Strain-dependent acute lung injury after intra-tracheal administration of a ‘refined’
aniline-denatured rapeseed oil. A murine model of the toxic oil syndrome?

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Abstract

Most attempts to reproduce the Toxic Oil Syndrome in animals, either with case-related oils or with refined rapeseed oils, have been unsuccessful. An aniline-denatured rapeseed oil that was subsequently refined according to a protocol yielding relevant markers of “toxic oil” (oil RSO160401) had led to possibly relevant lesions following oral administration in mice. Therefore, in the present study, RSO160401 was subjected to a more extended in vivo testing. To try and maximize the response, BALB/c, DBA/2, A/J, and C57BL/6 mice were administered RSO160401 oil by a single intra-tracheal instillation (1 ml/kg), with sacrifice 2 or 7 days post-exposure. Intra-tracheal administration led to a strain-dependent acute response: acute pulmonary damage in DBA/2 and A/J mice, and increases in blood eosinophilia in DBA/2 mice (6.5% vs 3.1% in controls). The pulmonary lesions regressed with time after exposure, being more complete in A/J than in DBA/2 mice. The observation of strain-dependent effects suggests that genetic susceptibility is an important factor in disease induction by the RSO160401 oil.

Introduction

The toxic oil syndrome (TOS) occurred in Spain, in 1981, as a result of the consumption of oil mixtures containing rapeseed oil that had been denatured with 2% aniline to be imported from France for industrial use, but was later illegally refined for human consumption and distributed by street vendors (Canas and Kilbourne, 1987). Over 20,000 people were affected by TOS, with approximately 300 dying shortly after the onset of the disease and a larger number developing chronic disease. Three clinical phases have been identified in TOS. The acute phase (1-2 months) was characterized by mild fever, rash, myalgia, dyspnea, cough, peripheral eosinophilia, elevated serum IgE, and pulmonary injury characterized by interstitial oedema with inflammatory mononuclear infiltrates (lymphocytes, monocytes and a few eosinophils). The intermediate phase (2-4 months) was characterized by severe myalgia, sensory neuropathy, liver damage, skin oedema, sicca syndrome, and weight loss. The chronic phase included peripheral neuropathy accompanied by muscle wasting, scleroderma, hepatopathy, and pulmonary hypertension (Kilbourne et al, 1983, 1991; Abaitua and Posada in WHO, 1992).

Despite considerable toxicological research the exact causal agent has never been identified. During the 1990s, progress was made regarding the identification of the possible causal agent and the interpretation of the biologic mechanisms of TOS. Studies led to the identification of a new family of compounds, the fatty acid esters of 3-(N-phenylamino)-1,2-propanediol (PAP). Concentrations of one specific member of the family, the 1,2-dioleoyl ester of PAP (OOPAP), were shown to be strongly associated with the occurrence of TOS (Posada et al., 1994, 1999; Hill et al., 1995). These PAP-esters are considered good markers for TOS-related oil, but based on

various studies they are unlikely to be the etiological agents (Gelpi et al., 2002). The inability to find an acceptable animal model has interfered with the identification of the actual etiologic agent(s) of TOS. All attempts to reproduce TOS in experimental animals failed either when oils believed to be case-related were tested or when refined rapeseed oils synthesized in the laboratory, containing high concentrations of aniline derivatives were tested (Gelpi et al., 2002). However, some promising results were obtained by Gallardo et al. (WHO, 2004) after oral administration of synthesized TOS oils with different concentrations of OOPAP for 8 days. Small increases in peripheral blood eosinophils were seen in BALB/c mice, whereas in DBA/2 mice, an increase in serum IgE was caused. These responses were not observed in other strains having different genetic background, particularly in terms of MHC (C57BL/6 and A/J). Genetic susceptibility seems to play an important role in the induction of the TOS both in humans (Abaitua Borda et al., 1998) and apparently also in animals (Bell et al., 1992, 1999a,b; Berking et al., 1998; Gallardo et al., 2005). Thus, significant excesses were found in the prevalence of HLA-DR2 among women affected by the acute stage of TOS (Arnaiz-Villena et al, 1982), and of HLA-DR3 and –DR4 among women affected by the chronic stage of TOS (Vicario et al, 1982). The genetic restriction found in humans was also tested using HLA transgenic mice (Gallardo et al., 2005). Mice expressing human DR2 and DQ6, had a higher percentage of blood eosinophils (DQ6) and IgE (DR2) after administration of toxic oil than other transgenic mice tested (DR3 and DR4). Also, a Th2-shift was found in DR2 transgenic mice when toxic oil was administrated with ovalbumin.

The objective of our study was to contribute to the development of a suitable mouse model that would reflect the acute pathology found in humans affected by TOS. In a

preliminary experiment the supplemented refined oil “RSO160401” did cause increased thickness of the small pulmonary arteries with swelling of endothelial cells in C57BL/6 mice given five daily doses of 5 ml/kg bw orally, while this was not observed in the other groups (controls and mice given 1.25 or 2.5 ml/kg bw) (Tarkowski and Nemery in WHO, 2004). Additionally, a moderate increase in blood eosinophils was observed in BALB/c mice given seven daily oral doses of 5 ml/kg bw (unpublished data). Consequently, the test oil “RSO160401”, produced in the laboratory to have a composition resembling that of toxic oil with regard to levels of marker anilides and PAP-esters, was subjected in this study to a new protocol to try and maximize the response. RSO160401 was administered by a single intra-tracheal instillation to four strains of mice (BALB/c, DBA/2, A/J, C57BL/6), with sacrifice two or seven days post-exposure.

The rationale for assessing the toxicity of oils when given via intra-tracheal administration is not that the oils reached the lungs of human victims as a result of inhalatory exposure. There is no evidence that this route of exposure was relevant in the affected subjects. However, it is conceivable that the physiology of absorption of critical lipid-related agents differs substantially between rodents and humans. The physiology of absorption of the unusual aniline-modified compounds related to the TOS is not known. It was hypothesised that these lipid-related agents may not reach the lungs to the same extent in rodents compared to humans, in whom lymphatic absorption via the thoracic duct (and hence without hepatic first-pass effect) may be more substantial. A convenient way to assess the direct pulmonary toxicity of foreign agents consists in their direct administration into the lungs via intra-tracheal instillation.

These intra-tracheal instillation experiments were a continuation of our previous efforts in which candidate toxic oils had been administered orally to various strains of mice (Tarkowski and Nemery, in WHO 2004). The rationale for choosing female mice of four different strains and for focusing on acute lung injury and blood eosinophilia, which were both features of the early human disease, was discussed and approved by the TOS-CISAT scientific committee (WHO, 2004).

Materials and methods

Test material

The test oil coded as 'RSO160401' was provided by Dr. M. Posada de la Paz (CISAT, Toxic Oil Research Centre). RSO160401 is a rapeseed oil first denatured with aniline and then refined according to Ruiz-Méndez et al. (2001), it was supplemented with anilides (10,701 ppm) and OOPAP (124 ppm). The test oil was kept at -80°C upon arrival until the first day of dosing. Then it was stored at 4°C.

Rapeseed oil (from *Brassica rapa*, Sigma-Aldrich (Fluka), Bornem, Belgium) was used as a control oil, and also stored at 4°C. Before intra-tracheal instillation, the oils were warmed to $\pm 30^{\circ}\text{C}$.

Animals

Female BALB/c, DBA/2, A/J, and C57BL/6 mice (6-8 weeks of age, Harlan, The Netherlands) were maintained in the animal facilities of the KULeuven on a 12-h light/dark cycle, in polyethylene cages with stainless steel lids and covered with a filter cap. They received standard rodent food and acidified tap water ad libitum.

Experimental design

The treatment protocol was performed following recommendations of the TOS WHO-CISAT Committee (Tarkowski and Nemery in WHO, 2004).

This study consisted of 3 experimental groups of 10 animals each, from 4 different strains (BALB/c, DBA/2, A/J, C57BL/6): untreated controls (only pentobarbital anaesthesia); intra-tracheal instillation of rapeseed oil, 1 ml/kg bw; intra-tracheal instillation of RSO160401 oil, 1 ml/kg bw. Half of the animals (n=5 per group) were sacrificed on day 2 and the other half on day 7 post-exposure. (Deviations in group size: BALB/c mice time point 2 days: n=7 for anaesthesia control and n=8 for oil-instilled animals; C57BL/6 mice time point 2 days: n=6 for RSO160401-instilled animals).

Animals were anaesthetized with Nembutal[®] (pentobarbital i.p.) and received, via a cannula inserted into the trachea via the mouth, a bolus injection of the test compound (1 ml/kg bw), followed by a bolus of 0.1 ml of air.

Body weights for each animal were recorded prior to exposure and daily until the day of sacrifice. Sacrifice (pentobarbital i.p.) took place on day 2 or day 7 post-exposure.

Determination of peripheral blood eosinophilia by flow cytometry

On the day of sacrifice, blood was sampled from the retro-orbital plexus for counting eosinophils by flow cytometry. Eosinophils were detected by counting double stained cells using Gr-1 and VLA-4 antibodies (BD Biosciences, Erembodegem, Belgium), adding 3 µl of each antibody to 100 µl of cells. Nonspecific fluorescence was detected by using control samples labeled with isotype matched control antibodies. After a 15 min incubation at room temperature, lysing solution was added to lyse red blood cells. Flow cytometry (CellQuest, BD Biosciences) was performed using at least 10⁴ cells.

Mouse total IgE

Total serum IgE was quantified by using the OptEIA™ Mouse IgE Set from Pharmingen (San Diego, CA) (serum dilution 1/20 or 1/80). Measurements were performed according to the manufacturer's instructions, with the use of anti-mouse IgE, biotinylated anti-mouse IgE, and horseradish peroxidase conjugate.

Autopsy, lung weight, bronchoalveolar lavage, histology

The gross macroscopic appearance of lungs, heart and major abdominal organs was assessed. The left bronchus and pulmonary vessels were clamped and the left lung was removed. This was weighed fresh (wet lung weight) and after 48 hours of drying at 60°C (dry lung weight); lung weights were expressed in relation to body weight, and the ratio of wet to dry lung weight was also determined. The right lung was lavaged 3 times with 0.4 ml of warm (37°C) sterile 0.9% NaCl. Each time saline remained in the lung for 15 s. The bronchoalveolar lavage fluid (BALF) was pooled, kept on ice and centrifuged at 1200 g for 10 min. The supernatants were kept at 4°C for lactate dehydrogenase (LDH) and protein assays. The cell pellet was resuspended in 200 µl phosphate-buffered saline (PBS) and total cell number was counted using a hemocytometer. Cytocentrifuge slides (Cytospin 3, Shandon; 1400 x g for 6 min; 100,000 cells/ml) were prepared for differential cell typing. After the bronchoalveolar lavage, the right lung was instilled via the trachea with formaldehyde (10%), the trachea was tied and the heart-lung complex was placed in formaldehyde (10%) for histological analysis. Additionally, liver, kidney, thymus, spleen, muscle, skin, and any visually abnormal tissue or organs were also preserved in formaldehyde. An experienced pathologist (Erik Verbeke) evaluated lung injury without knowledge of the animal's treatment, by examining 5 µm paraffin sections, stained with hematoxylin and eosin, from all lung lobes. The term 'major abnormalities'

comprises acute centrilobular inflammation characterised by neutrophils and intra-alveolar macrophages, whereas subacute centrilobular inflammation is characterised by fewer neutrophils, some tissue reaction and a mononuclear interstitial infiltration.

Biochemical and cytological evaluation of BALF

LDH activity (index of cytotoxicity) and total protein concentration (index of changes in permeability of the alveolar-capillary membrane) were determined by monitoring spectrophotometrically the reduction of pyruvate (Vassault, 1983), and by the Bradford method (Bradford, 1976), respectively.

Pulmonary inflammation was evaluated by cytologic characterisation of BALF cells. Differential cell counts (DCC) were performed (3 x 100 cells) on stained (Diff-Quick, Dade Behring AG, Switzerland) cytocentrifuge slide preparations.

Statistics

Data are presented as means and standard deviations (SD). Comparisons between groups were analysed by one-way analysis of variance followed by the Newman-Keuls test, or analysed by the non-parametric Mann-Whitney test, as appropriate. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed using the GraphPad Prism 3.0 software.

Results

The results of the single dosing by intra-tracheal instillation are presented in Table 1 (body weight, lung weight parameters, lung injury, lung inflammation, peripheral blood eosinophilia, serum IgE) and Table 2 (macroscopic appearance, histopathological effects).

Mortality, clinical observation, and body weight

In preliminary experiments, it had been observed that mice from the A/J strain suffocated after the instillation of the RSO160401 test oil. Therefore, to decrease their viscosity, oils were warmed to $\pm 30^{\circ}\text{C}$ before instillation. Eventually, A/J mice survived the instillation.

There was no treatment-related mortality, once the animals had recovered from anaesthesia.

No significant effect of the treatment on body weight was seen in BALB/c and C57BL/6 mice. In DBA/2 and A/J mice, two days post-exposure, the final body weight of the RSO160401 group was significantly decreased compared to anaesthesia- and rapeseed oil- control (Fig. 1a). Seven days post-exposure, no effects of treatment were detected.

Macroscopic appearance

The macroscopic observations have been summarised in Table 2. Pulmonary abnormalities seen at autopsy, were restricted to mice treated with RSO160401.

Lung weights

BALB/c mice showed significant changes in lung parameters 7 days post-exposure only. Wet and dry lung weights were significantly increased for the rapeseed oil treated group compared to the anaesthesia control group, but also compared to the RSO160401 treated group. Wet-to-dry lung weight ratio of the rapeseed oil treated group was significantly decreased compared to the anaesthesia control and RSO160401 treated group.

In DBA/2 mice, two days post-exposure, wet lung weight (Fig. 1b) was significantly increased for the RSO160401 treated group compared to the anaesthesia and rapeseed oil control groups; dry lung weight (Fig. 1c) was only significantly increased

compared to the anaesthesia control. Wet-to-dry lung weight ratio was not altered. Seven days post-exposure, there was still a non-significant trend in increased wet and dry lung weight.

The A/J strain (Fig. 1b,c) showed, two days post-exposure, significant increases in wet and dry lung weight for the RSO160401 treated group compared to anaesthesia control and rapeseed oil. Wet-to-dry lung weight ratio was significantly decreased for both the rapeseed oil group and the RSO160401 group compared to the anaesthesia control group. Seven days post-exposure, no significant changes were seen.

In C57BL/6 mice, two and seven days post-exposure, no significant changes were seen in lung weight parameters.

Bronchoalveolar lavage – Cytology

Seven days post-exposure Total Cell Count (TCC) findings for DBA/2 and A/J mice could not be taken into consideration because of a technical error.

In BALB/c (seven days post-exposure) and C57BL/6 (two days post-exposure) mice, TCC in the RSO160401 group and the rapeseed oil control group were significantly increased compared to the anaesthesia control group. RSO160401 oil treated C57BL/6 (two days post-exposure) mice showed also an increase in neutrophils (25.6%) compared to the anaesthesia control (0.3%).

In A/J mice, neither TCC nor cell distribution were altered for the RSO160401 treated mice.

In DBA/2 mice, two days post-exposure, TCC was significantly increased in the RSO160401 oil group (19.3×10^4 cells) compared to the rapeseed oil group (10.5×10^4 cells) and the anaesthesia control group (8.5×10^4 cells). Eosinophils were only found in RSO160401 treated mice (two days post-exposure: up to 3%, seven days post-exposure: up to 2.7%). Additionally, seven days post-exposure, neutrophils were

significantly increased for both the RSO160401 (12.5%) and rapeseed oil group (10.8%) compared to the anaesthesia control group (1.4%).

Bronchoalveolar lavage fluid – pulmonary toxicity endpoints

Some data were excluded, because of technical errors (not treatment related).

BALB/c mice did not show any significant changes in LDH activity and protein level from controls at any time point.

In DBA/2 mice (Fig. 2), two days post-exposure, the RSO160401 group showed significantly higher LDH activity (relative value 0.511) and protein level (24.4 µg/ml) in the BALF than the anaesthesia control group (LDH: relative value 0.274; protein: 4.5 µg/ml). Protein levels of the RSO160401 group were also significantly different compared to the rapeseed oil group (6.9 µg/ml). Seven days post-exposure, LDH activity and protein levels were no longer significantly altered.

In A/J mice (Fig. 2), two days post-exposure, the RSO160401 group showed a significantly higher LDH activity (relative value 0.447) and protein level (13.2 µg/ml) than the anaesthesia control group (LDH: relative value 0.246; protein: 4.2 µg/ml). Protein levels of the RSO160401 group were also significantly different compared to the rapeseed oil group (5.7 µg/ml). Seven days post-exposure, no significant changes in LDH activity and protein level were found.

Mice of the C57BL/6 strain did not show significant changes in LDH activity and protein level at two and seven days post-exposure.

Peripheral blood eosinophils

In BALB/c, A/J, and C57BL/6 mice no significant changes from controls in peripheral blood eosinophils were found at any time point.

In DBA/2 mice (Fig. 3), however, seven days post-exposure, peripheral blood eosinophils became significantly elevated for the RSO160401 group (6.5%) compared

to the anaesthesia control group (2.0%) and compared to the rapeseed oil group (3.1%).

Serum total IgE

The variability within groups, including control, was generally high. No increases in serum IgE levels were found at any time point in BALB/c, DBA/2, A/J, and C57BL/6 mice.

Histopathology

Histopathological major abnormalities observed during the lung injury evaluation are summarised in Table 2. Acute centrilobular inflammation was characterised by neutrophils and intra-alveolar macrophages (Figs. 4A, C). Subacute centrilobular inflammation was characterised by fewer neutrophils, some tissue reaction and a mononuclear interstitial infiltrate (Fig. 4B, D). Major abnormalities were clearly associated with RSO160401 oil treatment. Foamy alveolar macrophages were observed in both RSO160401- and rapeseed oil-treated animals in the C57BL/6 strain.

Discussion

In 1999, after a “successful” refining procedure had been achieved (Ruiz-Mendez et al., 2001), a new series of animal studies was started using case-related and reconstituted oils enriched with PAP-esters. The detailed results of these experiments done in different strains of mice have been reported to the WHO/CISAT Committee and published in part (Gelpi et al., 2002; Tarkowsky and Nemery in WHO, 2004). In none of these experiments was any major pulmonary toxicity observed. However, in a preliminary experiment with C57BL/6 mice, the supplemented refined oil “RSO160401” administered by oral gavage did cause increased thickness of the small

pulmonary arteries with swelling of endothelial cells (Tarkowski and Nemery in WHO, 2004). In another preliminary experiment performed in our laboratory, a moderate increase in peripheral blood eosinophils was observed in BALB/c mice given 5 ml RSO160401/kg bw by oral gavage for seven consecutive days (6.2% vs 4.0% in controls) (unpublished data). These findings are consistent with those of Gallardo et al. (in WHO, 2004), who showed that oral administration of synthesized TOS oils with different concentrations of OOPAP caused an increase in peripheral blood eosinophils in BALB/c mice. However, in contrast to these authors who found an increase in serum IgE levels in DBA/2 mice, no significant changes in serum IgE level were found in our experiments.

In the present study, RSO160401 was subjected to a protocol consisting of a single intra-tracheal instillation to four strains of mice (BALB/c, DBA/2, A/J, C57BL/6), with sacrifice two or seven days post-exposure. The rationale for using intra-tracheal administration was that this route might lead to a more pronounced response than the oral route.

The control oil, rapeseed oil, caused an influx of neutrophils and lymphocytes in all four mouse strains tested. In BALB/c mice, intra-tracheal instillation of rapeseed oil even resulted, seven days post-exposure, in significant increases in wet and dry lung weight, total cell counts in BAL fluid, and subacute centrolobular inflammation in addition to minor combined peribronchial and perivascular infiltrates. It is known that rapeseed oil can modulate immune responses (Calder, 1998). In a study by Koller et al. (2002), canola oil even modulated autoantibody production. According to Parke and Parke (1999), rapeseed oil contains one of the smallest lipid macromolecule resistant to digestion and metabolism, accumulating progressively in

tissues, with toxic effects. The present findings seem to warrant that the toxicity of rapeseed oil be reassessed.

The further discussion focuses on the effects evoked by the RSO160401 oil in comparison with rapeseed oil.

In *BALB/c mice*, little or no pulmonary response was seen. The few histological changes observed, were not well reflected in changes in the other measured parameters.

In contrast, *DBA/2 mice* clearly showed adverse responses to RSO160401 instillation. Acute centrilobular inflammation seen by histopathological examination of the lung tissue two days post-exposure, was accompanied by alterations in body weight, wet lung weight, total cell counts, eosinophils, and total protein levels in BAL fluid. Seven days post-exposure, pathologic features were more pronounced showing clear subacute centrilobular inflammation. However, these were no longer reflected in significant changes in the other measured lung parameters. Of interest, the increase in peripheral blood eosinophilia was significant.

As in *DBA/2 mice*, there was clear evidence of pulmonary damage in *A/J mice*. In the RSO160401 treated group acute as well as subacute centrilobular inflammation was seen two days post-exposure. The overall findings were consistent with the histopathological picture. However, in contrast to *DBA/2 mice*, seven days post-exposure, most parameters had recovered to control levels, with only one animal showing major abnormalities.

In *C57BL/6 mice*, tissue histopathological manifestations were mild, if any, and in line with the other reported observations two and seven days post-exposure.

Interestingly, both rapeseed oil and RSO160401 oil treatment resulted in the detection of foamy alveolar macrophages. Like in the other mouse strains, some individual

response to oil treatment was high, resulting in severe reactions, not only in RSO160401 but also in rapeseed oil treated animals.

A disadvantage of this mouse model was that severity of injury varied greatly from individual to individual. The power of the study was such that it was not possible to confirm the interesting trends observed.

The choice of the four mouse strains used in our experiments, was based on presumed differences in genetic susceptibility. DBA/2 and C57BL/6 mice are Th-1 biased immune responders, known to evoke auto-immune reactions. A/J and BALB/c mice were chosen as Th-2 biased responders. A/J mice have the H-2a MHC haplotype, C57BL/6 are H-2b, BALB/c and DBA/2 are H-2d. A hierarchy in the strength of the immune response dependent on the MHC haplotype was proposed with H-2s classified as high, H-2b as low, and H-2a as nonresponder (Mirtcheva et al., 1989 in Berking et al., 1998). Berking et al. (1998) classified H-2s and H-2b as chronic responders with autoimmune-like reactions and H-2a as acute responders with toxic reactions. Unfortunately, nothing is known about H-2d responders in relation to the TOS. In a study by Berking et al. (1998), the intraperitoneally administration of oleic acid anilide to C57BL/6 mice (H-2b) led to splenomegaly and a humoral immune response indicating a polyclonal activation of B cells. In the same study, most oleic acid anilide-treated A/J mice developed an acute wasting disease with weight loss, cachexia, and death. Few A/J mice survived, having serologic changes similar to the C57BL/6 strain. All oleic acid anilide-treated C57BL/6 mice showed a strong reduction of the Th1-cell-derived cytokine Tumor Necrosis Factor-beta (TNF- β), together with elevated IgE serum levels, suggesting a Th-2 mediated mechanism. As postulated by Berking et al. (1998), these divergent reactions in A/J and C57BL/6

mice might parallel the human disease in TOS, which was characterized by acute severe symptoms, including respiratory failure and cachexia, or chronic autoimmune-like manifestations with increase in immunoglobulins and antibody formation. This is in line with our findings, where A/J mice responded to RSO160401 with acute lung injury. The response of C57BL/6 mice was characterized by a mild histopathological manifestation of the disease, but without increase in IgE serum levels (other immunoglobulins not measured).

In parallel with the genetic restriction and shift toward Th-2 pattern, the ability to biotransform toxic agents is probably also involved in the Toxic Oil Syndrome.

However, this was not further investigated in the scope of this study.

So, we hypothesize that the strain-dependent toxicity observed in our study is caused by the combination of genetic factors, including MHC genotype and the ability to clear toxic agents.

According to Bell et al. (2002), another mechanism is possibly involved. In our study, acute pulmonary inflammation was characterized by the influx of neutrophils and eosinophils (DBA/2 only), together with elevated total protein (and LDH) levels in the BAL fluid. Neutrophils play a prominent role in the host defense against pathogens, and they are considered to be responsible for pulmonary injury by the release of toxic contents such as proteases and oxygen radicals. Instillation of RSO160401 oil resulted in infiltration of neutrophils associated with acute lung injury, characterized by increased pulmonary vascular permeability. As suggested by Bell et al. (2002), the resulting inflammation together with the cell damage might lead to an increased exposure of the immune system to antigenic material followed by T cell stimulation. Further, Bell et al. (2002) speculated that toxic oils are transformed through macrophages, into reactive metabolites which bind to Antigen-presenting-

cells (APCs) with subsequent transformation of molecules capable of activating T cells and/or that toxic oils cause cell membrane damage and modification of self protein, thus possibly leading to the presentation of cryptic peptides that may elicit autoimmune T cell reaction. In our study, there is histopathological evidence for lipophages (DBA/2) and foamy alveolar macrophages, even giant cells (C57BL/6), after only one single intra-tracheal instillation of RSO160401 in the Th1-biased strains.

In conclusion, these studies show that intra-tracheal administration of the reconstructed RSO160401 oil led to a strain-dependent acute response, being most pronounced in the DBA/2 and A/J strains but much less in BALB/c and C57BL/6 mice. There was clear evidence of acute pulmonary damage in DBA/2 and A/J mice and increases in blood eosinophilia in DBA/2 mice. Lung inflammation and lesions were observed with a regression of acute pulmonary lesions with time post-exposure, being more complete in A/J than in DBA/2 mice. Taken together, as different effects between mouse strains are demonstrated, genetic susceptibility seems to be an important factor in injury induction by the RSO160401 oil.

Probably, repeated intra-tracheal instillations inducing a continuous encounter with small amounts of reactive metabolites of RSO160401 might provoke chronic autoimmune-like reactions in C57BL/6 and DBA/2 mice.

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Footnote to the title page

Abbreviations: BALF, bronchoalveolar lavage fluid; DCC, differential cell count; i.p. intraperitoneal; LDH, lactate dehydrogenase; PAP, 3-(N-phenylamino)-1,2-propanediol; PBS, phosphate buffered saline; OOPAP, 1,2-diolester of PAP; SD, standard deviation; TCC, total cell count; TNF- β , tumor necrosis factor-beta; TOS, toxic oil syndrome; WHO-CISAT, World Health Organization- Toxic Oil Research Center;

Legends

Table 1: Body weight, lung weight parameters, lung injury, lung inflammation, peripheral blood eosinophilia and serum IgE, two and seven days after a single intra-tracheal instillation in 4 strains of mice.

Legend Table 1:

Data are presented as mean (standard deviation); n=5, except 2 days post-exposure: C57BL/6 RSO160401 oil (n=6), and for body weight, lung weight parameters, LDH, Protein, TCC, DCC, serum IgE, BALB/c Anaesthesia control (n=7), Rapeseed oil and RSO160401 oil (n=8), except 7 days post-exposure: A/J RSO160401 oil (n=4), and for TCC, LDH, Protein, BALB/c Rapeseed oil and A/J Anaesthesia control (n=4). Statistically significant differences * $p < 0.05$, ** $p < 0.01$ (Newman-Keuls). **Bold font:** Statistically significant differences from rapeseed oil control. ¹ Statistically significant different from RSO160401 oil. Body weights have been expressed as a percentage of the respective starting weight.

Table 2: Macroscopic findings at autopsy and histopathology of the lung two and seven days after a single intra-tracheal instillation in 4 strains of mice.

Legend Table 2:

n=5, except 2 days post-exposure: C57BL/6 RSO160401 oil (n=6), BALB/c Anaesthesia control (n=7), Rapeseed oil and RSO160401 oil (n=8). Macroscopic findings: a) brownish spots on lung surface, b) patchy distributed haemorrhagic areas on lungs, c) diffuse haemorrhage on lungs, d) suppurating collection to the lungs, e)

shrinkage of the thymus, f) enlarged spleen, g) swollen, translucent kidney, h) bald area on skin. Histopathological findings: MA) major abnormalities, acute centrolobular inflammation characterised by neutrophils and intra-alveolar macrophages, MS) major abnormalities, subacute centrolobular inflammation characterised by fewer neutrophils, some tissue reaction and a mononuclear interstitial infiltration, ML) major abnormalities with lipophages, centrolobular inflammation with lipophages, although almost no infiltrate present, MM) major alveolar mononuclear, perivascular infiltration with granules and/or giant cells, MQ) foamy alveolar macrophages, P) focus of pleuritis, lung not affected.

Figure 1: Body weight, expressed as the percentage of initial body weight (panel A), wet left lung weight (panel B), and dry left lung weight (panel C) in DBA/2 mice (left panel) and A/J mice (right panel), 2 days post-exposure. Groups of mice (n=5) were anaesthetized, or received a single transoral intratracheal administration of rapeseed control oil (1 ml/kg bw) or RSO160401 test oil (1 ml/kg bw). Data points represent individual animals; horizontal scale bars represent means; Statistics: Newman-Keuls, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2: Lung injury as indicated by lactate dehydrogenase (LDH) activity (relative values) and total protein concentrations ($\mu\text{g/ml}$) in bronchoalveolar lavage fluid supernatant, 2 days (left panel) and 7 days (right panel) after a single transoral intratracheal instillation of RSO160401 test oil (1 ml/kg bw) or rapeseed control oil (1 ml/kg bw), or anaesthesia only, in DBA/2 and A/J mice. Data points represent individual animals; horizontal scale bars represent means; Statistics: Newman-Keuls, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3: Flow cytometry analysis of peripheral blood eosinophilia. Groups of BALB/c, DBA/2, A/J and C57BL/6 mice (n=10; except the RSO160401 oil group in A/J mice, n=9 and in C57BL/6 mice, n=11) were administered RSO160401 test oil (1 ml/kg bw) or Rapeseed control oil (1 ml/kg bw) by 1 single transoral intratracheal instillation, or anaesthesia only. Half of the animals (n=5 per group; except the A/J mouse RSO160401 group, 7 days post-exposure: n=4; except the C57BL/6 mouse RSO160401 group, 2 days post-exposure: n=6) were sacrificed two and seven days post-exposure. Results are obtained individually in each animal, and are expressed as % of positive cells (double labeled VLA/4 and Gr-1) out of total cells counted (10,000). Data points represent individual animals; horizontal scale bars represent means; Statistics: Newman-Keuls, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4: Histopathology (hematoxylin-eosin stain) of the lungs of DBA/2 mice having received a single intratracheal instillation of RSO160401 (1 ml/kg bw). Left panels A and C: two days post-exposure; acute centrolobular inflammation, characterized by neutrophils and intra-alveolar macrophages. Right panels B and D: seven days post-exposure; subacute centrolobular inflammation, characterized by fewer neutrophils, some tissue reaction and mononuclear interstitial infiltration. Magnification: A and B x50, C and D x200.

Table(s)

		Body weight		Lung weight		Lung injury			Lung Inflammation (BAL)			Blood	Serum
			Wet	Dry	Ratio	LDH	Protein	TCC	Lymphocytes	Neutrophils	Eosinophils	Eosinophils	IgE
		%	mg/100g	mg/100g		relative value	µg/ml	x 104	%	%	%	%	ng/ml
BALB/c													
Control, anaesthesia	2 days	97.3 (2.1)	188 (14)	42 (8)	4.6 (0.8)	0.172 (0.038)	5.0 (0.7)	13.0 (4.9)	0.8 (0.7)	5.9 (6.2)	0.1 (0.2)	2.1 (0.4)	908 (418)
	7 days	101.0 (2.0)	198 (10)	50 (3)	4.0 (0.1)	0.153 (0.038)	7.2 (3.3)	5.5 (2.3)	2.7 (2.7)	5.4 (3.4)	0.1 (0.1)	1.1 (0.5)	625 (392)
Control, rapeseed oil	2 days	99.4 (3.0)	225 (25)	60 (17)	4.0 (1.0)	0.258 (0.087)	7.0 (2.4)	14.1 (4.6)	0.8 (0.8)	11.3 (10.4)	1.6 (3.4)	2.8 (1.0)	1383 (1621)
	7 days	100.8 (1.3)	255 (29)**	74 (11)**	3.4 (0.3)**	0.277 (0.077)	9.5 (2.1)	12.1 (1.6)*	5.3 (1.6)* 1	14.7 (8.7)	1.9 (2.2)	1.6 (0.6)	434 (71)
RSO160401	2 days	96.0 (4.2)	236 (67)	59 (23)	4.1 (0.6)	0.252 (0.127)	6.7 (4.8)	18.5 (3.7)	0.4 (0.8)	3.7 (4.2)	0.4 (1.1)	2.0 (1.4)	1151 (363)
	7 days	99.8 (1.3)	208 (25)	53 (9)	3.9 (0.2)	0.159 (0.087)	6.6 (1.4)	16.6 (5.8)**	1.5 (1.7)	8.5 (8.2)	0.5 (1.2)	1.1 (0.6)	649 (339)
DBA/2													
Control, anaesthesia	2 days	99.2 (3.9)	157 (11)	40 (1)	3.9 (0.2)	0.274 (0.066)	4.5 (0.4)	8.5 (2.5)	0.1 (0.1)	1.7 (1.1)	0.0 (0.0)	2.6 (2.3)	1686 (1273)
	7 days	100.4 (4.5)	156 (10)	40 (1)	3.9 (0.3)	0.190 (0.015)	8.3 (1.3)	n/a	1.1 (1.1)	1.4 (0.7)	0.0 (0.0)	2.0 (0.5)	2589 (1936)
Control, rapeseed oil	2 days	98.0 (4.7)	181 (11)	51 (7)	3.6 (0.3)	0.350 (0.046)	6.9 (2.9)	10.5 (2.1)	0.8 (0.6)	13.7 (13.1)	0.0 (0.0)	1.5 (0.9)	941 (510)
	7 days	104.2 (4.7)	190 (40)	50 (13)	3.8 (0.3)	0.295 (0.064)	12.4 (2.1)	n/a	1.2 (0.5)	10.8 (6.4)**	0.0 (0.0)	3.1 (1.5)	1044 (1541)
RSO160401	2 days	90.2 (4.9)*	223 (39)*	63 (15)**	3.6 (0.3)	0.511 (0.189)*	24.4 (18)*	19.3 (5.8)**	0.5 (0.7)	21.9 (17.6)	1.4 (1.2)**	1.9 (1.2)	3119 (4313)
	7 days	102.4 (11.0)	226 (75)	57 (23)	4.0 (0.3)	0.323 (0.126)	15.2 (10.3)	n/a	3.9 (4.2)	12.5 (4.2)**	0.8 (1.1)	6.5 (2.0)**	1773 (1373)
A/J													
Control, anaesthesia	2 days	96.6 (2.7)	189 (8)	46 (2)	4.1 (0.1)	0.246 (0.066)	4.2 (0.9)	8.3 (3.1)	0.3 (0.4)	4.1 (2.3)	0.0 (0.0)	0.5 (0.3)	107 (65)
	7 days	98.2 (0.8)	183 (7)	45 (9)	4.0 (0.3)	0.156 (0.044)	6.6 (0.4)	n/a	1.5 (0.5)	3.3 (3.1)	0.0 (0.0)	2.9 (1.2)	44 (37)
Control, rapeseed oil	2 days	96.4 (3.4)	214 (25)	60 (11)	3.6 (0.3)*	0.328 (0.098)	5.7 (2.4)	10.8 (4.6)	1.9 (0.8)*	16.7 (6.6)	0.8 (1.8)	0.9 (0.6)	474 (509)
	7 days	102.0 (2.6)	204 (40)	54 (17)	3.9 (0.4)	0.170 (0.061)	7.9 (3.2)	n/a	1.3 (0.8)	12.1 (15.0)	0.0 (0.0)	2.4 (0.9)	93 (62)
RSO160401	2 days	89.4 (5.4)*	294 (51)**	83 (14)**	3.6 (0.4)*	0.447 (0.167)*	13.2 (8.7)*	24.2 (18.6)	0.7 (1.3)	26.7 (25.8)	0.9 (0.7)	0.9 (0.8)	518 (223)
	7 days	101.8 (3.1)	210 (40)	21 (10)	4.0 (0.1)	0.167 (0.054)	7.8 (2.8)	n/a	1.2 (1.7)	17.7 (19.0)	0.1 (0.2)	3.7 (2.0)	139 (56)
C57BL/6													
Control, anaesthesia	2 days	99.2 (2.8)	183 (7)	46 (2)	4.0 (0.1)	0.259 (0.105)	4.2 (0.9)	9.6 (1.8)	1.2 (0.5)	0.3 (0.4)	0.0 (0.0)	1.9 (0.3)	115 (58)
	7 days	105.6 (5.0)	179 (5)	46 (2)	3.9 (0.1)	0.270 (0.064)	5.1 (1.1)	11.4 (2.6)	1.5 (0.9)	0.3 (0.3)	0.1 (0.1)	2.1 (2.4)	312 (250)
Control, rapeseed oil	2 days	99.2 (3.3)	210 (40)	60 (26)	3.8 (0.6)	0.303 (0.088)	5.4 (3.4)	14.8 (4.8)*	0.5 (0.3)	9.3 (19.6)	0.4 (0.5)	2.7 (1.0)	143 (56)
	7 days	104.0 (2.3)	209 (24)	57 (14)	3.7 (0.4)	0.234 (0.036)	4.6 (0.6)	11.0 (2.1)	1.0 (0.5)	4.8 (4.7)	0.3 (0.3)	4.3 (3.4)	200 (127)
RSO160401	2 days	98.2 (2.6)	225 (26)	61 (11)	3.7 (0.3)	0.309 (0.068)	7.8 (4.9)	17.6 (3.5)**	1.1 (0.7)	25.6 (16.3)*	0.5 (1.1)	4.0 (3.1)	206 (124)
	7 days	98.6 (7.0)	202 (27)	55 (9)	3.7 (0.1)	0.238 (0.059)	4.0 (0.3)	15.4 (5.5)	0.4 (0.4)*	2.0 (2.1)	0.1 (0.1)	2.0 (1.3)	238 (145)

Table(s)

	Control				RSO160401	
	Anaesthesia		Rapeseed oil			
	2 days	7 days	2 days	7 days	2 days	7 days
Macroscopic appearance						
BALB/c					1 a, 1 b	
DBA/2					2 b	2 b
A/J			1 b		4 c	
C57BL/6	2 g	1 h			1 a, 1 b	1 d, 1 e&f
Histopathology						
BALB/c				1 MS	2 MA	
DBA/2					2 MA	3 MS, 1 ML
A/J			1 MA		3 MA, 2 MS	1 MS
C57BL/6			1 P, 3 MQ	2 MM, 1 MQ	1 MM, 3 MQ	1 MM, 1 MQ

Fig 1

A

B

C

DBA/2

Fig 2

A/J

Fig 3

Figure(s)
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