Studies on Toluene Diisocyanate (TDI)-Induced Delayed Type Hypersensitivity

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Abstract—The toluene diisocyanate (TDI)-induced delayed type hypersensitivity reaction (DTH) in the ear of male mice was investigated and compared with the picryl chloride (PC)-induced one. The results obtained were as follows: 1) When 1% TDI solution (20 μl/ear) as a challenging concentration was used for 7 weeks-old ICR mice, distinct ear swelling was observed in every group sensitized with various concentrations (1–5%, 100 μl/animal) of TDI solution, and the swelling rate was the same or higher than that of PC-induced DTH. 2) Five, 7 and 13 weeks-old ICR mice showed a similar high response in TDI-induced DTH, whereas the reactivity of 16 weeks-old ICR mice was significantly lower than that of the above-mentioned younger mice. 3) In both TDI- and PC-induced DTH, ICR and BALB/c mice showed a similar high response, whereas the reactivity of ddY mice was significantly lower. The relationship between increase of the ear swelling and the amount of Evans’ blue dye leaked being regarded as the intensity of its vasopermeability was also studied. The results obtained were as follows: 1) The dye leakage reached the maximum at 20 hr after challenge in sensitized mice. This peak preceded slightly that of the ear swelling (25 hr after challenge). 2) A positive correlation was observed between ear swelling and dye leakage (r=0.87, P<0.01). The effects of dexamethasone (DX) and indomethacin (IM) were investigated with the TDI-induced DTH model. The DTH reaction was suppressed significantly by both drugs, but the suppressive effects of DX were higher than that of IM. All the above results indicate that the TDI-induced DTH model in mice taking dye leakage as an index may be useful for evaluation of drugs for disorders derived from DTH reactions.

For the study of cell-mediated immunity, picryl chloride (PC)-induced delayed type hypersensitivity (DTH) in the ears of mice has been frequently used (1, 2). However, in this method, the following drawbacks have been pointed out: 1) Preparation for the sensitizing antigen solution requires more than 24 hr, and PC crystals often come out from the solution. 2) The sensitizing antigen solution dropped onto the abdomen of mice remained on the surface of the skin and stuck to various other sites such as body sites other than the applied site, the cage and on the bodies of other animals. 3) Exact measurement of increased ear thickness, generally used as an index of the DTH reaction, may be difficult because extraneous materials such as hair and so on adhere on the challenging sites of the ear due to the non-volatile and viscous challenging antigen solution, and furthermore, it does not seem to be reproducible because it is difficult to make measurements at exactly the same site on the ear.

Recently, toluene diisocyanate (TDI), which is widely used in industry, has also
been found to induce DTH in the ears of mice as PC does (3, 4).

In this paper, TDI-induced DTH was studied and compared with the PC-induced one in several strains and ages of mice to solve the above-mentioned difficulties of PC-induced DTH.

The intensity of increased vasopermeability due to the TDI-induced DTH was estimated by the dye leakage, and its use as an index of the DTH reaction was examined.

Materials and Methods

1. Reagents

Reagents and their sources were as follows: Toluene-2,4-diisocyanate (TDI) and picryl chloride (PC) (Nakarai Chem., Kyoto); ethyl acetate (HPLC grade), acetone (HPLC grade) and olive oil (Wako Pure Chem., Osaka); Evans' blue (Merck, Darmstadt); dexamethasone (DX, Sigma Chem., St. Louis); indomethacin (IM, Merck, Darmstadt). Other reagents used were of the highest commercial grade available.

2. Animals

Seven weeks-old male ICR mice were mainly used. Five, 13 and 16 weeks-old male ICR mice and 7 weeks-old male BALB/c and ddY mice were also employed for the examination of age and strain differences, respectively. These animals were from the Shizuoka Agric. Coop. Assoc. for Lab. Animals, Hamamatsu.

3. Sensitizing antigen

TDI was used at concentrations of 0.01, 0.1, 1, 3, 5 and 10% in ethyl acetate as the sensitizing antigen. These solutions were prepared just before use. PC was dissolved in ethanol at 7% by stirring overnight and stored in sealed containers, in the dark, at room temperature.

4. Challenging antigen

TDI at a concentration of 0.1% or 1% in ethyl acetate and 1% PC in a mixture of acetone: olive oil=4:1 were used.

5. Sensitization

Mice were sensitized with TDI or PC according to the method of Asherson and Ptak (1). Briefly, onto the abdomen which had been shaved on the previous day, 100 or 500 μl of the antigen solutions were dropped, and the mouse was held until the solution had spread and evaporated. Control animals received the vehicles.

6. Challenge

Seven days after the first or second sensitization, the animal was challenged with 20 μl/ear of challenging antigen solutions on the left ear or on both ears.

7. Measurement of DTH reaction

Intensity of the DTH reaction was assessed by the following 2 methods.

1) Increased ear thickness: According to the method of Asherson and Ptak (1), the ear thickness was measured at a loading weight of 5 g/0.2 cm²/site with a dial thickness gauge (Ozaki Factory, Tokyo) at varying times after challenge. The increased ear thickness by the reaction was calculated by subtracting the thickness before challenge.

2) Dye leakage: The left ear of the sensitized mouse was challenged by the antigen, and the right one served as the control. Unless otherwise stated, 0.1 ml/animal of 1% Evans' blue in physiological saline was i.v. administered at 15 hr after the challenge. At 5 hr after the dye administration, animals were exsanguinated and the ears were removed.

Dye leaked in each ear was extracted and measured according to the method described by Katayama et al. (5) with some modifications. Dye leakage by the reaction was calculated by subtracting that of the control right ear from that of the antigen-challenged left ear.

8. Effects of dexamethasone (DX) and indomethacin (IM)

Drugs were suspended in 0.5% tragacanth solution and were orally given to mice at 2 hr before and at 5 and 12 hr after challenge. The administered doses of both drugs were 0.02, 0.05 and 0.2 mg/10 ml/kg/time for DX and 0.1, 0.3 and 1.0 mg/10 ml/kg/time for IM. The control mice received the vehicle.

Results

1. Primary and secondary DTH reaction by various concentrations of antigen

Primary reaction: Figure 1 shows the results of increased ear thickness of 7 weeks-old ICR mice in the primary reaction when 0–10% of TDI or 0 and 7% PC were employed for sensitization and challenged with 0.1 or
1% TDI or 1% PC. The ear thickness was measured at 24 hr after challenge.

Twenty μl/ear of 1% TDI as a challenging dose induced increased ear swelling in proportion to antigen doses of sensitization (1–5% TDI, 100 μl/animal), and the swelling rate was the same or higher than that of the PC-induced DTH reaction. However, sensitization with the highest dose of 500 μl/animal of 5% TDI in this experiment conversely decreased the reaction.

The animals challenged with 0.1% TDI (20 μl/ear) scarcely responded even when higher concentrations of the antigen were employed.

With regard to the condition of mice after sensitizing by antigen, TDI of up to 1% or 7% PC did not induce any visible disorder; however, 3% or higher concentration of TDI caused some lesion on the sensitizing sites such as reddening, erosion, desquamation and laceration. In addition to these, 500 μl of 5% TDI caused a decrease in body weight.

From the above results, 100 μl/animal and 20 μl/ear of 1% TDI were employed as the antigen dose for the primary sensitizing and challenging, respectively, for further experiments, except for secondary reaction. The reference antigen PC was used at doses of 7% for sensitization and 1% for challenge throughout the experiments.

Secondary reaction: Three days after the primary challenge, mice were sensitized again and challenged at 7 days after the resensitization.

As shown in Fig. 2, in any of the sensitizing and challenging concentrations of antigen, the reaction by secondary challenge was generally more increased than that by primary challenging.
2. Influence of age on DTH reaction

Five, 7, 13 and 16 weeks-old male ICR mice were employed for detecting the influence of age on the TDI-induced DTH reaction.

Results are shown in Fig. 3. Five, 7 and 13 weeks-old mice showed a high response with a decreasing tendency with age, and in 16 weeks-old mice, a considerably low response was induced.

Similarly, in the PC-induced DTH reaction, 7 and 13 weeks-old mice showed a similar high response, whereas the reactivity of 5 and 16 weeks-old mice was lower.

In the TDI-induced DTH reaction, the response of nonsensitized animals was significantly lower than that of sensitized animals; however, in the PC-induced DTH reaction, the response of nonsensitized animals came to 45% that of the sensitized animals.

From the above results, ICR mice were used at 7 weeks of age for subsequent experiments.

3. Influence of strain on DTH reaction

The results of the DTH reaction to TDI of ICR, BALB/c and ddY mice are shown in Fig. 4. BALB/c mice showed highest response to TDI, and ICR mice were comparable, but ddY mice were the lowest responders. Similar results were obtained from the PC-induced DTH reaction.

In the TDI-induced DTH reaction, the response to the challenging antigen of nonsensitized animals was obviously lower than the responses of the sensitized animals for all three strains. However, when PC was used for the antigen, nonsensitized BALB/c and ddY mice showed quite high responses, especially the ddY mice. Accordingly, a large part of the PC-induced DTH reaction in BALB/c and ddY mice was attributable to...
4. Time-course study of ear swelling and dye leakage

**Time-course at 10 hr intervals:** The time-course of the TDI-induced DTH reaction using ICR mice was examined. They were i.v. administered Evans’ blue saline solution at 0, 10, 20, 30 or 40 hr after challenge. At 10 hr after each administration, the ear swelling was measured, and then animals were exsanguinated, followed by removal of the ears for extraction and measurement of dye leaked. These results are shown in Fig. 5.

In sensitized animals, both the dye leakage...
and the ear swelling reached the maximum at 20 hr after challenge, followed by a gradual decrease with time. The dye leakage in nonsensitized animals was consistently significantly lower than that in sensitized animals, and it was the maximum at 10 hr after challenge and decreased with time. The ear swelling in nonsensitized animals was also lower than that in sensitized animals and scarcely altered with time.

Time-course at 5 hr intervals: Because the peak of the ear swelling and the dye leakage was observed about 20 hr after challenge, the reaction time-course was examined in detail within 15–30 hr after challenge. The intervals of time between the dye administration and measurement of the swelling or exsanguination for dye extraction were 5 hr.

As shown in Fig. 6, the dye leakage reached the maximum at 20 hr after challenge, and the ear thickness increased until 25 hr after challenge and hardly altered between 25 and 30 hr after challenge in sensitized animals. Namely, the peak of the dye leakage preceded slightly that of the ear swelling.

From the above results, it was fixed that Evans’ blue was administered at 15 hr and that mice were exsanguinated at 20 hr after challenge.

5. Influence of i.v. administered doses of Evans’ blue

For the suitable doses of Evans’ blue, sensitized ICR mice were i.v. administered 0.1 ml/animal of 0.25 to 1% of Evans’ blue saline solution after challenge, and the dye leakage was estimated.

Amount of leaked Evans’ blue dye increased in proportion to the concentration of the administered dye between 0.25 and 1% (Fig. 7). From these results, Evans’ blue at a dose as high as 1 mg/animal is considered not to affect the TDI-induced DTH reaction.

6. Correlation between ear swelling and dye leakage

In order to ascertain whether dye leakage is useful as an index of the DTH reaction, the correlation between dye leakage and ear swelling was examined.

As a result, a positive correlation was observed between ear swelling and dye leakage (r=0.87, P<0.01) (Fig. 8). Therefore, these results prove that the measurement of
dye leakage is useful as a suitable index of the DTH reaction instead of ear swelling.

7. Effects of dexamethasone (DX) and indomethacin (IM) on DTH reaction

The effects of DX and IM on the TDI-induced DTH reaction which were measured as dye leakage was examined.

The DTH reaction was suppressed significantly in a dose-dependent fashion by DX (Fig. 9). IM also suppressed the DTH reaction significantly, but the degree of the suppressive effect of IM was lower than that of DX (Fig. 10).

Discussion

It has been known that TDI, which is widely used in the manufacture of polyurethane foams and other elastomers, is highly irritating to the respiratory organs and skin. Since Karol et al. demonstrated that tolyl-specific IgE antibodies were found in the blood of workers with hypersensitivity to TDI (6), extensive studies about the effect of TDI have been reported (7, 8, 9–11).

In 1980, it was also demonstrated that TDI can induce a typical DTH reaction judging from histological findings in experimental animals and that the TDI-induced DTH reaction could be passively transferred (3).

In this paper, the TDI-induced DTH was examined and compared with the PC-
induced one in several strains and ages of mice, and it was made clear that TDI is useful as a good antigen for the DTH animal model instead of PC.

First of all, the three major faults in experiments of PC-induced DTH were eliminated by using TDI as antigen: 1) It takes more than 24 hr to prepare the sensitizing antigen solution of PC and its crystals often come out from the solution during storage because of its low solubility. TDI is freely soluble in ethyl acetate, therefore fresh antigen solution can be prepared just before use. 2) The sensitizing antigen solution dropped onto the abdomen of mice remained on the surface of skin and then stuck to various other sites such as body sites other than the application site, the cage and to the bodies of other animals. Therefore, some of the applied sensitizing antigen would be lost. In the TDI-induced DTH, the solvent of antigen solution, ethyl acetate, evaporated rapidly after sensitization and the antigen solution hardly stuck to any other sites. Thus, TDI was found to be much more efficient than PC as a sensitizing and challenging antigen. 3) In the PC-induced DTH, because extraneous materials such as hair and so on adhere to the challenging sites of the ear due to the non-volatile and viscous challenging antigen solution, exact measurement of increased ear thickness may be interfered with. In the TDI-induced DTH, because the ethyl acetate used for its solvent evaporates rapidly after challenge, extraneous materials hardly adhere on the challenging sites.

From the results using various concentrations of antigen solution, 1% TDI was most suitable for both sensitization (100 μl/animal) and challenge (20 μl/animal) (Fig. 1). Under this condition, the swelling rate was the same as that of animals sensitized with 7% PC, indicating that TDI has the ability to be a sensitizer at lower concentrations than PC.

In ICR mice, fairly good TDI-induced DTH reactions occurred in mice of a wide range of ages (5–13 weeks old), and particularly, 5 weeks-old mice were sensitive. On the other hand, the PC-induced one was relatively weak, especially in 5 weeks-old mice, with the drawback of non-immunologically increased ear thickness by the compound itself (Fig. 3).

It is generally recognized that there are high and low responders among strains of mice. In the TDI-induced DTH, ICR and BALB/c were found to be high responders, whereas ddY was a low responder (Fig. 4). This agrees with the result of the experiments in PC-induced DTH by Tajima et al. (12). Using these different strains properly, the TDI-induced DTH animal model may be useful for screening of anti-inflammatory agents, antiallergics of type IV or immunomodulators.

Measurements of the thickness of the ear do not seem to be reproducible because it is difficult to do repeated measurements at exactly the same site on the ear. Jegasotly and Waksman (13) reported that vasopermeability at the reaction site is increased by DTH. Therefore, the relationship between increases of ear swelling and vasopermeability was studied. Amount of Evans' blue dye
leaked was indexed as the intensity of vasopermeability. Evans’ blue is widely used as an index of vasopermeability because of its high affinity to plasma protein and low toxicity. According to Miyazawa et al. (14), Evans’ blue may affect the immune responses. However, 1 mg/animal of Evans’ blue used in these experiments did not affect the TDI-induced DTH reaction (Fig. 7). The peaks of the dye leakage and the ear swelling were 20 hr and 25 hr after challenge, respectively (Fig. 6). That is thought to be a matter of course; judging from the mechanism, a swelling is caused by the leakage of the blood’s components into the tissue as a result of the increase of vasopermeability.

The amounts of dye leaked in non-sensitized animals were small enough compared with those in sensitized animals when challenged by TDI (Figs. 5 and 6). In addition, a clearly positive correlation was observed between ear swelling and dye leakage ($r=0.87$, $P<0.01$) (Fig. 6).

The above results indicate that the dye leakage can be a suitable index of the DTH reaction instead of ear swelling.

Using TDI as an antigen and taking dye leakage as an index of DTH, effects of DX and IM were examined. The DTH reaction in this model was found to be suppressed significantly in a dose-dependent fashion by DX (Fig. 9). Although IM also suppressed the reaction significantly (Fig. 10), its potency was much less than that of DX. This suggests lymphokines as well as prostaglandins may be concerned with TDI-DTH.

All the above results suggest that the TDI-induced DTH mouse model taking dye leakage as an index may be very useful for drug evaluation.

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