Oral Exposure to Drugs with Immune-Adjuvant Potential Induces Hypersensitivity Responses to the Reporter Antigen TNP-OVA

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Immune-mediated drug hypersensitivity reactions are important causes of black box warnings and drug withdrawals. Despite the high demand for preclinical screening tools, no validated in vitro or in vivo models are available. In the current study, we used a previously described oral administration model using trinitrophenyl-ovalbumin (TNP-OVA) as an antigen to report immuno-adjuvating effects of the analgesic drug acetaminophen (APAP) and its nonhepatotoxic regioisomer 3'-hydroxyacetanilide (AMAP), the antibiotic ofloxacin (OFLX), the antiepileptic drug carbamazepine (CMZ), and the antidiabetic drug metformin (MET). Furthermore, APAP and AMAP were tested in a popliteal lymph node assay (PLNA) combined with TNP-OVA as reporter antigen (RA). C3H/HeOuJ mice were dosed by oral gavage with diclofenac (DF), APAP, AMAP, OFLX, MET, or CMZ. On the first exposure day, the mice received an ip injection with TNP-OVA. Fifteen days later, they were ear challenged with TNP-OVA and delayed-type hypersensitivity (DTH) responses were assessed 24 h later. One week after challenge, the ear-draining lymph node was removed and TNP-specific antibody-secreting cells were determined. DF, APAP, CMZ, and OFLX showed a significant increase in DTH responses to ear injection with TNP-OVA, whereas AMAP and MET did not. C57BL/6 mice were slightly less responsive to APAP and DF after oral gavage, and importantly both AMAP and APAP were negative in the RA-PLNA. The present work shows that the oral exposure model using RA and the RA-PLNA may serve to screen the immune-adjuvant potential of new chemical entities during preclinical drug development.

Key Words: immune-mediated drug hypersensitivity; preclinical testing; allergic drugs; oral administration; reporter antigen.

Immune-mediated drug hypersensitivity reactions (IDHR) are among the most frequent reasons for failure of new drugs. IDHR may cause patients to be admitted to hospital with life-threatening effects like drug-induced liver injury or toxic epidermal necrolysis. Unfortunately, due to their low frequency, IDHR are often unnoticed until a drug has been marketed and used in the general population for sometime (Nisbitt et al., 2000; Pourpak et al., 2008). Clearly, there is a need for predictive models to test the sensitizing capacity of new drugs during early stages of development.

The etiology of IDHR is very complex. Low molecular weight drugs as such are generally not immunogenic, i.e., are unable to cause drug-specific sensitization. However, these compounds or metabolites thereof may be able to bind to proteins to form drug-protein adducts or otherwise induce neoantigens, which can subsequently be presented to T cells by antigen-presenting cells or recognized by B cells. In addition, costimulatory help that may arise from immune-adjuvant effects is indispensable for optimum immunization.

The popliteal lymph node assay (PLNA) in its original version measuring increases in draining lymph node weight or cellularity upon sc footpad injection of a drug appears to enable prediction of a drug’s immune-stimulating capacity (Pieters, 2001; Weaver et al., 2005). Addition of reporter antigens (RA) in the PLNA further improves this assay by allowing discrimination between the adjuvant (using trinitrophenyl-ovalbumin [TNP-OVA]) or sensitizing (using TNP-Ficoll) capacity of drugs (Gutting et al., 1999; Pieters, 2001). However, although the RA-PLNA can be used as a straightforward screening test for new compounds, chemicals are injected sc and induce a local response. Hence, this way excludes major influences of gastrointestinal and hepatic metabolism on the local response.

Previous studies demonstrate the ability of an oral mouse model using RA to identify the immune-adjuvant or sensitizing capacity of drugs (Gutting et al., 2002a; Nierkens et al., 2005). In the current study, we extended these data by testing an additional selection of drugs in combination with TNP-OVA as RA. As positive compounds, the analgesic drug acetaminophen (APAP), the antibiotic ofloxacin (OFLX), the nonsteroidal anti-inflammatory drug diclofenac (DF), and the antiepileptic drug carbamazepine (CMZ) were used. As control compound for APAP, its nonhepatotoxic regioisomer 3'-hydroxyacetanilide (AMAP) and as an adverse drug reaction (ADR)-negative
compound the antidiabetic drug metformin (MET) were selected.

Although some IDHR have been reported for APAP, this compound was mainly chosen for its well-described adverse reactions in the liver. At therapeutic doses, APAP is metabolized by cytochrome P450 to N-acetyl-p-benzoquinone imine (NAPQI) and subsequently detoxified by glutathione. When overdosed, NAPQI depletes the liver of glutathione, allowing binding of metabolites to hepatic proteins causing toxicity (James et al., 2003). The isomer of APAP, AMAP, has also been shown to form reactive metabolites. However, it does not induce liver toxicity and glutathione depletion to the extent APAP does and therefore was used as control compound for APAP (Rashed et al., 1990; Salminen et al., 1998).

DF is a widely used drug, which incidentally induces severe hepatotoxicity. This compound is capable to form drug-protein adducts (Boelsterli, 2003; Naisbitt et al., 2007). Furthermore, being a nonselective cyclooxygenase (COX) inhibitor, DF can cause gastrointestinal irritation and ulceration, possibly resulting in an inflammatory environment. Moreover, DF has previously been tested in the (RA)-PLNA and oral models using either TNP-OVA or TNP-Ficoll and is regarded as positive control compound in these models (Gutting et al., 2002b,c; Nierkens et al., 2005).

Adverse reactions caused by CMZ involve the liver but are often also skin related. This compound has been shown to form reactive intermediates and drug-protein adducts (Ju and Uetrecht, 1999; Lillibridge et al., 1996; Njoku, 2010). In addition, CMZ and CMZ metabolite-specific T cells have been identified in CMZ-sensitized patients (Mauri-Hellweg et al., 1995; Wu et al., 2006).

Fluoroquinolones, e.g., OFLX, exhibit gastrointestinal and skin-related side effects (Rubinstein, 2001). As for CMZ, fluoroquinolones-specific T cells have also been observed in allergic patients (Schmid et al., 2006).

We have so far mainly used C3H/HeOuJ mice for the oral TNP-OVA approach and furthermore found that BALB/c mice appeared to be less suitable. However, because an important readout parameter appeared to be Thelper 1 (Th1)-dependent delayed-type hypersensitivity (DTH) responses, we compared the C57BL/6 strain, claimed to be biased toward cellular immune responses, with the C3H/HeOuJ strain, known to be biased toward humoral responses.

The results described here show that the combination of an RA-PLNA and an oral administration protocol using RA may be able to determine immune-adjuvant effects of new chemical entities and can be a useful tool in preclinical screening.

**MATERIALS AND METHODS**

**Mice.** Four- to 6-week-old female C3H/HeOuJ (C3H) or C57BL/6Jco (B6) mice from Charles River (France and Germany, respectively) were used. Mice were specific pathogen free and maintained under barrier conditions in filter-topped macronol cages with wood chip bedding at a mean temperature of 23 ± 2°C, 50–55% relative humidity, and a 12-h light/dark cycle. Drinking water and standard laboratory food pellets were provided ad libitum.

FIG. 1. Oral exposure protocol to assess the immune-adjuvant capacity of drugs. The immunosensitizing capacity of DF, APAP, AMAP, OFLX, CMZ, and MET to TNP-OVA was assessed by treating C3H mice orally with the specified drugs. On the first day of exposure, mice were injected ip with TNP-OVA (10 µg). After 15 days, mice were ear challenged with TNP-OVA. The same protocol was used to compare strains.

**Chemicals.** Chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. TNP-OVA and TNP-bovine serum albumin (BSA) was obtained from Biosearch Technologies (Novato, CA).

All compounds for sc injection were dissolved in saline and compounds for oral administration were dissolved in distilled water (Aqua B. Braun, Melsungen, Germany). CMZ was dissolved in 1% carboxymethylcellulose in H2O.

**Reporter antigen-popliteal lymph node assay.** Naïve B6 mice were injected sc into the hind footpad (toe-to-heel) with a freshly prepared mixture of APAP (0.3 or 1 mg) or AMAP (0.3 or 1 mg) together with a subsensitizing dose (10 µg) of TNP-OVA in 50 µl saline. Seven days after drug injection, mice were sacrificed by cervical dislocation and the popliteal lymph node (PLN) was removed and separated from adherent fatty tissue. PLNs were placed in ice-cold complete RPMI 1640 supplemented with 2.5% fetal calf serum and 1% penicillin/streptomycin (RPMI/2.5%), and single-cell suspensions were prepared, washed, and resuspended in 0.5 ml RPMI/2.5%. Cell numbers were determined using a Coulter Counter (Beckman Coulter) and adjusted to 1 × 10^6 cells per ml.

**Oral exposure protocols.** C3H mice received OFLX, APAP, AMAP, CMZ, or MET by oral gavage (200 µl) in different regimens chosen using the maximum tolerable dose as described in the datasheet of the compounds and used in literature. OFLX was dosed at 100, 300, or 1000 mg/kg, MET was dosed at 50, 100, or 500 mg/kg, and APAP and AMAP were administered at 30, 100, or 300 mg/kg for 7 consecutive days. CMZ was dosed 50 or 100 mg/kg for 7 days or at 250 mg/kg on day 1 (Fig. 1).

To study strain differences, C3H mice and B6 mice received APAP (150 mg/kg) for 7 days or a single dose (75 mg/kg) of DF on day 1 (Fig. 1).

On the first day of drug exposure, mice were injected ip with a subsensitizing dose (10 µg in 200 µl saline) of TNP-OVA to assure systemic responses. Mice were challenged with TNP-OVA (10 µg in 20 µl saline) in the ear on day 15 to assess DTH responses. Ear thickness was measured under isofluran anesthetics using a digital microcalliper (Mitutoyo, Japan).

One week after challenge, the mice were sacrificed by cervical dislocation. The auricular lymph node (ALN) was removed and separated from adherent fatty tissue. Single-cell suspensions of the ALN were made as described for the PLN.

**T-cell depletion.** C3H mice were treated with DF or APAP according to the oral administration protocol described in Figure 1. In addition, mice received ip...
injections with 250 μg T-cell depleting antibody (anti-CD3 clone 17A2) in 200 μl saline on days 13 and 14. The antibody was purified from culture supernatant using ammonium sulfate gradient on thiophilic agarose resin.

**ELISpot assay.** ELISpot assay, to detect TNP-specific antibody-secreting cells, was performed as described previously (Schielen et al., 1995). In brief, 5 × 10⁵ cells were added to TNP-BSA–coated Immobilon-P membranes (Immobilon polyvinylidene fluoride transfer, Millipore, Etten-Leur, The Netherlands). After cell incubation (4 h, 37°C), alkaline phosphatase-conjugated immunoglobulin G1 (IgG1) or IgG2a antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) were added and incubated overnight. After several washing steps, membranes were stained with para-nitroblue tetrazolium (Sigma-Aldrich) and 5-brome-4-chloro-3-indolylphosphate toluidine salt in dimethylformamide to visualize TNP-specific antibody spots. Spots were counted by sight by at least two independent observers.

**Statistical analysis.** Multiple comparisons of group means were analyzed using one-way ANOVAs with Bonferroni as post hoc test. A value of p < 0.05 was considered significantly different compared with controls (Graphpad prism 4 for Windows, Graphpad software Inc.)

### RESULTS

**Neither APAP nor AMAP Stimulate Antibody Responses to TNP-OVA in the RA-PLNA**

The immune-adjuvant capacity of APAP and AMAP was examined in the RA-PLNA using TNP-OVA as RA. Neither APAP nor AMAP was able to induce a significant increase in PLN cellularity (Fig. 2A). Furthermore, the amount of TNP-specific IgG1-producing antibody secreting cells (ASC) was not increased by either chemical (Fig. 2B).

The lack of a response to APAP and AMAP observed in the RA-PLNA indicates that the metabolizing capacity in the footpad is insufficient to detect the adverse immune effects of this compound in this assay.

**APAP, CMZ, DF, and OFLX, but not AMAP and MET, Stimulate CD3-Dependent TNP-OVA–Specific DTH Responses**

In contrast to the RA-PLNA, the described oral exposure model does take metabolism into account. Four compounds known to cause ADR and two ADR-negative compounds were administered orally to C3H mice. To determine RA-specific DTH responses, mice treated to drugs and TNP-OVA received an sc TNP-OVA injection into the ear. Twenty-four hours later, the increase in ear thickness was determined.

Animals exposed to APAP showed a significant dose-dependent increase in DTH responses (Fig. 3A), whereas its nonhepatotoxic regioisomer AMAP did not cause an increase in DTH responses at any dose (Fig. 3B). Oral OFLX treatment showed a significant increase in the RA-specific DTH response after exposure to 300 mg/kg but not after exposure to 1000 mg/kg OFLX (Fig. 3C). The negative compound MET did not induce a DTH response at any dose (Fig. 3D), although the control group did have a high background response. Exposure to DF and the highest concentration of CMZ caused a significant increase in RA-specific ear swelling when compared with control animals (Figs. 3E and 3F).

T-cell depletion (using anti-CD3) resulted in a diminished DTH response in DF- or APAP-treated mice, which is indicative of the T-cell dependence of the DTH response measured (Fig. 4). These results show that of the selection of compounds tested, only those linked to any kind of toxicity induce a T-cell–dependent DTH response.

**Oral Exposure to Drugs May Result in TNP-OVA–Specific Antibody-Secreting Cells**

Seven days after ear challenge with the RA, TNP-OVA–specific IgG1 antibody responses in the ALN were determined using the ELISpot assay.

Exposure to APAP resulted in increases in RA-specific IgG1 ASC in some animals (Fig. 5A), whereas AMAP exposure did not show changes in IgG1 levels (Fig. 5B). Of all other compounds, only DF caused a significant increase in the amount of IgG1 ASC (Figs. 5C–F).

**FIG. 2.** Immune-adjuvant capacity of APAP and AMAP. B6 mice were injected sc with 0.3 or 1 mg APAP or AMAP in the hind footpad together with a subsensitizing dose of TNP-OVA (10 μg). After 7 days, the PLN was removed, and subsequently lymph node cellularity (A) and the amount of IgG1 ASC (B) were determined.
Oral Administration of a Drug Induces Higher DTH Responses in C3H Mice Compared with B6 Mice

To compare RA-specific responses of different mouse strains, B6 mice and C3H mice were subjected to the oral model described in Figure 1.

In C3H mice, the DTH response toward TNP-OVA was significantly increased compared with controls when mice were treated with either DF or APAP (Fig. 6A). B6 mice also displayed a DTH response toward the RA after oral administration of DF or APAP, but this response was not significant. Treatment with DF and APAP resulted in increased numbers of IgG1-secreting cells, which were only significant in B6 mice (Fig. 6B). In contrast to the results described in Figure 5F and previous experiments (data not shown),

**FIG. 3.** DTH response measured by ear swelling. C3H mice were orally treated with 30/100/300 mg/kg APAP/AMAP (A and B), 100/300/1000 mg/kg OFLX (C), 50/100/500 mg/kg MET (D), or 50/100 mg/kg CMZ (E) for 7 consecutive days. DF (75 mg/kg) and 250 mg/kg CMZ were given only on day 1. Administration of the drug was performed by oral gavage and the RA by ip injection. At day 15, ear thickness was measured after which mice received an sc injection in the ear with TNP-OVA. After 24 h, ear thickness was measured again to determine the DTH response. *p < 0.05, **p < 0.01, and ***p < 0.001 significantly different compared with vehicle controls.
exposure of C3H to DF in this experiment did not result in significantly increased numbers of IgG1-secreting cells, which is indicative of the variability of this parameter. These results suggest that the strength of drug-induced responses are strain dependent.

**DISCUSSION**

In this study, the PLNA and a previously described oral sensitization model, both in combination with TNP-OVA as RA, have been used to test the immune-adjuvant capacity of an additional selection of pharmaceuticals (Nierkens et al., 2005; Pieters, 2001; Weaver et al., 2005). Previously, it has been discussed that immunostimulation of TNP-specific responses in these models allows assessment of adjuvant or danger-inducing activity of (new) compounds.

Of the pharmaceuticals used in this study, APAP, OFLX, DF, and CMZ, but not MET and AMAP, were clearly able to induce increased responses toward TNP evidenced in particular by moderate dose-dependent increases of DTH responses to TNP-OVA. Importantly, these responses were T-cell dependent and hence can be regarded as relevant DTH responses (Posadas and Pichler, 2007; Rozieres et al., 2009).

In contrast to the DTH responses, TNP-specific antibody responses measured by ELISpot were less pronounced, and although the ELISpot as such is regarded a sensitive assay, the high variability of this assay hinders drawing clear conclusions. The use of TNP-Ficoll as RA to register neoantigen-specific T cells or the inclusion of other readout parameters might be an improvement to the oral exposure model. Importantly, the use of an RA allows testing numerous drugs using the same readout system, but the presence of drug-specific (T-cell) responses remains undetermined. Of the compounds we tested here, DF, OFLX, and CMZ have previously been shown to be able to induce drug-specific T cells in drug-sensitized humans (Beeler et al., 2006; Naisbitt et al., 2003; Schmid et al., 2006; Wu et al., 2006). Isolated T cells from sensitized patients were stimulated with the culprit drug or its metabolite, resulting in the production of a specific set of cytokines secreted by these cells (Lochmatter et al., 2009). Furthermore, the expression of certain T-cell activation markers like CD69 were found to be upregulated on T cells of sensitized patients when stimulated with the specified drug (Beeler et al., 2008). In addition to the DTH and number of antibody-secreting cells, the production of cytokines or expression of T-cell activation markers after incubation of cells with drugs or their metabolites could be included as complementary parameters in the oral administration model. To identify effects caused by the drug itself or by metabolites thereof, mice could be challenged with proteins conjugated with the different compounds or their metabolites.

The use of DF in this study was based on previous studies, and our results confirmed that this drug is able to induce robust DTH responses toward the RA upon oral administration (Nierkens et al., 2005). However, the mechanism by which DF stimulates responses toward the RA is yet unknown. DF is able to increase cell numbers in the draining lymph node after sc injection into the footpad, despite the fact that metabolism is absent in the paw (Gutting et al., 1999, 2002a,b). Furthermore, incubation of isolated splenocytes or several hepatocyte cell lines with DF resulted in the induction cell death (data not shown and Wang et al., 2002). This indicates that the parent compound is able to induce cell stress or inflammation, possibly attracting metabolizing macrophages. Whether gluco-coordination of DF by liver metabolism and the subsequent enterohepatic circulation of protein-coupled metabolites are also involved after oral exposures remains to be established. Furthermore, the direct pharmacological effects of oral exposure to DF might be of influence on the response. Recently, we have shown that DF stimulates the allergic response to peanut in a mouse model. In that study, we observed that DF, being a COX inhibitor, causes damage to the epithelial lining of the gut and the stomach, thereby possibly causing a local response (Bol-Schoenmakers et al., 2011).

The immune-adjuvant effects of APAP appeared to be dependent on the route of exposure. APAP was not able to enhance TNP-OVA responses in the RA-PLNA, but the compound showed a dose-dependent DTH response to TNP-OVA when used in the oral exposure model. This indicates that metabolism is required for the toxic effect of APAP, which is lacking in the paw. In line with this, APAP (up to 2mM) was not able to induce direct cell death in H1G1 mouse hepatocytes (data not shown). AMAP did not induce a response in the RA-PLNA or the oral model, despite the fact that AMAP is also metabolized. Metabolites of AMAP and APAP have
been shown to bind to different proteins in hepatocytes, with APAP derivatives binding to mitochondrial proteins and AMAP derivatives binding merely to cytosolic proteins (Rashed et al., 1990; Roberts et al., 1990; Tirmenstein and Nelson, 1991). In addition, APAP is able to deplete mitochondrial glutathione and cause disruption of calcium homeostasis to a higher extent than AMAP (Tirmenstein and Nelson, 1989). Apparently, the metabolites of APAP and AMAP are toxicologically different, resulting in their different adjuvant effects. Notably, AMAP has not been tested as a drug in the general population, and we can therefore not completely rule out the possibility of this compound to induce adverse reactions. However, in present experiments, it does not act as an adjuvant and is therefore considered a negative compound.

Similar to DF, also OFLX was able to induce a response in the PLNA and also in a modified lymph node assay using the ear-draining lymph node as readout (Nierkens et al., 2004). Therefore, this compound most likely does not require metabolism to become immune stimulating in the PLNA. In contrast to all other positive compounds studied, OFLX showed a higher DTH response at a dose of 300 mg/kg than after a dose of 1000 mg/kg. Similar results have been shown...
previously for DF and D-Pen in combination with TNP-Ficoll (Gutting et al., 2003; Nierkens et al., 2005). This illustrates that determination of the correct dosing and duration of administration of the compound is of crucial importance in further exploration of this model.

CMZ injection into the paw resulted in increased cellularity in the PLNA, indicative of an adjuvant capacity of this drug (Kammüller et al., 1989). CMZ is also metabolized after oral administration, and similar to DF and OFLX, both metabolites and the parent compound could act as immune adjuvant. As described previously, the dosing and subsequent challenge with TNP-OVA is very delicate, and this may be the reason of the high background DTH response observed after MET exposure (Nierkens et al., 2005). Consequently, these high background levels hamper the conclusions that can be drawn following oral administration of this compound. However, because no significant differences were observed between treatment groups, MET is regarded a negative compound.

As shown in the present and in previous studies using the (RA)-PLNA or oral administration models, the mouse strain influences on the outcome of the experiments (Kammüller et al., 1989; Nierkens et al., 2005). When exposed to DF or APAP, B6 mice showed a DTH response but C3H mice showed higher DTH responses toward the same compounds. In contrast, the number of TNP-specific antibody-secreting cells was higher in B6 compared with the number of antibody-secreting cells in C3H mice. Although strain-specific responses have been found frequently in studies on drug-induced immune responses, it is remarkable that a strain claimed to be a typical Th1 responder shows higher antibody responses than typical Th2-responding C3H mice (Pieters and Nierkens, 2007).

In addition to effects due to genetic background, we have previously demonstrated that the chemical itself might be as important in determining the immunological outcome (Albers et al., 1998). For example, DF is capable of inducing intestinal damage and COX inhibition, and e.g., APAP in high doses results in liver damage. Although the doses for all compounds have been chosen carefully, possible pharmacological effects of the compounds should be taken into account when analyzing the data.

In conclusion, the present work shows that the oral exposure model using RA and the RA-PLNA may serve to screen the immune-adjuvant potential of new chemical entities during preclinical drug development.

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