



Use of the local lymph node assay in assessment of immune function

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Abstract

The murine local lymph node assay (LLNA) was originally developed as a predictive test method for the identification of chemicals with sensitizing potential. In this study we demonstrated that an adapted LLNA can also be used as an immune function assay by studying the effects of orally administered immunomodulating compounds on the T-cell-dependent immune response induced by the contact sensitizer 2,4-dinitrochlorobenzene (DNCB). C57Bl/6 mice were treated with the immunotoxic compounds cyclosporin A (CsA), bis(tri-*n*-butyltin)oxide (TBTO) or benzo[*a*]pyrene, (B[*a*]P). Subsequently, cell proliferation and interferon- γ (IFN- γ) and interleukin (IL)-4 release were determined in the auricular lymph nodes (LNs) after DNCB application on both ears. Immunosuppression induced by CsA, TBTO and B[*a*]P was clearly detectable in this application of the LLNA. Cytokine release measurements proved valuable to confirm the results of the cell proliferation assay and to obtain an indication of the effect on Th1/Th2 balance. We believe to have demonstrated the applicability of an adapted LLNA as an immune function assay in the mouse.

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1. Introduction

The murine local lymph node assay (LLNA) was originally developed as a predictive test method for the identification of chemicals that have the potential to cause sensitization (Kimber et al., 1986; Kimber and Weisenberger, 1989). This assay has been validated

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extensively and is nowadays commonly used to uncover allergenicity (Kimber et al., 2002). In the LLNA, lymphocyte proliferation in draining lymph nodes is used as a measure for the immune response to an applied allergen. As such, the LLNA can be seen as a functional assay of immune reactivity to T-cell-dependent antigens, which is suggested to be applicable in the study of direct effects on the immune system after exposure to (toxic) compounds (Albers et al., 2002, 2003).

In this study, we applied the LLNA in combination with cytokine enzyme-linked immunosorbent assays (ELISAs) to demonstrate immunomodulating effects of various orally administered substances. For this purpose, groups of mice were exposed to control or test diets prior to sensitization with a T-cell-dependent, low molecular weight antigen. The lymphocyte activation in the animals exposed to the test agents was then compared to that in the control animals. An immunosuppressive or -stimulating effect of the test agent would thus be expressed by a diminished or enhanced proliferative and cytokine response of lymphocytes after application of the sensitizer.

The T-cell-dependent low molecular weight antigen used to induce immune responses was 2,4-dinitrochlorobenzene (DNCB). DNCB is a well-known contact sensitizer that induces a T helper (Th) 1 response and interferon- γ (IFN- γ) and interleukin (IL)-2 production (Dearman et al., 1992). Cyclosporin A (CsA), bis(tri-*n*-butyltin)oxide (TBTO) and benzo[*a*]pyrene (B[*a*]P) were the test substances used. These compounds are known immunotoxicants with different modes of action and employed here as model compounds to show the usefulness of the LLNA as an immune function assay. The immunosuppressive drug CsA is used after organ and bone marrow transplantation and in treatment of autoimmune diseases. CsA primarily interferes with T-cell proliferation by affecting transcription of several genes encoding growth and differentiation factors (such as cytokines), cell surface receptors and a transcription factor (Kiani et al., 2000). Some of these genes are under control of the transcription factor nuclear factor of activated T-cells (NFAT) that needs to be dephosphorylated by the protein phosphatase calcineurin to be able to translocate to the nucleus and activate target genes. CsA binds to cyclophilin, after which this complex binds and thereby inhibits calcineurin (Ho et al., 1996; Mascarell and Truffa-Bachi, 2003).

Furthermore, recent studies suggest that promotion of transcription of certain (partially immunologically relevant) genes by CsA or binding of CsA to specific surface receptors could contribute to its effects as well (Allain et al., 1996; Cacalano et al., 1992; Mascarell and Truffa-Bachi, 2003). In addition to the immunosuppressive effects, CsA treatment turned out to promote development of lymphoid and skin cancers (Shinozuka et al., 1986; Stewart et al., 1995).

TBTO is a persistent organotin compound that is used as a biocide, e.g. in anti-fouling paints, and accordingly occurs as an environmental pollutant. This chemical has been shown to cause immunosuppression in rats and consequently reduction of resistance to infections. This is mainly due to a decreased cellular immune response caused by a direct action of TBTO on cortical thymocytes, but also to the effect of TBTO on non-specific immune functions like NK cell and macrophage activity (Krajnc et al., 1984; Vos et al., 1984, 1990). Whether the observed thymus atrophy after TBTO exposure is caused by induction of apoptosis or antiproliferative effects is still unclear (Raffray and Cohen, 1993; Vos et al., 1984).

The combustion product B[*a*]P is the prototype immunotoxic polycyclic aromatic hydrocarbon (PAH) and also the first carcinogenic compound isolated from coal tar (White et al., 1994). The effect of B[*a*]P on the immune system manifests itself generally in atrophy or decreased cellularity in spleen, thymus and bone marrow and a reduction of circulating red and white blood cells and immunoglobulins (De Jong et al., 1999). The exact mechanism of B[*a*]P-induced immunosuppression still remains to be established. Potential effects of B[*a*]P or its reactive metabolites (formed by cytochrome P450 enzymes, possibly particularly in macrophages) that could be involved are binding to the aryl hydrocarbon receptor (AhR), entering cell membranes and interference with its function, affecting production of various interleukins or altering mobilization of intracellular calcium (White et al., 1994). Metabolic activation of B[*a*]P is also associated with its genotoxic effect in various tissues (Levin et al., 1982; Stowers and Anderson, 1985). In addition, B[*a*]P was shown to have sensitizing capacity after application to the skin in the standard LLNA (Ashby et al., 1995).

Our findings show that the LLNA offers a simple approach to demonstrate immunosuppression by orally administered agents.

2. Material and methods

2.1. Animals

Male and female C57Bl/6 mice of 6–9-week-old were obtained from Charles River Laboratories (Someren, The Netherlands). We performed the LLNA in the C57Bl/6 strain instead of the traditionally used CBA mice because these experiments were part of a larger study in which C57Bl/6 mice were used also. These mice are comparable to CBA mice with regard to Th1 responses, such as the response to DNCB, as shown by Woolhiser et al. (2000). The animals were bred specific pathogen free and housed in macrolon cages under conventional conditions.

2.2. Treatment

The mice were randomly divided over the treatment groups. For every treatment group, a separate control group was included. During 4 weeks, C57Bl/6 mice were fed a standard diet (SRM-A food, Hope farms Arie Blok, Woerden NL) or similar food mixed with 300 ppm of TBTO (Sigma–Aldrich, Zwijndrecht, The Netherlands) or with 500 ppm of CsA (Kindly provided by Novartis Pharma AG, Basel, Switzerland), both feed mixtures were manufactured at Altromin (Lage, Germany). B[a]P (purpose-made by Serva, Germany), dissolved in 0.1 ml sunflower oil, was given by gavage three times a week at a dose of 13 mg/kg body weight. The administered doses of CsA, TBTO and B[a]P corresponded to the maximum tolerated doses (MTDs), here defined as the dose that causes a 10% reduction in body weight gain along the growth curve compared to the control group.

Standard diet, the diets containing CsA or TBTO and water were given ad libitum. The mice that received B[a]P orally, had free access to standard diet during the treatment.

2.3. Sensitization

Immediately after the 4-week treatment period with CsA, TBTO and B[a]P, for every different treatment group four mice were topically exposed to DNCB (2,4-dinitrochlorobenzene 98%; Sigma–Aldrich) (25 μ l) dissolved in acetone:olive oil (4:1) (AOO) on the dorsum of both ears. The DNCB concentrations used were

1%, 0.66%, 0.33% and 0% (w/v). DNCB application was performed daily for three consecutive days. During these days the mice were given standard diet ad libitum.

2.4. Cell isolation

In contrast to the LLNA, we have not labeled lymphocytes in the auricular lymph nodes (LNs) with [3 H]-thymidine in vivo, but rather harvested the lymphocytes, and labeled them ex vivo. For this reason, we use the term adapted LLNA.

Three days after the last topical application of DNCB to the ears, the mice were euthanized by CO₂/O₂ exposure and the auricular lymph nodes were excised. The LNs were pooled for each animal, weighed and suspended in 5 ml RPMI 1640 (Gibco, Life Technologies, Breda, The Netherlands) with 5% heat inactivated fetal calf serum (FCS) (PAA, Linz, Austria), 100 U/ml penicillin and 100 μ g/ml streptomycin (standard medium). Single cell suspensions were prepared by pressing the LNs through a 70 μ m nylon cell strainer (Falcon, Franklin Lakes, USA). The cells were washed twice in standard medium (10 min, 300 \times g, 4 °C) and resuspended in 1 ml standard medium with 10% FCS. A Coulter Counter (Z2, Coulter Electronics, Mijdrecht, The Netherlands) was used to count the cells. Then the concentration of the cell suspensions was adjusted to 1 \times 10⁷ cells/ml. Some cell suspensions of AOO treated animals were pooled, because the number of cells per animal was insufficient to perform the subsequent assays. Then the averages of the cell counts of the pooled lymph nodes were used for further calculations.

2.5. Cell proliferation

Of each cell suspension of 1 \times 10⁷ cells/ml, 200 μ l was seeded in triplicate in a U-bottom 96-well plate (Greiner, Alphen aan den Rijn, The Netherlands). After addition of 10 μ l/well (=1 μ Ci) [methyl- 3 H]-thymidine (Amersham Biosciences, Buckinghamshire, UK) the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ during 20–24 h. The cells were harvested on glass-fiber filters (LKB-Wallac, Turku, Finland) using a multiple cell culture harvester (LKB-Wallac). The [3 H]-thymidine activity was determined using a liquid scintillation counter (1205 Betaplate TM, LKB-Wallac). For further calculations the

median of the triplicates was used. The [^3H]-thymidine incorporation is expressed per set of LNs, being the mean of the measured counts per minute (cpm) per group times the mean total LN cell number per group \pm S.E.M.

2.6. Cytokine release

Hundred microlitres of the cell suspension of 1×10^7 cells/ml was seeded in duplicate in a 24-well plate (Greiner). The cells were incubated at 37°C in a humidified atmosphere for 20–24 h in the presence of the mitogen concanavalin A (ConA, ICN Biochemicals, Irvine, USA). The supernatant was harvested and used in an ELISA to measure the amount of IFN- γ and IL-4. For the IFN- γ ELISA, MaxiSorp 96-well flat-bottom plates (Nunc-Immuno Plate, Denmark) were coated overnight at 4°C with $2 \mu\text{g/ml}$ rat anti-mouse IFN- γ (Pharmingen, San Diego, CA) in coating buffer (0.04 M carbonate buffer pH 9.6). Then the plates were incubated in blocking buffer (PBS with 0.1% Tween-20 (Merck, Amsterdam, The Netherlands) containing 1% (w/v) blocking reagent (NEN 3 GM) for 2 h at 37°C or overnight at 4°C and washed (PBS plus 0.1% Tween-20) on an Auto Plate Washer (ELx405, Bio-Tek, Instruments Inc.). Recombinant mouse IFN- γ (Pharmingen) was used as a standard. Serial dilutions of the standard as well as the culture supernatants were added to the plate ($100 \mu\text{l/well}$). Biotinylated rat anti-mouse IFN- γ ($0.1 \mu\text{g/ml}$) (Pharmingen) was added ($50 \mu\text{l/well}$) and the plates were incubated at 37°C for 2 h under continuous shaking. The plates were washed and poly horseradish peroxidase labeled streptavidin (10,000-fold dilution, CLB, Amsterdam, The Netherlands) was added, after which the plates were incubated for 45 min under continuous shaking. Plates were washed again and TMB/DMSO solution (1.6% tetramethylbenzidine (TMB, Sigma) in dimethyl sulfoxide (DMSO, Fluka), 9.8% sodiumacetate (1.1 M), pH 5.5, 0.02% perhydrol (30%, Merck)) was added. The reaction was terminated with H_2SO_4 ($50 \mu\text{l/well}$) (10%, Merck) and the absorbance was read at 450 nm, using an automated multichannel photometer (Titertek Multiscan MCC/340, Merlin Diagnostics System B.V.). Standard curves were used to calculate the cytokine release.

For IL-4, a similar protocol was used with $1 \mu\text{g/ml}$ rat anti-mouse IL-4 (Pharmingen) for coating, blocking

with blocking buffer containing 1% (w/v) dried milk, recombinant mouse IL-4 (Biosource, Camarillo, CA) as a standard and $0.4 \mu\text{g/ml}$ biotinylated rat anti-mouse IL-4 (Pharmingen) for detection.

Antibodies, standards, samples and streptavidin were diluted in dilution buffer containing PBS plus 0.1% Tween-20. A 0.5% blocking reagent (IFN- γ) or 0.5% dried milk (IL-4) were added.

The cytokine release is expressed per set of auricular LNs, being the mean of the measured picograms (pg) per group times the mean total LN cell number per group \pm S.E.M.

2.7. Statistical analysis

For the cell proliferation assay as well as the cytokine release assay, statistical analysis was performed using one-way analysis of variance (ANOVA). Significant differences of the control group were determined with the Bonferroni post hoc test, using a significance level of $p = 0.05$.

3. Results

3.1. Cell proliferation

The effects of the treatments with immunomodulating compounds on DNCB induced lymphocyte proliferation in draining LNs are shown in Fig. 1. Treatment of C57Bl/6 mice with CsA, TBTO or B[a]P resulted in a reduction of the [^3H]-thymidine incorporation in comparison with the control mice. Fig. 1a shows that the reduction of [^3H]-thymidine incorporation after CsA exposure was significant ($p = 0.032$) in the highest DNCB treatment group. After B[a]P exposure (Fig. 1c) the incorporation was also significantly reduced ($p = 0.008$) in the highest DNCB group. As shown in Fig. 1b, the TBTO exposed mice showed a similar pattern, but here the reduction was not statistically significant.

3.2. Cytokine release

Analyses of cytokine release in supernatant of the cultures of draining LN-cells were performed for IL-4 and IFN- γ . Fig. 2 shows that in the groups exposed to the highest DNCB concentration, both the IL-4 and

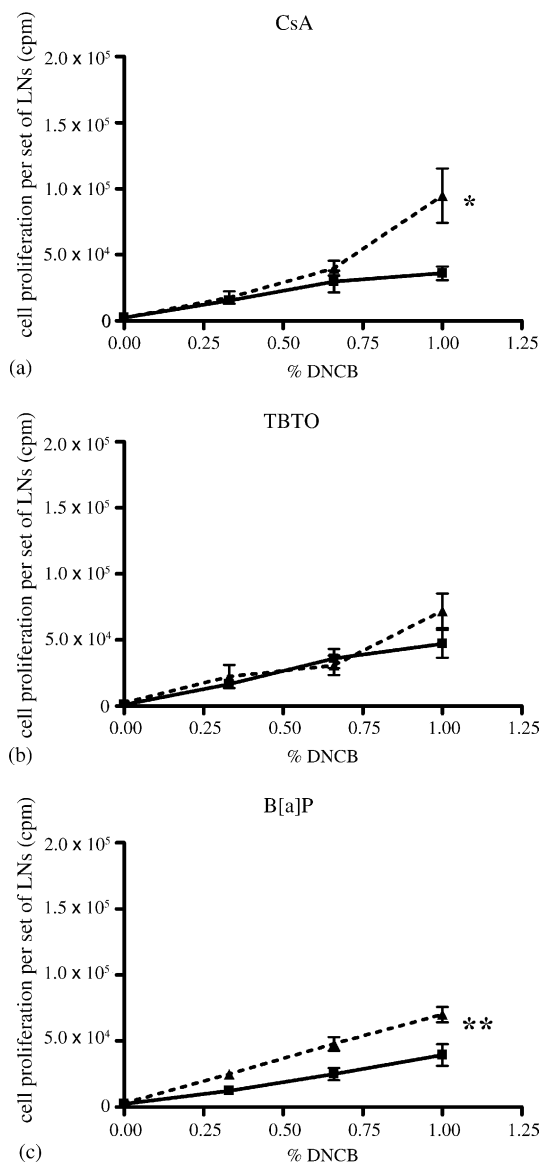


Fig. 1. [³H]-thymidine incorporation in auricular LN cells of C57Bl/6 mice after treatment with different immunosuppressive compounds. Mice were treated during 4 weeks with CsA (a); TBTO (b) or B[a]P (c) ($n = 16$, 8 females and 8 males). These mice (■) as well as the untreated animals (▲) (similar group sizes) were subdivided in groups ($n = 4$, 2 females and 2 males) for the different DNCB concentrations (0%, 0.33%, 0.66% and 1%). The [³H]-thymidine incorporation is expressed per set of auricular LNs, being the mean of the measured counts per minute (cpm) per group times the mean total LN cell number per group \pm S.E.M. Significance levels are * $p < 0.05$ and ** $p < 0.01$.

Table 1

The ratios of Th1 and Th2 response for every treatment group and matching control group

Compound	Control group	Treatment group
TBTO	1.4 \pm 0.6	0.7 \pm 0.1
CsA	2.1 \pm 1.7	3.4 \pm 1.0
B[a]P	1.1 \pm 0.3	2.1 \pm 2.3

The results are expressed as mean ratio of IFN- γ /IL-4 times 1×10^{-2} in the 1% DNCB group \pm S.E.M.

IFN- γ release were decreased for the mice treated with CsA or B[a]P compared to the non-treated control mice. TBTO only suppressed IFN- γ release at that concentration. Only the reduction of the IFN- γ release after B[a]P exposure (Fig. 2f) at 1% DNCB was statistically significant ($p < 0.001$).

Table 1 summarizes the ratios of IFN- γ and IL-4 release in the 1% DNCB group for every treatment group and matching control group. TBTO exposed mice had a lower ratio than the control group, whereas the CsA and B[a]P exposed mice showed a higher ratio than their control group.

4. Discussion

The murine LLNA is currently being used to identify compounds having a sensitizing capacity (Kimber et al., 2002). The aim of the present study was to demonstrate that the LLNA can also be employed as an immune function assay. By applying a contact sensitizer that induces a T-cell-dependent immune response, the effects of immunomodulating compounds on this response can manifest themselves in effects on the cell proliferation and cytokine release pattern in draining lymph nodes.

We treated C57Bl/6 mice with the known immunotoxic compounds CsA, TBTO and B[a]P. With regard to the results of the cell proliferation assay, the immunosuppressive effects of these compounds were demonstrated by reduction of the proliferative response in auricular LNs after DNCB application. These findings were confirmed by the cytokine release assays, which showed that IFN- γ and IL-4 release in the LNs are reduced after treatment with the immunotoxic compounds. This effect was most overt in the group that was exposed to 1% DNCB. This finding was as expected, since the course of the proliferatory response after

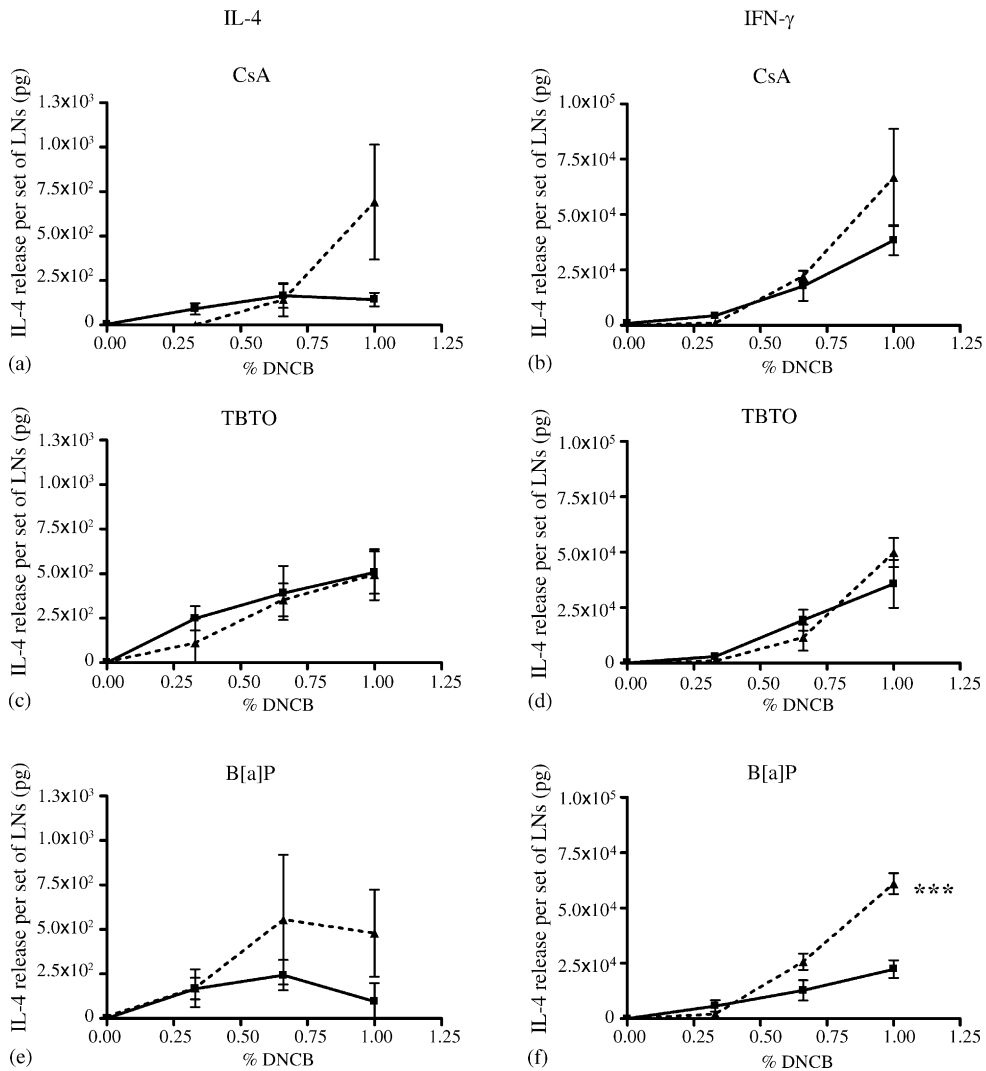


Fig. 2. The release of IL-4 and IFN- γ in auricular LN cells of C57Bl/6 mice after treatment with different immunosuppressive compounds. Mice were treated during 4 weeks with CsA (a and b); TBTO (c and d); B[a]P (e and f) ($n = 16$, 8 females and 8 males). These mice (■) as well as the untreated animals (▲) (similar group sizes) were subdivided in groups ($n = 4$, 2 females and 2 males) for the different DNCB concentrations (0%, 0.33%, 0.66% and 1%). The cytokine release is expressed per set of auricular LNs, being the mean of the measured picograms (pg) per group times the mean total LN cell number per group \pm S.E.M. Significance level is *** $p < 0.001$.

application of the allergen showed dose dependency. The effect of the highest DNCB concentration is thus the most pronounced and can consequently be influenced the most by treatment with immunomodulating compounds. This finding demonstrates that use of the most effective concentration of the applied allergen is important in detection of an effect of the treatment. It

is therefore recommended to use a suitable dose-range of the allergen to rule out false-negative results.

We also used cytokine release patterns to determine whether a shift in the Th1/Th2 balance took place after exposure to the immunotoxic compounds compared to the control groups. IFN- γ was used to measure Th1 response and IL-4 for Th2 function. According to our

results, CsA and B[a]P treatment caused a relative shift towards Th1-dependent immunity whereas TBTO treatment led to a relatively more pronounced Th2 character. This may indicate that although all compounds showed overall immunosuppression, the nature of the effects that are involved may be different. Occurrence of such shifts in Th1/Th2 balance is of clinical importance with a view on induction or exacerbation of allergies or autoimmunity.

It is imaginable that the effects of orally administered immunosuppressive chemicals on the immune function and Th1/Th2 balance could be somewhat different when the animals are triggered to generate, for example, a Th2 response rather than a Th1 response, as induced by DNCB. Therefore, the results of our experiments could have been different if another sensitizer than DNCB would have been used. This is an interesting aspect that should be investigated in future experiments.

In the standard LLNA, B[a]P was found to have a sensitizing effect in auricular LNs when topically applied to the ear, as published by Ashby et al. (Ashby et al., 1995). One could wonder if this allergenic activity of B[a]P has influenced our test results. However, whereas B[a]P elicits sensitizing effects after application to the skin, oral administration of B[a]P would more readily lead to stimulatory effects in the mesenteric than in the auricular LNs. Although it cannot be ruled out that this sensitizing activity might have some impact on the process of sensitization to another allergen, it is not very likely that the draining lymph node response to a different compound (DNCB) that is applied locally (on the ear) will be influenced by the potential sensitizing effect of B[a]P after oral administration. It is exactly this determination of the local immune response to sensitizers in the LLNA that makes this test relatively insensitive to systemic allergenic influences of compounds on these immune responses. The systemic immunosuppressive effect of B[a]P, though, is expected to influence the response in the auricular LN draining the ear on which DNCB is applied, and we indeed detected a reduced immune response to DNCB.

We administered MTDs of the immunotoxicants because these experiments were part of a larger study, performed with the same compounds. The aim of that study was to characterize patterns of gene expression in several tissues of the same mouse strain exposed to CsA, TBTO or B[a]P, in order to define the mecha-

nisms that are involved in carcinogenesis. The results of the adapted LLNAs in the current study can now serve as proof of the immunosuppressive action of the compounds administered at this MTD. We are aware of the fact that lower doses of the chemicals would probably also have resulted in a diminished immune response to DNCB.

We conclude that the adapted LLNA was successfully applied here to detect immunosuppression after oral exposure to the immunotoxic agents CsA, TBTO and B[a]P. In contrast to Ravel et al., 2004, we showed that the addition of cytokine release measurements proved valuable to confirm the results of the cell proliferation assay and to obtain an indication of the effect on Th1/Th2 balance. Therefore, we believe to have demonstrated the applicability of the adapted LLNA as an immune function assay in the mouse, which could as such be employed in hazard identification of potential immunomodulating compounds.

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