Development of an Oral Exposure Mouse Model to Predict Drug-Induced Hypersensitivity Reactions by Using Reporter Antigens

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The capability of certain drugs to cause immune-mediated drug hypersensitivity reactions in susceptible individuals has initiated a search for pre-clinical screening tools to identify immunosensitizing drugs. Since most drugs are taken orally, hazard assessment of their immunosensitizing potential should include oral exposure models. In this study, the predictive value of the reporter antigen (RA) approach was investigated in combination with oral or intraperitoneal (ip) exposure to a selection of allergenic drugs, i.e., D-penicillamine (D-Pen), Diclofenac (DF), or Nevirapine (Nevi). The RA trinitrophenyl-Ovalbumin (TNP-OVA) was used to assess the capacity of the drugs to stimulate systemic immune responses to a bystander antigen, whereas the RA TNP-Ficoll was used to indicate whether the drugs were able to induce specific anamnestic T-cell responses. TNP-OVA was injected (ip) in C3H/HeOuJ mice that were subsequently exposed (orally or ip) to one of the drugs via different exposure protocols. All three model drugs used resulted in delayed type hypersensitivity reactions to TNP-OVA after ip and oral exposure. In addition, TNP-specific serum antibody levels were increased after ip exposure to Nevirapine, and after both oral and ip exposure to D-Pen and DF. These data indicate that the present drugs are able to stimulate immune responses to bystander antigens. Responses to TNP-Ficoll were measured in the popliteal lymph node of BALB/c mice three weeks after they received a single oral dose of D-Pen or DF. Results of this approach show that orally pre-treated mice responded with enhanced responses (TNP-specific IgG1 and IFN-γ production) to sub-optimal injection of the test compound, seems a promising tool for this purpose (Pieters et al., 2002). Many compounds have been tested over the past 20–25 years and responses in the PLNA show good correlation with human clinical data (Pieters and Albers, 1999b). Together, the RA-approach allows assessment of systemic sensitization upon oral and/or ip exposure to the selected drugs. To further evaluate the utility of these models, more drugs, including non-allergic drugs and those that require metabolic conversion to become allergenic need to be studied in the present models.

Key Words: drug-induced hypersensitivity; reporter antigen; mouse model; oral; relevant route; antibodies.

Some drugs cause allergic and autoimmune reactions in a considerable proportion (5–20%) of patients (Bigazzi, 1997). Moreover, immune-mediated drug hypersensitivity reactions (IDHR) have been reported to be the most frequent cause (64% of cases) of failure of drugs during clinical development (Dean, 2000). The accompanying costs and potential severe morbidity of these unexpected idiosyncratic reactions are of concern in clinical practice and drug development. Patients suffering from IDHR often display clinical symptoms that resemble the characteristics of idiopathic autoimmune derangements (Pichler, 2003) and the underlying etiology comprises a similar multifactorial complexity, including genetic predisposition factors (MHC haplotype, metabolic polymorphisms, complement deficiencies, gender, etc.) and a range of environmental factors (food intake, ongoing infections, etc.) (Adkinson et al., 2002). As a result, the exact mechanisms of drug-induced immunosensitization are mostly unknown, which hampers the development of a generalized screening tool to assess drug-induced immunosensitization. Consequently, a standardized and validated screening test to assess the hazard of orally taken drugs to induce systemic hypersensitivity does not exist.

The popliteal lymph node assay (PLNA), which focuses on lymphocyte activation in the draining lymph node upon footpad injection of the test compound, seems a promising tool for this purpose (Pieters et al., 2002). The use of well-defined bystander antigens in the PLNA, so-called ‘reporter antigens’ (RA), have further improved the assay with respect to robustness and capacity to discriminate sensitizing from non-sensitizing chemicals (Albers et al., 1997; Pieters and Albers, 1999b). In the RA-PLNA, the chemical of interest dictates the formation of RA-specific antibody secreting cells (ASC) to a co-injected sub-sensitizing dose of RA (Albers et al., 1997; Nierkens et al., 2002; Pieters and Albers, 1999b). This enables measurement of RA-specific ASC as a fast and simple read-out parameter for immunogenic potential. More specifically, the RA used in the RA-PLNA allow discrimination of chemicals that induce non-cognate T-cell activation (i.e., IgG formation to the
RA trinitrophenyl (TNP)-Ficoll) and chemicals that stimulate cognate T-cell activation (i.e., IgG responses to the RA TNP-Ovalbumin [OVA]). (For a review see Pieters and Albers, 1999a.)

Efficient immune responses to TNP-OVA require cognate T-B cell interactions and costimulatory adjuvant signals (Nierkens et al., 2002). Therefore, any reactive drug that induces inflammatory mediators, including non-sensitizing irritants or adjuvants, will induce or enhance the formation of TNP-specific IgM and IgG ASC. Alternatively, the sugar-antigen TNP-Ficoll is unable to cause T-cell sensitization, but it is well capable of stimulating B cells to produce IgM isotypes. However, when TNP-Ficoll is injected together with a sensitizing drug that is able to induce the formation of neo-T-cell epitopes, the TNP-specific B cells will receive soluble help from these non-cognate neo-antigen-specific T cells and will elicit TNP-specific IgG responses. Hence, the formation of TNP-specific IgG ASC to TNP-Ficoll indicates that the drug induces neo-antigen specific T-cell help. Together, the RA-approach with well-defined bystander antigens may provide a robust and specific T-cell-dependent read-out parameter to assess the immunosensitizing potential of a wide variety of drugs.

Although the (RA-)PLNA is very well suited as a fast and simple pre-clinical screening assay to detect immunosensitizing potential of chemicals, it has the important drawback that the chemicals are injected subcutaneously (sc) in the footpad, whereas most drugs are taken orally. Evidently, for more relevant hazard identification it would be important to assess whether chemicals can induce systemic immunological changes via the oral route of exposure. However, the development of oral exposure models for screening purposes is hindered by insufficient knowledge about mucosal and systemic immunoregulatory processes in relation to oral antigen exposure. Of particular relevance for the present study is that antigens generally induce immunological tolerance when administered orally, of which the underlying mechanisms are only poorly understood (Strobel and Mowat, 1998). In addition, the antigen-specificity of drug-induced immunological responses can vary from hapten-specific to auto-antigen-specific, which further impedes analyses of specific responses. However, this latter difficulty may possibly be circumvented by using the immune response to RA as a standard read-out for sensitization.

In the present study, we evaluated whether oral administration of IDHR-associated drugs are able to stimulate systemic immunosensitization to a bystander antigen, TNP-OVA. In these experiments C3H/HeJ mice were ip injected with a sub-sensitizing concentration of TNP-OVA on day 1. Mice were gavaged with different model allergenic drugs in multiple doses. The capacity of the orally administered drugs to induce specific immune responses to TNP-OVA was monitored by measuring TNP-OVA-specific serum antibodies and delayed type hypersensitivity (DTH) upon a challenge with TNP-OVA. As model compounds, we used D-penicillamine (D-Pen), Diclofenac (DF), and Nevirapine (Nevi), because these drugs are associated with autoimmune-like derangements in humans (Boelsterli, 2003; Emery and Panayi, 1989; Pollard et al., 1998) and animal models (Brik et al., 1995; Donker et al., 1984; Gutting et al., 2002; Shenton et al., 2003; Ware et al., 1998). Intrapерitoneal (ip) injections with these drugs were used as a positive reference for the induction of systemic sensitization.

In addition, D-Pen and DF were used in a model with TNP-Ficoll to elucidate whether oral administration of the drugs primes T cells to respond to a secondary stimulation, as was recently presented by Gutting et al. (2002). In this model, BALB/c mice receive a single gavage of the drug and are challenged three weeks later with a suboptimal dose of the drug together with TNP-Ficoll. The detection of TNP-specific IgG ASC in the draining lymph node indicates that the drug is able to stimulate specific T cells.

Present results show that the TNP-OVA model identifies D-Pen, DF, and Nevirapine as compounds with systemic immuno-sensitizing capacity when administered orally or ip. In addition, the model with TNP-Ficoll provides evidence for priming of specific T cells in case of oral exposure to D-Pen or DF. These findings indicate that the RA approach may be useful to assess the hazard of immunostimulating and/or immunosensitizing capacity of drugs administered via the oral route of exposure.

MATERIALS AND METHODS

Mice. Female C3H/HeOuJ mice from Charles River (Lyon, France) and BALB/c mice from Harlan (Horst, The Netherlands) were used. All mice were 6–8 weeks old, specific pathogen-free and maintained under barrier conditions in filter-topped Macrolon cages with wood chips bedding, at a mean temperature of 23 ± 2°C, 50–55% relative humidity, and 12 h light/dark cycle. Drinking water and standard laboratory food pellets were provided *ad libitum*. Mice were allowed to settle for a week before random assignment to specific treatment groups. The experiments were conducted according to the guidelines of the animal experiments committee of the Faculty of Veterinary Medicine, Utrecht University.

Chemicals. Chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless stated otherwise. D-Pen and DF were diluted in saline (0.9%; B. Braun, Melsungen, Germany). Nevirapine was kindly provided by Dr. J. Weaver (Food and Drug Administration, Division of Applied Pharmacology Research, Office of Testing & Research). TNP-Ficoll and TNP-OVA were prepared as previously described (Albers et al., 1996).

Oral Exposure Models

Oral exposure model with TNP-OVA. C3H/HeOuJ mice were gavaged or ip injected with D-Pen, DF, or Nevirapine using different oral exposure regimens for the different drugs. For D-Pen, mice were exposed to 50 or 150 mg/kg each day during 7 or 21 days (Fig. 1A). These doses were chosen based on a publication by Brik et al. showing that 50 mg/kg and/or 150 mg/kg D-Pen induced elevated levels of autoantibodies (Brik et al., 1995). Based on the positive antibody responses in our study after only seven days of exposure to D-Pen, we decided to test whether similar doses and time courses of exposure could be used for Nevirapine. Nevirapine (150 mg/kg) was administered for six consecutive days. The dose for Nevirapine was also similar to the one used by Shenton et al., who showed that 150 mg/kg/day
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Determination of TNP-specific IgG1, IgG2a, IgG2b, and IgE in serum.

TNP-specific IgG1, IgG2a, and IgG2b in serum were determined using ELISA. Plates (Highbond 3590; Costar, Cambridge, MA) were coated overnight at 4°C with TNP-BSA (20 μg/ml), washed, and blocked with PBS/Tween/1% BSA. For IgG1, IgG2a, and IgG2b, wells were incubated with two-step dilutions of individual mouse serum samples for 1 h at room temperature. For measurement of IgE, pooled sera were used. Further incubation was performed with alkaline-phosphatase (AP)-conjugated goat anti-mouse IgG1, IgG2a, or IgG2b (Southern Biotechnology Associates [SBA], Inc., Birmingham, AL) for 1 h at room temperature. After washes with PBS/Tween and diethanolamine buffer, 4-nitrophenylphosphate was added (30 min, room temperature) and the reaction was stopped with EDTA in bidistilled water. Substrate conversion was measured as optical density at 405 nm using an automated reader ELx800 (Bio-Tek Instruments, Winooski, VT).

To measure TNP-specific IgE, ELISA plates were coated with 2 μg/ml rat anti-mouse IgE (BD Pharmingen, Hamburg, Germany) overnight at 4°C. Plates were washed and sera were added without blocking the plates first. Samples were added in two-step dilutions in PBS/Tween/1% BSA and incubated overnight at 4°C. After washes, wells were incubated with biotinylated TNP-BSA (2.5 μg/ml) (1 h at room temperature) and additionally with streptavidin-conjugated horseradish peroxidase (CLB, Amsterdam, The Netherlands). Color development was performed with 3,3′-5,5′-tetramethylbenzidine substrate and the reaction was stopped with 2 M H2SO4. OD-values were determined using an automated reader at 450 nm.

ELISPOT assay. The ELISPOT assay was performed based on the procedure described by Schielen et al. (1995). Cells were added to TNP-BSA-coated Immobilon-P membranes (Immobilon PVDF Transfer, Millipore, Etten-Leur, The Netherlands). After cell incubation, AP-conjugated goat anti-mouse, human-adsorbed IgG1, IgG2a, and IgM antibodies were added and membranes were stained with para-nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidine salt in dimethylformamide (BDH Laboratory Supplies, Poole, England) diluted in Tris (100 mM Trizma base, 100 mM NaCl, 5 mM MgCl2, pH 9.5) to visualize development of TNP-specific antibody spots. Spots were counted by two independent observers using a stereomicroscope.

Cell culture and cytokine measurement. Cell suspensions were prepared from PLN from individual mice and cytokine production from individual mice were measured separately. Cell suspensions (3.75 × 106 cells) in complete RPMI-1640 with GlutaMAX-I (Life Technologies, Paisley, Scotland) supplemented with 10% FBS (ICN Pharmaceuticals, Costa Mesa, CA) and 2% penicillin-streptomycin were incubated with 200 μg/ml TNP-BSA or TNP-BSA in IgE-negative culture plates (Costar) overnight at 37°C, 5% CO2. Supernatant was collected and stored at −20°C until analysis. IFN-γ and IL-4 were determined by sandwich ELISA as previously described (Nierkens et al., 2002).

Statistics. To statistically evaluate IgG1 measurements, 2log serum titers were calculated for individual animals. The means of individual titers were compared with controls using Student t-test analyses. Levels of IgG1 ASC, IFN-γ, and footpad swelling are expressed as group means (±standard error of the mean). Each individual treatment group consisted of at least 4–8 mice. Multiple comparison of group means were analyzed using one-way analyses of variance with Bonferroni as post hoc test. A value of p < 0.05 was considered statistically significant.

RESULTS

TNP-Specific IgG1 and IgE Levels Are Elevated in Serum of Drug-Exposed Mice

Oral administration of 150 mg/kg D-Pen to C3H/HeOuJ mice for 7 or 21 days resulted in increased serum levels of TNP-specific IgG1 on day 10 (Fig. 2A). But mice that were treated with 150 mg/kg for up to 10 days (note that this group is treated until day 21 for further analyses) showed lower levels

FIG. 1. Exposure protocols to assess the immunosensitizing capacity of drugs. (A) The capacity of D-Pen, DF, and Nevi to induce immunosensitization to TNP-BSA was assessed by treating mice orally or ip with the specific drug. D-Pen (50 or 150 mg/kg) was administered for 7 (oral and ip) or 21 days, DF was administered once (75 mg/kg) or during three days (25 mg/kg), Nevi was administered for six days. In addition, mice received an ip injection with the RA TNP-BSA on day 1 and were challenged in the hind footpad with TNP-BSA on day 15 to measure DTH responses. At day 0, 10, and 21, blood was drawn to monitor TNP-specific antibody production. Note that mice received a double challenge on day 15 (in paw and neck) in case of D-Pen. (B) To elucidate whether sensitization was drug-specific, D-Pen and DF were given orally by gavage and, 21 days later, mice were sc injected in the hind footpad with a suboptimal dose of the drug together with TNP-Ficoll (10 μg).

introduced Nevi-induced skin rash in rats (Shenton et al., 2003). In case of DF, mice received a single dose of 75 mg/kg on day 1 or 25 mg/kg on three consecutive days. Higher doses or longer exposure protocols with DF resulted in mortality or severe morbidity.

On day 1, the first day of drug exposure, mice were also injected with 10 μg TNP-BSA (100 μl ip in saline). For D-Pen, mice were double-challenged on day 15 with 10 μg TNP-BSA in the hind footpad (total volume: 20 μl/mouse) and sc in the neck (total volume 100 μl/mouse). D-Pen-exposed mice were sacrificed on day 21. Serum was obtained by orbita puncture on days 0, 10, and 21. This protocol resulted in increased serum levels of TNP-specific IgG1 on day 21 for control animals. Therefore, it was decided to adjust the protocols for DF and Nevi: mice were only challenged in the footpad on day 15 to assess DTH responses and were also killed on day 21.

RA-PLNA with TNP-Ficoll in orally pre-treated mice. In the next experiments (based on Gutting et al., 2002), BALB/c mice were exposed to a single dose of D-Pen (50 or 150 mg/kg) or DF (50 mg/kg) by gavage on day −21, and challenged on day 0 with a suboptimal dose (0.5 mg) of either drug together with 10 μg TNP-Ficoll (Fig. 1B). This sup-optimal dose was determined in the PLNA in previous unpublished experiments. Challenge was performed in the hind footpad (50 μl, sc) in toe-to-heel direction using a 25-gauge needle. To check whether sensitization was drug-specific, D-Pen and DF were given orally by gavage and, 21 days later, mice were sc injected in the hind footpad with a suboptimal dose of the drug together with TNP-Ficoll (10 μg).
than mice that were treated for only 7 days. Also ip administration of 150 mg/kg D-Pen (for only seven days) caused an increase of TNP-specific IgG1 on day 10 (Fig. 2B). The 50 mg/kg D-Pen-dosed group only showed increased serum levels of TNP-specific IgG1 when gavaged for 10 days or ip exposed.

Additionally, ASC numbers in spleen, bone marrow, mesenteric lymph nodes, and Peyer’s patches were determined using ELISPOT and shifts in cell subpopulations (CD4+ and CD8+ T cells, B cells and DC) and expression of costimulatory molecules (CD80, CD86, CD54) were assessed with flow cytometry on day 21. However no significant changes in any of these parameters were observed (data not shown).

DF administered in a single oral dose of 75 mg/kg, and to a lesser extent when administered in three doses of 25 mg/kg on consecutive days, resulted in increased TNP-specific IgG1 levels on day 10 (Fig. 3A) and day 21 (Fig. 3B), but only 75 mg/kg DF resulted in elevated TNP-specific IgE titers on day 21 (Fig. 3C). Parenteral (ip) administration of 75 mg DF/kg body weight only moderately enhanced TNP-specific serum levels on day 21, whereas 25 mg DF/kg was ineffective (Figs. 3D–3F). In contrast, ip exposure to Nevi, but not oral exposure, resulted in elevated levels of TNP-specific IgG1 levels on days 10 and 21, and of TNP-specific IgE levels on day 21 (Figs. 4A–4C). In addition, IgG2a and IgG2b levels were determined to assess whether a difference in Th1- (IgG2a and IgG2b) and Th2- (IgG1 and IgE) associated antibodies could be observed for the different drugs or the different exposure protocols. However, the results of TNP-specific IgG2a and IgG2b antibodies were similar to those of IgG1.

D-Pen-exposed mice were challenged with TNP-OVA in the footpad and in the neck on day 15 in order to reveal local as well as systemic anamnestic immune responses. This double challenge, resulted in increased TNP-specific IgG1 levels in control animals on day 21 (data not shown). Only the 150 mg/kg-dosed groups (both the oral and the ip seven-day exposure groups) were still significantly increased compared to controls in that experiment. In order to prevent this increase in further experiments, DF- and Nevi-exposed mice as well as the control mice received a single sc challenge in the footpad on day 15 and no additional injection in the neck. This resulted in clearly less increased TNP-specific IgG1 levels on day 21 in control animals compared to the earlier performed D-Pen-study.

**DTH Responses to TNP-OVA Are Elevated in Drug-Exposed Mice**

On day 15, drug-treated mice received a sc footpad injection of TNP-OVA (10 μg) to determine RA-specific DTH responses. As mentioned above, D-Pen-exposed mice received an additional sc injection of TNP-OVA in the neck.

Oral exposure to all drugs induced significant RA-specific DTH reactions (footpad swelling) upon a challenge in the footpad with the RA (Fig. 5A). In case of D-Pen, footpad swelling was considerably larger in mice that were treated continuously until challenge (now 15 days of total 21 days) compared to mice that received D-Pen for only seven days. Also ip-treated groups, except the 50 mg/kg D-Pen-treated group, showed significant DTH responses (Fig. 5B).

**BALB/c Mice Are Not Susceptible for Drug-Induced Sensitization to TNP-OVA**

To study strain differences, BALB/c mice were subjected to the same protocol with D-Pen as the C3H/HeOuJ mice, i.e., ip or oral treatment by gavage with 50 mg/kg or 150 mg/kg D-Pen for seven consecutive days and a single ip injection with 10 μg TNP-OVA/mouse on day 1. In contrast to C3H/HeOuJ, BALB/c did not display DTH responses upon sc footpad challenge with TNP-OVA and serum levels of TNP-specific IgG1 and IgE were not significantly different from controls (data not shown).
Oral Exposure to D-Pen or DF Elicits Specific Immunosensitization

We also assessed the influence of oral pre-sensitization with DF or D-Pen on responses to TNP-Ficoll in combination with a footpad injection of a sub-optimal dose (0.5 mg/mouse) of the drug in the RA-PLNA. TNP-specific IgG1 ASC levels in the PLN were clearly increased in mice that were pre-exposed orally with 50 mg/kg DF or D-Pen compared to naive BALB/c mice.

FIG. 3. TNP-specific antibody levels in serum on days 10 and 21 after oral or ip exposure to Nevi. C3H/HeOuJ mice were ip injected with 10 μg TNP-OVA on day 1. Nevi (150 mg/kg) was administered orally or ip for six consecutive days. On days 10 and 21, blood was drawn and TNP-specific IgG1 (individual samples) and IgE (pooled samples) were determined with ELISA. Statistics were performed on 2log serum titers. *Significant differences when compared with controls.

FIG. 4. TNP-specific antibody levels in serum on days 10 and 21 after oral or ip exposure to DF. C3H/HeOuJ mice were ip injected with 10 μg TNP-OVA on day 1. Mice received a single dose of 75 mg/kg or 25 mg/kg on three consecutive days by gavage (A–C) or ip (D–F). On days 10 and 21, blood was drawn and TNP-specific IgG1 (individual samples) and IgE (pooled samples) were determined with ELISA. Statistics were performed on 2log serum titers. *Significant differences when compared with controls.

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In addition, mice that were pre-treated with DF or D-Pen and cross-challenged with D-Pen or DF, respectively, showed no or lower increases in TNP-specific ASC numbers. This indicates that the drugs induce specific non-cognate T-cell help. In addition, orally pre-treated mice showed increased IFN-\(\gamma\) levels in the RA-PLNA compared to non-pre-treated animals (only significant for 50 mg/kg D-Pen; Fig. 6B). Remarkably, oral pre-treatment with 150 mg/kg D-Pen did not show increased IFN-\(\gamma\) levels in the RA-PLNA compared to non-pre-treated animals (only significant for 50 mg/kg D-Pen; Fig. 6B).

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not result in changes in TNP-specific IgG1 ASC levels in response to TNP-Ficoll and the sub-optimal dose of D-Pen.

**DISCUSSION**

In this study we show that drugs that are associated with IDHR in humans can be identified by using an oral exposure model in combination with the RA-approach. The present research reveals promising data in the development of oral exposure models that are urgently needed for hazard identification of drugs with regard to their potential to induce systemic hypersensitivity.

The TNP-OVA model, using DTH responses and serum levels of RA-specific antibodies as read-out parameters, shows that the selected model allergenic drugs are stimulating systemic immune responses to a bystander antigen. Oral and ip administration of D-Pen, DF, and Nevi caused systemic sensitization to a sub-sensitizing dose (10 μg/mouse, ip) of TNP-OVA, which was evidenced by DTH responses upon sc footpad challenge with this RA. These delayed type reactions to the RA may be mediated by both Th1 and Th2 cells (Lerch and Pichler, 2004) and may thus be independent of the type of drug-induced immune response. Accordingly, DTH reactions may be independent of the specific mechanisms and hence a particularly valuable parameter to detect drug-induced hypersensitivity. Although DTH responses may not necessarily reflect the clinical manifestation of IDHR, they might be indicative of the development of skin effects that are frequently encountered in IDHR. The immunohistological examination of infiltrates at the challenge site may provide more information about the type of clinical effector response (Lerch and Pichler, 2004).

Differentiation of immune responses may also be analyzed using Th1- and Th2-associated isotypes of the RA-specific serum antibody responses. In the present experiment oral exposure to D-Pen and DF resulted in increased serum levels of TNP-specific IgG1 on day 10. In case of DF, levels of TNP-specific IgG1, IgG2a, IgG2b, and IgE on day 21 were increased after an additional challenge with TNP-OVA in the footpad on day 15. Notably, Nevi caused an elevation of both TNP-specific IgG1, IgG2a, IgG2b, and IgE, but only after ip exposure to the drug.

The kinetics of the serum IgG isotypes (IgG1, IgG2a, and IgG2b) that were determined in the present research were very similar. Such results were previously reported for a murine oral food allergy model for peanut allergens. Both ip and oral administration of a peanut extract increased peanut allergen-specific IgG1, IgG2a, and IgE (Van Wijk et al., 2004). Together, these data implicated that both Th1 and Th2 phenomena are involved in the development of immune-mediated hypersensitivity. As such, it remains to be established whether serum IgG isotypes can be predictive for the type of clinical response that is elicited by certain drugs.

Important to note is that the use of higher doses or repeated dosing of TNP-OVA is also able to induce specific T-cell memory. Hence, the TNP-specific response can be elevated by each extra dose of antigen, as was observed for the double challenge of TNP-OVA on day 14 in case of D-Pen (not shown). For an optimal sensitivity and selectivity of the TNP-OVA model a single challenge with RA is preferred, as was done in case of DF and Nevi.

Based on the particular characteristics of the immune responses to the RA, responses to TNP-OVA indicate that the drugs have immunostimulatory potential, which may not necessarily be T cell-mediated. The TNP-Ficoll model, based on the detection of drug-induced memory responses upon footpad injection with a suboptimal dose of the drug in combination with TNP-Ficoll, is particularly suitable to prove that T cells are involved. Oral exposure to D-Pen or DF increased the formation of TNP-specific IgG1 ASC and, in case of D-Pen, secretion of IFN-γ. For DF, this was shown before by Gutting et al. (2002). Our present results of cross-over challenges with D-Pen and DF confirm that the induced responses are specific for the particular drug, i.e., oral exposure to DF sensitized for DF and not for D-Pen and vice versa. Data with the RA TNP-Ficoll indicate that both D-Pen and DF are able to induce systemic immunosensitization upon a single oral exposure. The response may be specifically directed to (epitopes of) the drug or its metabolites, or to fragments from the reactive compound (hapten) and a carrier. Alternatively, T cells may be specific for sequestered, or cryptic, non-tolerated antigens that are released by a particular drug.

Clearly, more drugs, including sensitizing but also non-sensitizing ones as well as those that require bio-activation before becoming sensitizing, need to be tested. In both models, dose-response relationships need to be assessed for various periods of exposure to prevent non-immune mediated toxic effects, such as gastrointestinal hemorrhages in case of DF (Melarange et al., 1991). In addition, dose and treatment regimens need to be optimized to allow the induction of sensitization rather than the induction of tolerance or non-responsiveness. For instance, in the TNP-Ficoll model a single gavage with D-Pen (50 mg/kg) was sufficient to induce systemic sensitization, whereas 150 mg/kg D-Pen did not induce sensitization, suggesting the induction of high-dose tolerance (Strobel and Mowat, 1998). Studies of others (Gutting et al., 2003) showed similar dose regimen-related tolerance induction in the TNP-Ficoll model in case of DF. Also, administration of low doses (40 or 75 mg/kg/day) of Nevi were shown to induce tolerance in rats, while these rats developed skin rash after exposure to 100 mg/kg/day (Shenton et al., 2003). These latter phenomena may be attributed to induction of low-dose oral tolerance (Strobel and Mowat, 1998) and indicate that the development of (high or low dose) tolerance vs. sensitization occurs within a small dose range.

Dose and treatment regimens also seem associated with the type of effector response. For instance, in comparison with seven-day exposure, continuous exposure to D-Pen caused lower serum Ig levels (production of IgG1 and IgE are regarded as humoral Th2 responses), but higher DTH responses...
(associated with Th1 responses) in the model with TNP-OVA. And in case of Nevi, oral administration increased DTH responsiveness but failed to increase TNP-specific Ig serum levels, whereas ip dosing clearly induced IgG1 and IgE isotype production as well. So, the preferential immunological response to Nevi via the oral route could be via Th1 cells, which would be in agreement with the development of rash and CD8+ T cells and macrophages in skin infiltrates in BN rats (Shenton et al., 2003). Probably, the exposure route influences the availability of the antigen in lymphoid tissues by processes, such as antigen exclusion in the gut. This may eventually affect the immunological mechanisms leading to tolerance or sensitization (Brandtzaeg, 2002).

DF induced increased TNP-specific serum antibody levels in orally exposed mice, but not in ip-treated animals. DF specifically causes gastrointestinal toxicity and immune-mediated injury after oral administration (Boelsterli, 2003) but may cause less toxicity and subsequent stimulation of responses to TNP-OVA when administered ip. These data indicate that the ip exposure route might not always be suitable as a positive control for oral sensitization in case of drug exposure.

Care should also be taken with regard to selection of specific susceptible strains. For instance, the immunostimulating capacity of D-Pen as observed in the TNP-OVA model in C3H/HeOuJ mice was not observed in BALB/c mice. Previously, it was already shown that D-Pen caused formation of autoantigens in C3H/HeJ mice but not in BALB/c or C57BL/6 mice (Brik et al., 1995). Similarly for rat, D-Pen- and Nevi-induced reactions occur in Brown Norway (BN) but not in Lewis or Sprague-Dawley rats (Donker et al., 1984; Shenton et al., 2003). Interestingly however, the TNP-Ficoll model showed positive responses for D-Pen in BALB/c mice and this model might therefore have the important advantage that a broad range of chemicals may be evaluated using only one strain. This has to be confirmed with more drugs.

In all, more drugs, including non-allergenic drugs and those that require metabolic conversion to become allergenic, need to be studied in the present models. In addition, our future experiments will focus on the further development of these models for screening purposes, but also on the mechanisms of drug-induced sensitization via the oral route. Topics that require elucidation are the role of metabolism, the mechanisms of oral tolerance, and the influence of specific genetic backgrounds (Pieters et al., 2002).

In conclusion, data obtained with the presented oral and ip exposure models hold clear promise for the RA approach as a means to assess the hazard of immunosensitizing drugs via systemic exposure. The use of RA and RA-specific read-out parameters enables the measurement of standardized endpoints and makes these models suitable tools to screen a range of different drugs in routine immuno toxicological research. Systemic exposure models may be regarded as a second tier following fast pre-screening tests with local lymph node assays, like the RA-PLNA.

REFERENCES


