blinding' of the data. Standard statistical tests were used to analyse study parameters for the Pollinex[®] Quattro Grass-treated groups vs control.

RESULTS

Ames Test

Pollinex[®] Quattro Grass showed negative results with all five *S. typhimurium* strains in both the absence and presence of S9, when tested using a range of concentrations up to the maximum feasible level of 2000 SU per plate. For each strain, the negative controls showed the expected background levels of mutant colonies and positive control chemicals induced significant increases in mutant colonies, both in the absence and presence of S9. The results obtained with Pollinex[®] Quattro Grass in the Ames test are presented in Table 3.

Mouse Lymphoma Assay

Pollinex[®] Quattro Grass is formulated as an insoluble tyrosine suspension and, owing to precipitation of Pollinex[®] Quattro Grass in the treatment medium, the highest test concentrations were 100 or 125 SU ml⁻¹. There was some evidence of moderate cytotoxicity as shown by reductions in relative total growth (RTG, of up to approximately 50% reduction) at concentrations ≥80 SU ml⁻¹ following 3 h treatment in the presence of S9 and after 24 h treatment in the absence of S9. There were no increases in mutant frequency (MF) seen following 3 h treatment in the absence of S9, and although small increases in MF were seen after 24 h treatment in the absence of S9, these were less than the GEF (126 mutants per 10⁶ viable cells; Moore *et al.*, 2006), indicating a negative result. However, in the presence of S9, reproducible increases in MF that exceeded the GEF were observed. The results from treatment of mouse lymphoma L5178Y cells with Pollinex[®] Quattro Grass are presented in Table 4.

For the concentrations giving positive responses, colony sizing was performed and showed that the frequencies of both large and small colony mutants increased, indicating that both gene mutagenic and clastogenic mechanisms may be involved.

Micronucleus Test

The frequencies of MN PCE observed in the Pollinex[®] Quattro Grass-treated groups were similar to those seen in the vehicle control group. There was no reduction in the percentage of PCE that would be indicative of bone marrow toxicity and could indicate exposure of the bone marrow to the test agent. Groups of satellite animals given either saline or Pollinex[®] Quattro Grass at 40 000 SU kg⁻¹ per day × 2 days were used to provide blood samples for analysis of antibodies using immunochemical methods. Although the results of this analysis could not determine systemic exposure to Pollinex[®] Quattro Grass, the data provided evidence of increased serum protein content following administration of the test vaccine compared with the control, indicating the production of antibodies to Pollinex[®] Quattro Grass. Hence, it was concluded that Pollinex[®] Quattro Grass immunogens did enter the systemic circulation, and therefore the bone marrow was exposed. The positive control animals exhibited significantly increased numbers of micronucleated PCEs compared with the vehicle control group, confirming the sensitivity of the assay. The results from the micronucleus test with Pollinex[®] Quattro Grass are presented in Table 5.

Comet Assay

The data showed that no animals with >30% clouds and/or >30% 'diffused cells' were observed in rat liver or kidney preparations after treatment with Pollinex[®] Quattro Grass or vehicle control. It was therefore concluded that no excessive fragmentation of the DNA owing to cytotoxic effects was caused by the method used to isolate the cell suspensions or following treatment with Pollinex[®] Quattro Grass. The positive control group showed significant increases (5-fold in kidney and 10-fold in liver) in the mean tail moment over the concurrent controls. There were no significant increases in tail moment or tail intensity in the liver or kidney cells of animals treated with Pollinex[®] Quattro Grass at 20 000 or 40 000 SU kg⁻¹ per day. The results

from the comet assay with Pollinex[®] Quattro Grass are presented in Table 6.)

No blood samples for analysis of systemic exposure were included in this study. However, by extrapolation from the satellite animals used in the rat micronucleus test (which used the same dose route and a dose of 40 000 SU kg⁻¹ per day), the available

data indicate that the liver and kidney would have been exposed to the test agent in this comet assay. It was concluded that Pollinex[®] Quattro Grass did not induce DNA damage in the liver or kidney of male rats, following s.c. administration up to a maximum achievable of 40 000 SU kg⁻¹ per day for two consecutive days.

Table 3. Ames test: summary of mean revertant colonies with Pollinex Quattro Grass

Concentration (SU per plate)	TA98	TA100	TA1535	TA1537	TA102
<i>Experiment 1 –S9</i>					
0 (water)	18 ± 3	114 ± 4	21 ± 4	20 ± 5	282 ± 19
0.64	23 ± 3	118 ± 5	25 ± 5	19 ± 7	302 ± 20
3.2	21 ± 4	123 ± 11	26 ± 2	16 ± 3	305 ± 11
16	22 ± 3	126 ± 15	26 ± 4	17 ± 5	306 ± 6
80	20 ± 7	117 ± 18	24 ± 1	17 ± 4	291 ± 24
400	20 ± 3	112 ± 16	25 ± 2	17 ± 3	292 ± 12
2000	23 ± 1	130 ± 17	20 ± 2	22 ± 4	301 ± 14
5 µg 2NF	1193 ± 79	—	—	—	—
2 µg NaN ₃	—	944 ± 39	773 ± 27	—	—
50 µ AAC	—	—	—	476 ± 71	—
0.2 µg MMC	—	—	—	—	659 ± 14
<i>Experiment 1 + S9</i>					
0 (water)	33 ± 9	154 ± 5	20 ± 8	24 ± 6	257 ± 21
0.64	32 ± 5	163 ± 7	17 ± 9	20 ± 6	232 ± 8
3.2	33 ± 6	156 ± 9	23 ± 8	22 ± 3	232 ± 6
16	36 ± 9	135 ± 3	27 ± 3	23 ± 6	238 ± 12
80	33 ± 5	134 ± 8	18 ± 3	19 ± 6	217 ± 1
400	26 ± 6	151 ± 9	21 ± 8	21 ± 2	159 ± 13
2000	34 ± 6	142 ± 16	16 ± 4	24 ± 6	173 ± 36
10 µg B[a]P	511 ± 41	—	—	—	—
5 µg AAN	—	1470 ± 101	335 ± 20	167 ± 49	—
20 µg AAN	—	—	—	—	1466 ± 57
<i>Experiment 2 –S9</i>					
0 (water)	26 ± 3	114 ± 15	15 ± 5	18 ± 4	308 ± 16
6.325	29 ± 4	124 ± 7	11 ± 4	21 ± 2	268 ± 56
20	21 ± 7	116 ± 14	11 ± 1	21 ± 1	282 ± 23
63.25	21 ± 3	104 ± 17	17 ± 16	20 ± 2	310 ± 34
200	22 ± 3	110 ± 9	15 ± 3	14 ± 13	300 ± 38
632.5	22 ± 8	113 ± 7	10 ± 4	17 ± 4	293 ± 14
2000	24 ± 8	119 ± 10	16 ± 4	18 ± 1	294 ± 19
5 µg 2NF	1404 ± 56	—	—	—	—
2 µg NaN ₃	—	1233 ± 59	916 ± 25	—	—
50 µ AAC	—	—	—	221 ± 44	—
0.2 µg MMC	—	—	—	—	693 ± 39
<i>Experiment 2 + S9</i>					
0 (water)	42 ± 1	149 ± 6	18 ± 6	19 ± 1	225 ± 8
6.325	40 ± 2	125 ± 9	15 ± 2	20 ± 1	218 ± 38
20	38 ± 7	115 ± 0	14 ± 2	19 ± 7	223 ± 6
63.25	39 ± 4	111 ± 1	21 ± 6	18 ± 7	213 ± 24
200	33 ± 5	117 ± 17	12 ± 4	19 ± 2	190 ± 29
632.5	34 ± 6	119 ± 8	16 ± 2	21 ± 3	219 ± 37
2000	41 ± 1	104 ± 7	12 ± 2	19 ± 4	214 ± 5
10 µg B[a]P	379 ± 122	—	—	—	—
5 µg AAN	—	1721 ± 15	262 ± 82	154 ± 6	—
20 µg AAN	—	—	—	—	1384 ± 322
2NF, 2-nitrofluorene; NaN ₃ , sodium azide; AAC, 9-aminoacridine; MMC, mitomycin C; B[a]P, Benzo[a]pyrene; AAN, 2-aminoanthracene.					

Table 4. Mouse lymphoma assay results with Pollinex Quattro Grass

Concentration (SU ml ⁻¹)	Endpoint	
	MF ($\times 10^{-6}$)	Reduction in RTG (%)
<i>Experiment 1 (-S9/3 h)</i>		
0	88	0
12.5	73	-7 ^a
25	84	5
50	53	-4 ^a
75	77	-14 ^a
100P, PP	78	1
NQO 0.15 µg ml ⁻¹	460	42
NQO 0.2 µg ml ⁻¹	735	62
<i>Experiment 2 (-S9/24 h)</i>		
0	61	0
20	89	19
40	97	34
60	118	40
80	136	47
100P	136	45
125P, PP	141	51
NQO 0.05 µg ml ⁻¹	745	
NQO 0.1 µg ml ⁻¹	341	
<i>Experiment 1 (+S9/3 h)</i>		
0	68	0
12.5	69	-4 ^a
25	92	5
50	172	38
75	192	37
100P	236	49
125P, PP	267	58
BP 2 µg ml ⁻¹	487	44
BP 3 µg ml ⁻¹	923	78
<i>Experiment 2 (+S9/3 h)</i>		
0	66	0
20	73	15
40	143	37
60	149	37
80	224	54
100P	286	61
125P, PP	214	65
BP 2 µg ml ⁻¹	442	45
BP 3 µg ml ⁻¹	525	55

Figures in bold indicate that the MF value exceeds sum of mean control MF plus GEF (126×10^{-6}).

BP, benzo(a)pyrene. NQO, 4-nitroquinoline 1-oxide.

P, Precipitate observed at time of treatment. PP, Precipitate observed at end of treatment incubation. MF, mutant frequency; GEF, global evaluation factor.

^aNegative values indicate an increase in %RTG (relative total growth).

Table 5. summary of rat bone marrow micronucleus test data for Pollinex Quattro Grass

Treatment (SU kg ⁻¹ per day)	Cell total	%PCE	MN PCE	%MN PCE
0 (vehicle)	12 000	50.88	21	0.18 ± 0.08
10 000	12 000	53.60	19	0.16 ± 0.10
20 000	12 000	53.21	12	0.10 ± 0.03
40 000	12 000	52.84	17	0.14 ± 0.04
CPA	12 000	50.76	248	2.07 ± 0.45***

CPA: 20 mg kg⁻¹ cyclophosphamide, single dose.

%PCE, percentage of erythrocytes which are polychromatic.

MN PCE, micronucleated polychromatic erythrocytes.

*** $P \leq 0.001$.

genotoxic effect was seen at concentrations that did not induce excessive cytotoxicity. Furthermore, assessments showed no significant changes in osmolality or pH at the highest concentration analysed (200 SU ml⁻¹), compared with the vehicle control, and although precipitation was observed at the highest concentration tested (125 SU ml⁻¹), significant increases in mutant frequency were observed at lower concentrations where there was no precipitation seen. Hence, the positive result is considered unlikely to be caused by extreme culture conditions. The response is also considered unlikely to be due to a genotoxic impurity or contaminant in Pollinex[®] Quattro Grass, since this would have been expected to give a positive response in the Ames test, which tested similar high concentrations. The S9 used in the *in vitro* genotoxicity assays with Pollinex[®] Quattro Grass was obtained from rat livers following enzyme induction with Aroclor 1254 and as such would have contained significantly higher levels of certain cytochrome P450 enzymes (e.g. CYP1A1, 1A2, 2B1, 2B2 and 2C11) than normal liver (Lubert *et al.*, 1989; Mirvish *et al.*, 1995). It is therefore possible that the positive response in the MLA could be attributed to use of the exogenous metabolizing system, which could have generated an *in vitro* specific metabolite from the Pollinex[®] Quattro Grass formulation. However, if this were the case a positive response would also be expected in the Ames test, which used the same exogenous metabolizing system.

Inclusion of *in vitro* mammalian cell tests in the standard battery of genotoxicity assessments for pharmaceuticals increases the sensitivity and broadens the spectrum of genetic events detected (in comparison to testing in bacterial cells only), but also decreases the specificity of prediction (i.e. increases the incidence of positive results that do not correlate with rodent carcinogenicity). Furthermore, comparative trials have shown that each *in vitro* test system generates both false negative and false positive results in relation to predicting rodent carcinogenicity (ICH, 2009). The test battery approach is designed to reduce the risk of false negative results for compounds with genotoxic potential, whereas a positive result in any single assay for genotoxicity does not necessarily mean that the test compound poses a genotoxic/carcinogenic hazard to humans.

For human pharmaceuticals that show positive genotoxicity results *in vitro* it is recommended that the battery of tests should include two (rather than only one) *in vivo* genotoxicity assays. Although positive *in vitro* data may indicate intrinsic genotoxic properties of a drug, appropriate *in vivo* data determine the biological significance of these *in vitro* signals in most cases (ICH,

DISCUSSION

Negative results were obtained in bacterial cells (Ames test), and in the absence of metabolic activation in mammalian cells *in vitro* (MLA), but a reproducible positive response was seen in mammalian cells in the presence of S9. This reproducible

Table 6. Summary of rat comet assay data for Pollinex Quattro Grass

Treatment (SU kg ⁻¹ per day)	Liver		Kidney	
	Tail moment	Tail intensity	Tail moment	Tail intensity
0 (vehicle)	0.14 ± 0.01	1.21 ± 0.11	0.80 ± 0.06	5.41 ± 0.32
20 000	0.13 ± 0.01	1.18 ± 0.11	0.85 ± 0.09	6.06 ± 0.34
40 000	0.17 ± 0.02	1.59 ± 0.17	0.54 ± 0.10	4.19 ± 0.53
EMS	1.66 ± 0.03***S + T	8.46 ± 0.11***S +	4.09 ± 0.49***S + T	18.20 ± 1.77***S +

Data shown as group means ± standard error of the mean.
 EMS, 250 mg kg⁻¹ ethylmethanesulphonate, single dose.
 S +, two-sample *t*-test, upper tail.
 T, log-transformed data.
 *** *P* ≤ 0.001.

2009). It has been reported that the MLA can give up to 60% 'false positive' results with substances that are not carcinogenic *in vivo* (Kirkland *et al.*, 2005). Furthermore, such 'false positives' can be caused by a number of indirect mechanisms with non-DNA targets such as effects on protein synthesis, lysosomes, receptors and signalling pathways (Scott *et al.*, 1991; Kirkland and Müller, 2000; Müller and Kasper, 2000) and may therefore have a threshold below which DNA damage would not be induced (Scott *et al.*, 1991; Thybaud *et al.*, 2007). Assessments were, therefore, conducted *in vivo* with Pollinex[®] Quattro Grass and comprised a micronucleus test in rat bone marrow cells, and a comet assay using cell preparations from the liver (since the MLA positive result was obtained in the presence of S9) and kidney of rats, following s.c. administration at a maximum feasible level of 40 000 SU kg⁻¹ per day. These *in vivo* studies showed no evidence of genotoxicity in cells that included metabolically competent (liver) cells and evidence of systemic exposure to the allergoid preparation. A summary of the results from the battery of genotoxicity studies on Pollinex Quattro Grass is presented in Table 7.

Although actual plasma exposure levels to Pollinex[®] Quattro Grass were not quantified in the *in vivo* genotoxicity studies, evidence of systemic exposure was obtained and both studies showed negative results at dose levels which were calculated to be approximately 1300- and 500-fold higher than the intended clinical dose of 2000 SU per person, based on adult and child body weight values of 65 and 25 kg, respectively.

The MPL component of Pollinex[®] Quattro Grass has been evaluated previously for genotoxicity and the results showed that it did not induce mutation in a reverse mutation bacterial test or induce chromosome aberrations in CHO cells (Baldrick

et al., 2002b). In addition, inclusion of MPL in the Pollinex[®] Quattro Grass formulation tested in the current battery of genotoxicity studies indicated no effect of this adjuvant material in the rat micronucleus test and comet assay in rat liver and kidney cells at levels calculated to be 500- to 1300-fold higher than the intended human dose. Furthermore, although the investigations were of limited design compared with modern standards, L-tyrosine (a formulation component of Pollinex[®] Quattro Grass) did not show any evidence of mutagenic activity when tested in two *in vitro* assays (reverse mutation test in bacteria and cell transformation assay in mammalian cells; Baldrick *et al.*, 2002a). A similar allergoid preparation, based on modified ragweed pollen extract, has also been reported to show no evidence of genotoxicity when tested in an Ames assay (Baldrick *et al.*, 2007).

Overall, although there is no clear requirement for genotoxicity data to support marketing of the Pollinex[®] Quattro Grass vaccine, genotoxicity data were generated for the product which showed a reproducible positive response in the mouse lymphoma assay in the presence of S9. However, when positive results have been obtained in an *in vitro* genotoxicity assay, negative results in appropriate *in vivo* assays with adequate justification for the endpoints measured, and demonstration of exposure, is generally sufficient to demonstrate absence of genotoxic activity (ICH, 2009). Furthermore, two *in vivo* studies on Pollinex[®] Quattro Grass (rat bone marrow micronucleus test and comet assay in rat liver and kidney cell preparations) showed negative results at dose levels which were calculated to be approximately 1300- and 500-fold higher than the intended clinical dose of 2000 SU per person (based on adult and child body weight values of 65 and 25 kg, respectively). Hence, taking a weight of evidence approach, it is concluded

Table 7. Results from standard regulatory genotoxicity test battery with Pollinex[®] Quattro Grass

Endpoint	Treatment conditions	Result
Ames (bacterial reverse mutation)	<i>Salmonella typhimurium</i> strains TA98, TA100, TA1535, TA1537 and TA102, plate incorporation -/+ S9, preincubation + S9, up to 2000 SU per plate	Negative
Mouse lymphoma assay	3 and 24 h treatment -S9, up to 200 SU ml ⁻¹ 3 h treatment + S9 up to 200 SU ml ⁻¹	Negative Positive
Micronucleus test (rat, bone marrow)	2 × s.c. doses of 10 000, 20 000 or 40 000 SU kg ⁻¹ per day, evaluation of MN in 2000 PCE/rat, %PCE based on 1000 total bone marrow erythrocytes. Satellite animals indicated bone marrow exposure.	Negative
Comet assay (rat, liver and kidney)	2 × s.c. doses of 20 000 or 40 000 SU kg ⁻¹ per day, evaluation of comets in 100 cells per animal. Exposure of target organs indicated by extrapolation	Negative

that the available genotoxicity test data indicate no biologically relevant cause for concern and support the proposed clinical use of Pollinex® Quattro Grass.

Disclosure Statement

Lesley Reeve and Paul Baldrick assisted in preparation of the manuscript under contract with Allergy Therapeutics Ltd.

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