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Immunotoxicological studies of genetically modified rice expressing PHA-E lectin or Bt toxin in Wistar rats

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Abstract

As part of the SAFOTEST project the immunmodulating effect of Cry1Ab protein from *Bacillus thuringiensis* (Bt) and PHA-E lectin from kidney bean (*Phaseolus vulgaris* erythroagglutinin) was examined in 28- and 90-day feeding studies in Wistar rats. PHA-E lectin was chosen as positive control. Rats were fed control rice, transgenic rice expressing Cry1Ab protein or PHA-E lectin, or transgenic rice spiked with the purified recombinant protein. Total immunoglobulin levels, mitogen-induced cell proliferation, T-dependent antibody response to sheep red blood cells and the antigen-specific antibody response in serum were examined at the end of the studies.

A dose-dependent increase in mesenteric lymph node weight and total immunoglobulin A was seen when feeding PHA-E transgenic rice alone or spiked with 0.1% purified PHA-E lectin for 90 days indicating a local effect of PHA-E in the intestine. No adverse effects of Cry1Ab protein were found. An anti-PHA-E and anti-Cry1Ab antibody response was induced both after inhalation (control groups) and after inhalation/ingestion (groups fed recombinant protein alone or together with transgenic rice).

In conclusion, only PHA-E lectin was found to have an immunomodulating effect when feeding rats for 90 days with approximately 70 mg PHA-E/kg bodyweight per day. As both PHA-E lectin and Cry1Ab protein were capable of inducing an antigen-specific antibody response it is important to make careful considerations when designing future animal studies to avoid intake of proteins from the other groups by inhalation as well as to examine the sensitization and elicitation potential of 'foreign' proteins before introduction to the world market.

Keywords: *Phaseolus vulgaris* erythroagglutinin; *Bacillus thuringiensis* Cry1Ab protein; Immunotoxicology; Genetically modified rice; Wistar rats; SAFOTEST

1. Introduction

For the last decades there has been a growing interest from the food crop industry to construct and produce genetically modified (GM) crops with the primary goal to significantly increase the yield and avoid the use of pesticides. At present, GM crops are grown and consumed by humans in many countries, for example corn or potatoes expressing the insecticidal genes (*cry* genes) from *Bacillus thuringiensis* (Bt) (High et al., 2004; Eizaguirre et al., 2006).

In spite of the extensive research in the field of GM crops there is a still increasing requirement from European consumers and regulative authorities for guideline tests to assess the safety of genetically modified foods (European Commission, 1997). The Organization for Economic Co-operation and Development (OECD) guideline study no. 408 ('Repeated Dose 90-Day Oral Toxicity Study in Rodents') is traditionally used for safety assessment of single chemical substances but needs technical adjustments, if at all useful for testing of whole foods. The European Commission (EC) project SAFOTEST ('New methods for the safety testing of transgenic food') was conducted to examine certain refinement of the study design as well as the potential of a broad range of *in vivo* and *in vitro* parameters to improve the sensitivity and specificity of the traditional 90-day study.

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One of the parameters included in the SAFOTEST project was immunotoxicological examination of rats fed a diet consisting of 60% of either conventional rice or transgenic rice expressing an insecticidal protein. In addition, the immunotoxicological effect of spiking of the purified insecticidal protein to the diet was examined.

Bt toxin (Cry1Ab protein) a well-known insecticide (Dorsch et al., 2002; Hua et al., 2004; Zhang et al., 2005) was chosen as a model protein as it has been widely used in commercial GM crops to control major insect pests (Betz et al., 2000; Mendelsohn et al., 2003; Romeis et al., 2006). Bt toxins are regarded to be non-toxic to mammals including humans (Betz et al., 2000; Siegel, 2001). In addition, Hammond et al. (2006a,b) concluded that genetically modified Bt corn is as safe and nutritious as existing commercial corn based on results obtained from 90-day feeding studies in rats.

The kidney bean lectin E-form (*Phaseolus vulgaris* erythroagglutinin, PHA-E) was selected as a positive control since PHA lectin is known to have a toxic effect in both insects and mammals (Carvalho and Sgarbieri, 1998; Habibi et al., 1998, 2000; Kunzelmann et al., 2004). PHA lectin exists in two isoforms, PHA-E and PHA-L (*P. vulgaris* Leucoagglutinin), which is able to agglutinate red blood cells and lymphocytes, respectively (Rüdiger and Gabius, 2001). Intake of PHA lectin has been found to induce extensive proliferation of epithelial cells in the small intestine (Bardocz et al., 1995; Otte et al., 2001) and enlargement of pancreas and colon in mammals (Grant et al., 1995).

The aim of this study was to investigate the immunotoxicological potential of transgenic rice expressing PHA-E lectin or Bt toxin in rats fed a diet with 60% transgenic or parental rice for 28 or 90 days according to the SAFOTEST approach. In addition, the effects of purified recombinant PHA-E lectin or recombinant Bt toxin spiked to the diet was investigated. The amount of purified recombinant PHA-E lectin and Bt toxin spiked to the diets was limited by the actual yield of the recombinant proteins following expression and subsequent purification. Therefore, the subsequent animal experiments were designed to achieve the highest concentration of spiked PHA-E lectin or Bt toxin as possible.

2. Materials and methods

2.1. Test material

The PHA-E transgenic rice line (PHA-E rice) was developed by Newcastle University (Poulsen et al., 2007b) and multiplied together with its parental rice line (EY1105) in Hangzhou, China. Bt rice (KMD1) was jointly developed by University of Ottawa (Canada) and Zhejiang University (China) (Shu et al., 2000), and were also bulked up together with its parent variety Xiushui 11 in Hangzhou, China. For further details of the rice lines and characterization of the test materials see Poulsen et al. (2007b) and Schrøder et al. (2007).

Purified PHA-E lectin (Baumgartner et al., 2002) and Bt toxin (Cry1A(b) toxic fragment) were provided by Scottish Crop Research Institute (U.K.). A construct containing the *kurdhI* gene (which encodes Cry1A(b) of *B. thuringiensis* sub-species *kurstaki* HD1) under the control of the tac promoter was provided by the *Bacillus* Genetic Stock Center (http://www.bgsc.org/Catalogs, catalogue number ECE54). The construct was expressed in *E. coli* JM103 and the recombinant protein was extracted from inclusion bodies as described in Ge et al. (1990). The 66 kDa toxic fragment was generated by incubation of the protoxin with trypsin (2 mg per 100 mg of protoxin) at 37 °C for 3 h. Following trypsin treatment, the toxin solution was dialysed against 10 mM sodium carbonate, 10.5 and purified by anion-exchange chromatography on Q-Sepharose Fast flow.

2.2. Animals

Male and female Wistar rats (specific pathogen free, SPF), 4–5 weeks old, were obtained from M&B Breeding centre, Ry, Denmark. The animals were kept in stainless steel wire cages (two/cage) at 22 ± 1 °C, relative humidity 55 ± 5%, air change 10 times/h, and electric light from 09.00 to 21.00. Animals were inspected twice daily and body weights recorded weekly. The weight of food supplied and the spill of food were recorded for each cage weekly throughout the treatment period. From these records, the mean weekly food consumption per animal was calculated. Using the compositional data from the feed and the animal weight data the daily intake of PHA-E lectin and Bt toxin per kilogram of body weight were calculated as a mean for the whole feeding period.

Animal studies were performed under conditions approved by the Danish Agency for Protection of Experimental Animals and by the in-house Animal Welfare Committee.

2.3. Experimental design

A 28- and 90-day study was performed with PHA-E lectin and Bt toxin, respectively. Animals were fed purified diets containing 60% rice together with different concentrations of purified PHA-E lectin or Bt toxin (Table 1). Diets were adjusted to assure the same amount of macronutrients in all groups and thereby avoid effects caused by compositional differences between the diets (for details see Poulsen et al., 2007b; Schrøder et al., 2007). Diets and acidified water (pH 3.5) were given *ad libitum*. One week after arrival animals were randomly assigned to control and experimental groups. At termination, all animals were anaesthetized by carbon dioxide inhalation and killed by exsanguinations.

Study 1 was performed as a 28-day dose-range finding study for toxicity of the PHA-E lectin. In addition the effect of immunization with sheep red blood cells (SRBC) on immunological parameters was investigated by comparison of groups 3 and 4 (Table 1). Groups of rats ($n = 6$–9/sex) were fed diets containing 60% control rice spiked with 0%, 0.005%, 0.01%, 0.02% or 0.08% purified PHA-E lectin.

Study 2 was performed as a 90-day study with control rice and PHA-E rice with or without spiking. Groups of 18 female rats were fed diets containing 60% control rice, 60% transgenic PHA-E rice or 60% transgenic PHA-E rice spiked with 0.1% purified PHA-E lectin.

Study 3 was performed as a 28-day study with control rice and Bt rice with and without spiking. Groups of 10 female rats were fed diets containing 60% control rice, 60% transgenic Bt rice or 60% transgenic Bt rice spiked with 0.1% purified Bt toxin.

Study 4 was performed as a 90-day study with control rice and Bt rice without spiking. Groups of 32 rats (16 males and 16 females) were fed diets containing 60% control rice or 60% transgenic Bt rice.

The mean daily intake of PHA-E lectin or Bt toxin from the inherent content of GM-rice in groups fed transgenic rice was approximately 30 mg PHA-E lectin and 0.6 mg Bt toxin/kg body weight (BW), respectively (Poulsen et al., 2007b; Schrøder et al., 2007). Spiking of PHA-E lectin or Bt toxin to the transgenic rice diets resulted in a mean daily intake of approximately 100 mg PHA-E lectin and 70 mg Bt toxin/kg BW.

Blood was collected at sacrifice and sera were stored at −20 °C until analysis. Sera were analyzed by enzyme-linked immunosorbent assay (ELISA) for concentrations of total IgM, IgG and IgA, anti-SRBC IgM and levels of Bt-specific and PHA-E-specific antibodies.

2.4. Immunization with SRBC

Sheep red blood cells (SRBC) from a single animal source (Statens Serum Institut, Copenhagen, Denmark) were used for all experiments. For immunization, SRBC in Alsevers' solution were washed three to four times (1000 × *g*, 20 min, +4 °C) in sterile saline, and the buffy coat removed. After the last wash, concentration of SRBC was measured using a Vet Abc, Animal Blood