

Appraisal of the sensitising potential of orally and dermally administered Mercaptobenzothiazol by a biphasic protocol of the local lymph node assay

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Abstract Mercaptobenzothiazole (MBT) is used while manufacturing natural rubber products. Our study deals with assessing its allergenic potential following dermal and oral routes of exposure, using a biphasic local lymph node assay (LLNA). Female Balb/c mice were treated with MBT (dermally 3, 10, 30% concentrations in DMSO; orally 1, 10, 100 mg/kg doses in corn oil) on the back (dermal study) or through oral administration (oral study) on days 1–3 followed by auricular application of 3, 10 and 30% concentrations, respectively, on days 15–17. End points determined on day 19 included ear thickness, ear punch weight, lymph node weight, lymph node cell count, and lymphocyte subpopulations (CD4+, CD8+, CD45+). After dermal application of 3% or 10% solution, a significant increase in cell count and lymph node weight along with significant decrease in CD8+ cells was observed. After initial oral administration of 1 mg/kg, we noticed a significant amplification in cell count. Following oral administration of 10 mg/kg, we observed a similar increase in cell count and lymph node weight. The results of our study show that the modified biphasic LLNA protocol can be used to study the sensitising potential of a compound also following the oral route of exposure.

Keywords Mercaptobenzothiazole · MBT · LLNA · Allergic contact dermatitis

Introduction

In recent years, there has been an increase in the occurrence of allergy due to the indiscriminate use of rubber products in everyday life. Allergic reactions against rubber can manifest itself both as a Type I (IgE mediated) and Type IV (cellular-mediated) response depending on the inducing compound, i.e. latex proteins or the containing chemicals, respectively (Warshaw 1998). Due to the chemicals present, irritant contact dermatitis is the most common clinical manifestation of an adverse reaction. These chemicals comprise accelerators such as 2-mercaptobenzothiazole, thiurams and carbamates, and antioxidants such as various amines. Mercaptobenzothiazole (MBT), a rubber additive, is used while manufacturing natural rubber products to accelerate vulcanisation, i.e. it speeds up the process and enables it to proceed at a lower temperature, which is considered to produce a more uniform product. It is prevalent in almost all the rubber products of common use. Other industrial applications of MBT include corrosion inhibition in cutting oils and greases, a floatation agent in metal ore processing, and in fungicides and bactericides (Reddy and Quinn 1997).

Local lymph node assay (LLNA) is the recommended test to assess the allergenic potential of chemicals having an objective end point being the proliferation of the activated lymph node cells (Kimber et al. 1995). We studied the allergenic potential of MBT after dermal exposure with the help of a ‘biphasic’ murine LLNA. The sensitising potential of MBT was also studied orally, keeping in mind a Dutch report regarding the presence of MBT in

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baby teats and soothers (Bouma et al. 2003). Systemic contact dermatitis induced by oral exposure to skin allergens was observed with some drugs and nickel (Veien et al. 2004). To the best of our knowledge, there are no studies on the sensitisation potential of MBT after oral exposure so far.

Materials and methods

Chemicals

The chemicals used in the investigation included MBT (2-mercaptobenzothiazol, Merck KGaA, Darmstadt, Germany) as the test substance, DMSO (Dimethylsulfoxide; Merck KGaA, Darmstadt, Germany) and corn oil (Corn oil; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as the vehicle.

Experimental animals

Female BALB/c mice, 7 weeks old were used, weighing approximately 17 to 22 g at the beginning of the experiments. The mice were kept in Macrolon® cages in groups of five at room temperature ($23 \pm 1^\circ\text{C}$, artificial light from 9:00 a.m. to 9:00 p.m., relative humidity 45–55%). Feed and water were given ad libitum. The animals were divided into various groups; the sample size was five, except for 10 for the 1 mg/kg and 100 mg/kg oral study.

The experiments were performed in accordance with approval from competent local authority (G 0250/05; LAG-eSo, Berlin).

Treatment protocol

The animals were treated using a “biphasic” or “sensitisation-challenge protocol”.

Day 1–3 “Sensitisation”: The animals were treated once daily, orally or dermally, to sensitise the animal. For dermal studies, the animals were shaved over a surface of approximately 2 cm^2 on their back. This area was treated once daily on days 1–3 with $50\text{ }\mu\text{l}$ of the test compound in DMSO, with the below mentioned concentrations. For oral studies, mice were administered the chemical in corn oil by oral gavage. All mice remained untreated on days 4–14. The control animals were treated with the vehicle alone, with the same protocol as for MBT.

Day 15–17 “Challenge”: In both studies, the treatment was repeated once a day for three consecutive days on the dorsum of both ears ($25\text{ }\mu\text{l}$ per ear).

Day 19 “End-point analysis”: The animals were euthanised with deep CO_2 anaesthesia, and the lymph nodes were prepared.

Dose selection

The dose selection was performed after reviewing earlier studies involving MBT (Ikarashi et al. 1993; van Och et al. 2000; Ulrich et al. 2001; De Jong et al. 2002).

Dermal study: The animals were treated with 3, 10 and 30% of MBT on the back for days 1–3, repeating the same doses on the ears also at days 15–17.

Oral study: The animals were treated with 1, 10 and 100 mg/kg of MBT by oral gavage on days 1–3, followed by application of a 3, 10 and 30% solution of MBT, respectively, on the back of the ears on days 15–17.

Endpoints

The ear thickness (mm) was measured with a spring-loaded micrometer, Oditest (Kroeplin, Schüchtern, Germany). A section was taken from both ears with a punch of 6 mm-diameter and weighed (mg). The draining auricular lymph nodes were excised and weighed (mg). The single cell suspension from each lymph node was prepared by gentle mechanical disaggregation through stainless-steel mesh filter, suspended in 2 ml phosphate buffered saline (PBS, Biochrom, Berlin, Germany) and counted (million/lymph node) using the automated cell counter Sysmex F 820 (Sysmex Europe GmbH, Norderstedt, Germany).

The single cell suspension from the two lymph nodes of each animal was pooled (10^6 cells) and conjugated with fluorochrome-conjugated monoclonal antibodies. Antibodies used were phycoerythrin (PE)-conjugated rat anti-CD8a, fluorescein isothiocyanate (FITC)-conjugated rat anti-CD4 and rat anti-CD45R/B220 (Pharmingen, Heidelberg, Germany). CD4 and CD8 are T-cell markers characterising T-helper and cytotoxic cells, respectively, while CD45R (B220) is a mouse-specific B-cell marker.

In brief, cells were incubated with the antibody at 4°C in dark for 30 min and washed twice in PBS and 0.02% sodium azide (Sigma, Heidelberg, Germany). With the FACScan flow-cytometer (Becton Dickinson, Heidelberg, Germany), the percentages of lymphocytes bearing particular surface antigen were calculated. Calibration was done by using Cal-iBRITE Beads (Becton Dickinson). At least 10^4 viable lymphocytes were counted per sample.

Analysis

The values for the individual cell count and lymph node are presented along with their mean values as scatter plots.

Mean values were calculated for the ear thickness, ear-punch weight and phenotypic marker expression for each animal. The results of the MBT group were compared to those obtained from vehicle control animals using *t*-test. The statistical analysis was done using SPSS 13.0 (SPSS Inc., Chicago, USA). Statistical significance was assumed when $P < 0.05$. The analysis of the FACscan data was carried out by using the WinList 5.0 software (Verity Software House, Topsham, USA).

Results

In all the experiments, none of the animals showed any abnormal clinical signs. We did not observe any significant changes in the ear thickness and ear-punch weight in any of our experiments (Table 1). In the study involving dermal application of MBT at 3% concentration, we found a significant increase in cell count (Fig. 1a) and weight of lymph node (Fig. 1b). Number of CD8+ cells were significantly decreased in the MBT-treated group as compared to the control. We observed a non-significant modulation in CD4+ and CD45+ cells (Table 1). Following 10% dermal application, a significant increase in the cell count (Fig. 1a) and lymph node weight (Fig. 1b) were observed. We also found a significant decrease in CD8+ cells, while a non-significant modulation in CD4+ and CD45+ cells was observed (Table 1). The cell count and lymph node weight increased non-significantly (Fig. 1a, b, respectively) in the

treated group as compared to the control group following 30% dermal application of MBT. No significant changes were observed in the phenotypic markers in this study (Table 1).

After an oral dose of 1 mg/kg and 3% auricular application of MBT, the cell count showed significant increase (Fig. 2a) while the lymph node weight showed non-significant increase (Fig. 2b) in the treated group as compared with the vehicle control group. We did not observe any significant changes in the phenotypic markers in these mice (Table 1). We observed a non-significant increase in cell count and lymph node weight (Fig. 2a, b), respectively, in the treated group as compared to that in the vehicle control group following 10 mg/kg oral treatment and 10% auricular application of MBT, while no significant changes in the phenotypic markers were seen (Table 1). In the 100 mg/kg oral study followed by 30% auricular treatment, we observed a significant increase in lymph node weight (Fig. 2b), while no significant changes were observed in the phenotypic markers studied (Table 1).

Discussion

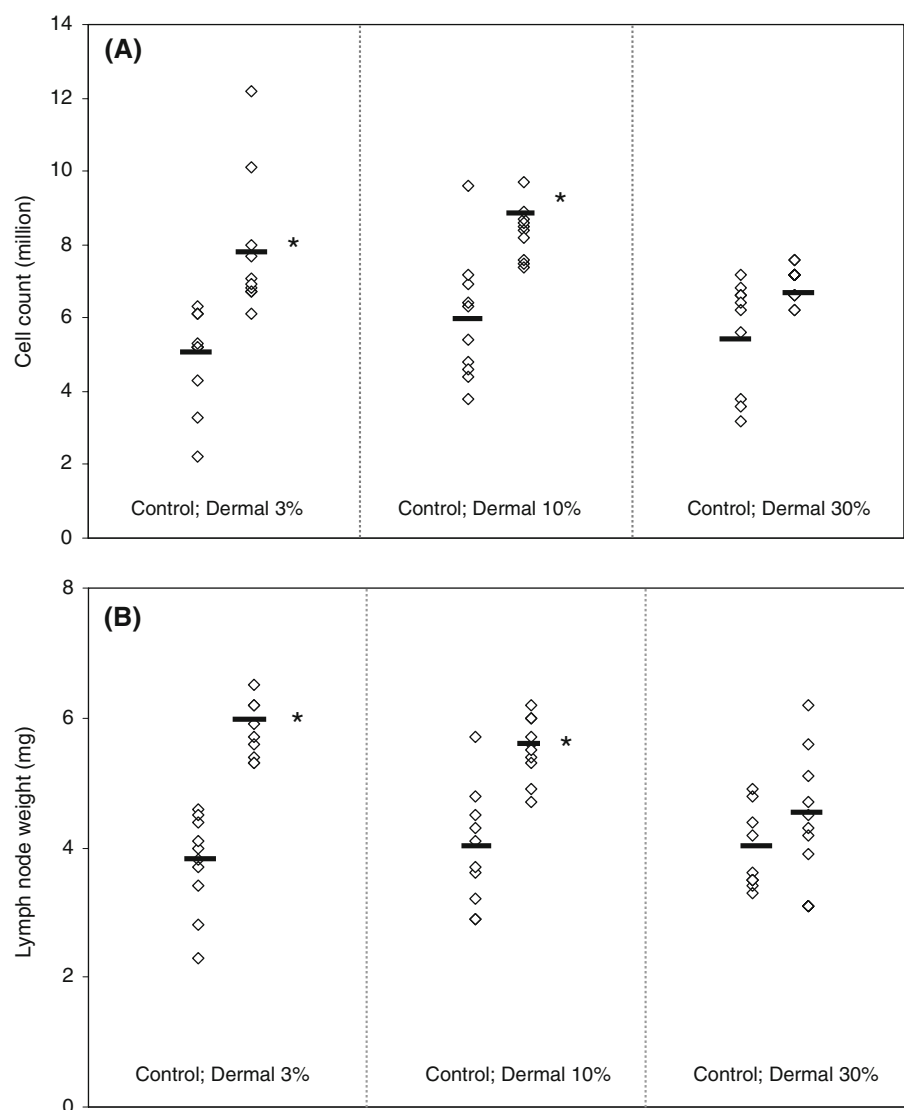
There have been reports of allergy due to presence of MBT in brassieres (Joseph and Maibach 1967), foot-wear (Lynde et al. 1982), socks (Rietschel 1984), condoms (Banerjee et al. 2006), sports equipments (Ventura et al. 2001), ear plug (Deguchi and Tagami 1996), bikini (Jung et al. 2006)

Table 1 End points (Mean $\pm$ SD) following exposure of mice to MBT in modified biphasic local lymph node assay

		Ear thickness (mm)	Ear punch wt. (mg)	CD4+ (%)	CD8+ (%)	CD45+ (%)
1.	3% dermal					
	Control	0.26 $\pm$ 0.01	8.6 $\pm$ 0.54	46.4 $\pm$ 3.8	24.5 $\pm$ 2.0	26.2 $\pm$ 4.7
	MBT	0.27 $\pm$ 0.01	9.1 $\pm$ 0.43	43.2 $\pm$ 3.3	20.8 $\pm$ 1.5*	30.4 $\pm$ 4.9
2.	10% dermal					
	Control	0.26 $\pm$ 0.01	9.0 $\pm$ 0.58	50.0 $\pm$ 2.2	25.3 $\pm$ 0.88	17.6 $\pm$ 2.4
	MBT	0.26 $\pm$ 0.02	8.8 $\pm$ 0.60	43.6 $\pm$ 6.1	23.0 $\pm$ 1.12*	22.8 $\pm$ 4.7
3.	30% Dermal					
	Control	0.24 $\pm$ 0.01	8.2 $\pm$ 0.52	47.5 $\pm$ 1.6	22.7 $\pm$ 1.4	19.8 $\pm$ 1.8
	MBT	0.24 $\pm$ 0.01	7.9 $\pm$ 0.36	45.0 $\pm$ 2.5	21.9 $\pm$ 1.0	23.0 $\pm$ 2.1
4.	1 mg/kg oral					
	Control	0.24 $\pm$ 0.01	8.3 $\pm$ 0.33	48.9 $\pm$ 3.8	25.0 $\pm$ 1.5	11.7 $\pm$ 3.4
	MBT	0.24 $\pm$ 0.01	8.4 $\pm$ 0.54	47.8 $\pm$ 4.5	24.4 $\pm$ 2.5	11.8 $\pm$ 2.9
5.	10 mg/kg oral					
	Control	0.24 $\pm$ 0.01	8.4 $\pm$ 0.20	55.1 $\pm$ 1.7	23.0 $\pm$ 0.82	16.7 $\pm$ 2.3
	MBT	0.24 $\pm$ 0.02	8.4 $\pm$ 0.41	56.0 $\pm$ 2.3	22.6 $\pm$ 1.2	17.0 $\pm$ 2.4
6.	100 mg/kg oral					
	Control	0.25 $\pm$ 0.02	8.3 $\pm$ 0.46	53.7 $\pm$ 2.4	24.7 $\pm$ 1.6	13.8 $\pm$ 2.1
	MBT	0.24 $\pm$ 0.01	8.3 $\pm$ 0.22	53.2 $\pm$ 3.7	23.8 $\pm$ 2.1	13.5 $\pm$ 2.0

Sample size was $n = 5$, except for $n = 10$ for the 1 mg/kg and 100 mg/kg oral study. * indicates significant difference at $P < 0.05$ (*t*-test) between vehicle control and treated animals

Fig. 1 Cell count (a) and lymph node weight (b) values from lymph nodes of mice treated dermally with MBT according to the sensitisation-challenge protocol. The right and left draining auricular lymph nodes were weighed (mg), and single cell suspension was prepared in PBS followed by cell counting (million/lymph node). Sample size was $n = 5$. The squares indicate individual values while the black line indicates mean value. *indicates significant difference at $P < 0.05$ (t -test) between vehicle control and treated animals

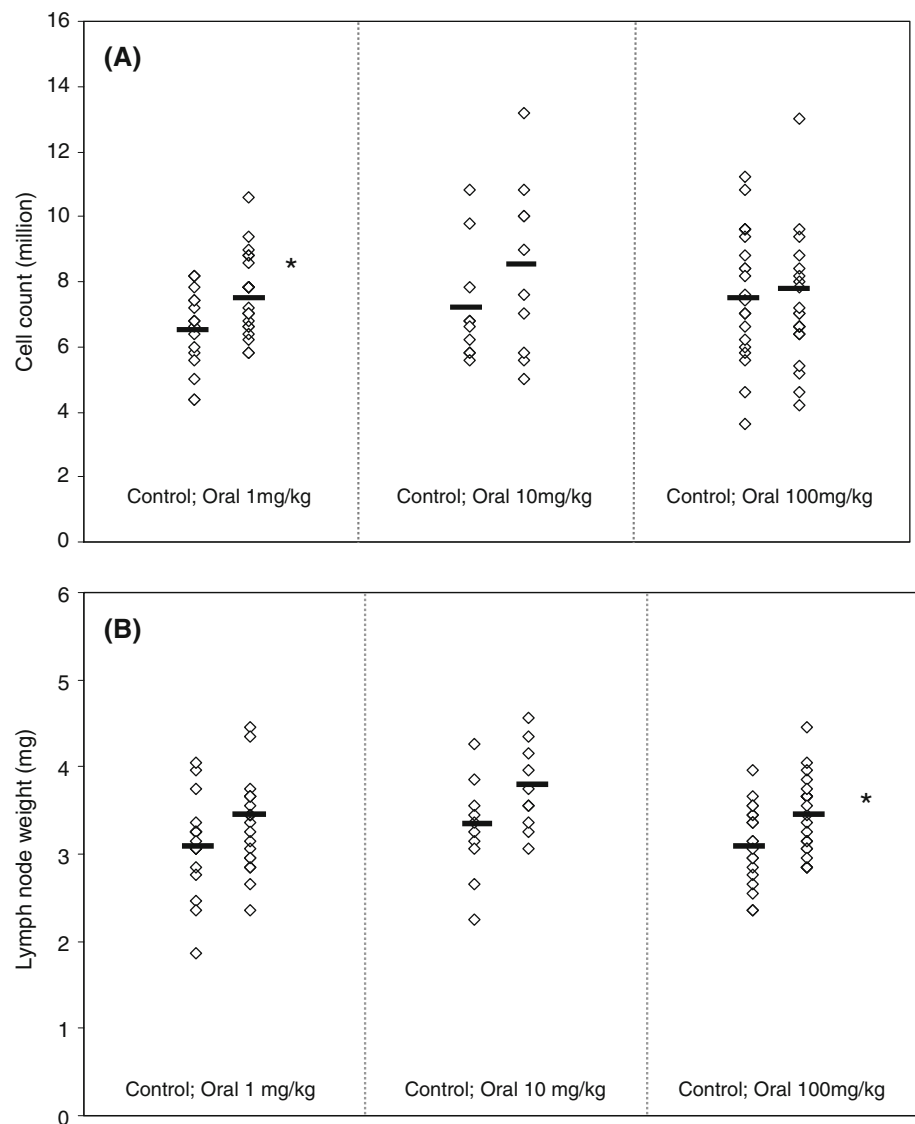


and numerous other rubber products. With regard to pacifiers as putative triggers of allergy it was discussed that MBT might be responsible (Schweisfurth 1995). In some cases, allergic reactions to pacifiers were attributed to type I sensitisation (Venuta et al. 1999; Kimata 2004). In an overview on contact dermatitis from Spain, it was deduced from patch test study results that MBT may also be a relevant allergen in children (Romaguera and Vilaplana 1998). In the opinion of the Scientific Committee on Consumer Products (SCCP) of the EU, a risk assessment was performed for the use of MBT in rubber soothers and teats, and it was concluded that the risk of sensitisation is negligible when migration of MBT from these articles is below the limit set by a CEN standard (SCCP 2005; European standard EN 2002). It was also noted by SCCP that no report of contact sensitisation to MBT from use of soothers or feeding teats has been reported.

The murine local lymph node assay (LLNA) was proposed by Kimber and coworkers to study allergic potential of chemicals (Kimber and Weisenberger 1989). However, it has some disadvantages, such as the use of radioisotopes to quantify lymph node cell proliferation. Meanwhile, various modifications have been proposed including measurement of both lymph node cell proliferation (cell counts and lymph node weights) and ear thickness or ear weight (Homey et al. 1998). Measurements of immunophenotypic end points have also been combined (Gerberick et al. 1999; Humphreys et al. 2003). In earlier studies, we have found the biphasic protocol to be proficient enough in assessing the sensitisation potential of the studied compounds (Stahlmann et al. 2006; Ahuja et al. 2009).

We used a biphasic LLNA protocol to study the sensitisation potential of MBT, including estimation of ear thickness, ear weight, lymph node weight and lymph node cell

Fig. 2 Cell count (a) and lymph node weight (b) values from lymph nodes of mice treated orally with MBT according to the sensitisation-challenge protocol. The right- and left-draining auricular lymph nodes were weighed (mg), and single cell suspension was prepared in PBS followed by cell-counting (million/lymph node). Sample size was $n = 5$ for the 10 mg/kg study, $n = 10$ for the 1 mg/kg and 100 mg/kg oral study. The squares indicate individual values while the black line indicates mean value. *indicates significant difference at $P < 0.05$ (t -test) between the vehicle control and the treated animals



count along with phenotypic markers. In the dermal study, we observed a more pronounced modulation in the various end points at the lower concentrations, while at higher concentrations, we observed less pronounced changes. This kind of response has also been observed by Ikarashi and coworkers while evaluating the contact sensitivity of rubber chemicals using local lymph node assay (Ikarashi et al. 1993). They observed inhibition of lymph node proliferative response at higher concentrations of N-isopropyl-N-phenyl-p-phenylenediamine (IPPD) and Tetramethylthiuram disulfide (TMTD), probably due to immunotoxic or immunosuppressive effects of these chemicals. In the study of phenotypic markers, we observed a decrease in CD4+ and CD8+ cells, while the CD45+ cells tended to increase. The changes in proportion of lymphocytes observed in our experiments have been previously described (Stahlmann et al. 2006; Sikorski et al. 1996; Hariya et al. 1999). After

oral treatment, we observed only slight changes in cell count and lymph node weight, indicating towards a weak allergenic response.

Vohr et al. studied the sensitising potential of MBT at 1, 3, 10 and 25% concentrations using a modified protocol of LLNA. They found that MBT up to 10% concentration did not induce a significant increase in cell count, and therefore used 25% concentration of MBT for dermal studies (Vohr et al. 2000). Van Och and coworkers performed a dose-response study using a single-phase protocol and pretreatment with 1% SDS to increase the response of the mice to the test substance. They tested five different concentrations of MBT ranging from 0.1–17.5% by dermal route, and found a significant dose dependent increase in lymph node weight and cell count. They concluded that the positive results for a weak allergen might be due to the fact that pretreatment with SDS increased the sensitivity (Van Och

et al. 2000). Ulrich and coworkers also observed a bell-shaped response curve while studying dermal sensitisation potential of MBT at 3, 12.5, 25 and 50% concentration, with maximum effect at 12.5% and subsequent reduction at higher concentrations (Ulrich et al. 2001). De Jong et al. performed a single-phase LLNA study, and reported that 10% concentration of MBT was needed to induce a positive response in cell proliferation. Inconsistent results for cell proliferation were obtained with a dose of 17.5%, while at 25% concentration the proliferation was found to be decreased. They further used SDS to increase the proliferative response. With respect to the inconsistent and sometimes negative results, they suggested MBT to be a mild to weak sensitiser (De Jong et al. 2002).

Our results show that the biphasic protocol seems to be more sensitive in studying the allergic potential of MBT even at lower concentrations. Keeping in view the results of our study and the report of previous investigators, it could be concluded that MBT is a mild to moderate allergen. The results also show that MBT exhibits a sensitising potential following dermal and oral application, and that the modified biphasic LLNA protocol can be used to study the sensitising potential of a compound after the oral route of exposure. However, supplementary studies with a panel of other chemicals including strong allergens are needed for further validation.

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