Dose and Timing Studies for the Optimization of Contact Sensitivity in the Mouse

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We investigated the effectiveness of very low doses of the contact sensitizer dinitrofluorobenzene in sensitizing BALB/cJ mice. Surprisingly, the ear swelling reactions were greater with lower dinitrofluorobenzene doses, down to one-twentieth of doses commonly used. Although it is common practice to use much lower doses at challenge than at sensitization, we found greater reactions with lower doses at sensitization than at challenge. We also studied the timing of the development and waning of reactivity to dinitrofluorobenzene, dinitrochlorobenzene and oxazolone. Reactivity peaked at day 5 for dinitrofluorobenzene and dinitrochlorobenzene, and at day 3 for oxazolone. Reactivity waned by 3 weeks with dinitrofluorobenzene and oxazolone, and by day 7 with dinitrochlorobenzene. Pretreatment with cyclophosphamide caused a delay in the development and waning of reactivity. ***Key words: Dinitrofluorobenzene: Dinitrochlorobenzene: Oxazolone: Ear swelling.***

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Contact sensitivity reactions in mice are often used in dermatological research, for example to study the effects of treatments such as UVB irradiation on the immune system. Since contact sensitivity reactions are used as a tool in such analyses, it is important to know the optimum doses and timing of the reactions. It is common practice to use sensitizing doses of contact sensitizers that are greater than the doses used at challenge.

However, in our studies on the contact photosensitizer tetrachlorosalicylanilide (TCSA), we found that optimum reactions were achieved with larger doses of TCSA and UVA at challenge than at sensitization (1). Because of this surprising finding with a photosensitizer, we decided to study low doses of the contact sensitizer dinitrofluorobenzene for this effect as well. We also investigated the optimum timing between sensitization and challenge, the time taken for sensitivity to wane, and the effect of cyclophosphamide on the development and waning of reactivity.

MATERIALS AND METHODS

Female BALB/cJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine, U.S.A. We obtained 2, 4 dinitro-1-fluorobenzene (DNFB), 1-chloro-2, 4-dintrobenzene (DNCB) and 4-ethoxymethylene-2 phenyloxazol-5-one (OX) from Sigma Chemical Company, St. Louis, MO. U.S.A. Cylcophosphamide (Cy) was purchased from Procytox, Montreal, Canada.

Mice were anesthetized with ethyl ether and a patch of fur 2×2 cm was removed from the dorsal skin with electric clippers. For dose-response studies, we sensitized groups of mice with various quantities (1 μl to 150 μl) of 0.5% DNFB in acetone applied to the shaved back skin on days 0 and 1. The total dose was therefore 10 μg to 1500 μg. For studies

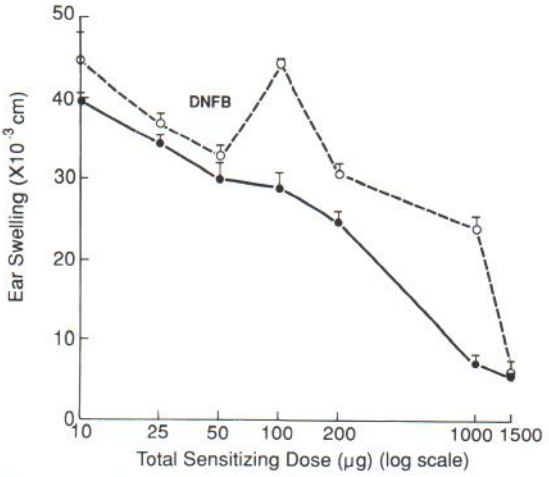

Fig. 1. Dose response for vaious sensitizing doses of DNFB. Mean values and standard errors of means. —— without cyclophosphamide; - - - - -, with cyclophosphamide.

on the timing of the development and waning of reactivity, we sensitized mice with 20μl of 0.5% DNFB, DNCB or OX in acetone, applied on days 0 and 1 (total 200 μg). For challenge in the dose-response studies, 10 μl of 0.2% DNFB (20 μg) in acetone was applied to both sides of the mouse ears on day 5. For challenge in the timing studies, 10 μl of 0.2% DNFB, DNCB or OX in acetone was applied the the ears at various times between 2 days and 9 weeks after sensitization. Non-sensitized mice challenged with DNFB, DNCB or OX served as controls. Some groups of mice were injected with 200 mg/kg of Cy in distilled water 3 days before sensitization. In a group of non-sensitized control mice, Cy was injected 8 days before challenge. Ear thickness was measured with a spring-loaded dial micrometer before challenge and at 24 and 48 h after challenge. Ear swelling data are reported for the maximum reactions whether they occurred at 24 or 48 h.

We used a total of 628 mice with 2 data points for each mouse (both ears). There was an average of 12 mice per group in the dose-response studies and an average of 8 mice per group in the timing studies. The Student's *t*-test was used to determine the statistical significance of differences between means. Differences were considered significant for $p < 0.05$.

RESULTS

Dose-response for DNFB

We started with a dose and timing protocol that has been commonly used: 20 μl of 0.5% DNFB applied to the shaved back skin on days 0 and 1 followed by challenge on day 5 with 10 μl of 0.2% DNFB applied to the ears. We varied the volume of DNFB at sensitization and found that lower doses produced higher reactions, even down to the lowest dose tested (Fig. 1). For each test dose we had a second

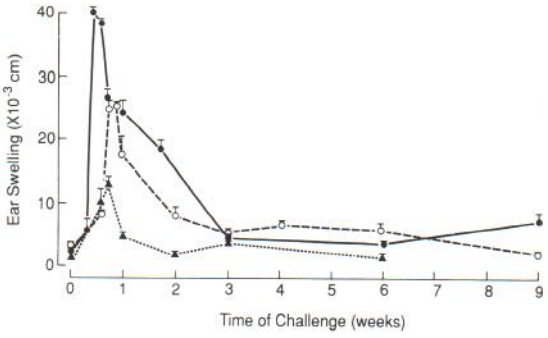

Fig. 2. Time course of development and waning of contact sensitivity to oxazolone, DNFB, and DNCB. Sensitization was with 50 μl of 0.5% on days 0 and 1. Challenge was with 10 μl of 0.2% at various times after sensitization. Mean values and S.E. ——, Oxazolone; - - - - -, DNFB; ....., DNCB.

group of mice that were treated with Cy 3 days before sensitization. The Cy-treated mice had generally higher responses than those not Cy-treated, although the increase was not always significant.

Time of development and waning of reactivity to DNFB, DNC and OX

To compare the timing of reactivity to the three antigens, we used common dosages for sensitization and challenge. The sensitization dose for all three antigens was 200 μg and the challenge dose was 20 μg. With these standard doses, OX produced the greatest response, about 50% greater than that to DNFB and about 200% greater than that to DNCB (Fig. 2). However, when the sensitizing dose of DNFB was optimized at 10 μg, as shown in Fig. 1, the reaction was about the same as that to the stan-

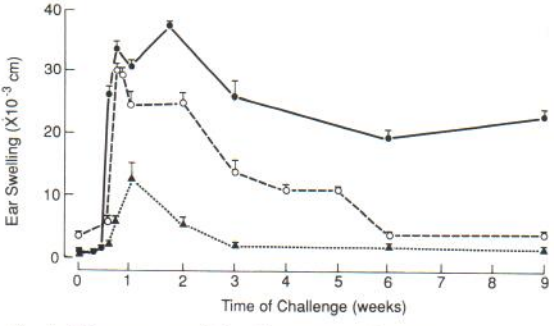

Fig. 3. Time course of development and waning of contact sensitivity to oxazolone, DNFB, and DNCB after injection with 200 mg/kg of cyclophosphamide. Sensitization was with 50 μl of 0.5% on days 0 and 1. Challenge was with 10 μl of 0.2% at various times after sensitization. Mean values and S.E. ——, Oxazolone; - - - - -, DNFB; ....., DNCB.

dard sensitization dose of 200 μg of OX. OX reactivity peaked early, at 3 and 4 days, while DNFB peaked at 5 and 6 days, and DNCB peaked at 5 days. The waning of reactivity was most rapid with DNCB (about 1–2 weeks), longer with DNFB (about 2–3 weeks), and longest with OX (about 3 weeks).

Cy treatment caused a delay in waning of reactivity with all three sensitizers (Fig. 3). This delay was most pronounced with OX, where reactivity was still significant at 9 weeks after sensitization. The initial development of reactivity was delayed by Cy treatment of mice sensitized to OX and DNCB, but not to DNFB.

DISCUSSION

The dose of sensitizer used at induction has usually been larger than that used at challenge, both for contact sensitivity (2, 3) and for contact photosensitivity (4). However, we found greater reactions when doses of the contact sensitizer DNFB were smaller at induction than at challenge (10 μg versus 20 μg). In studies on contact photosensitivity to topically applied TCSA (1) we also found greater reactions with lower doses of UVA and lower concentrations of TCSA at induction than at challenge. Wirestrand & Ljunggren (5) also found greater reactions with lower doses at induction than at challenge in studies on photoallergy to quinidine in mice, but the quinidine was systemically administered, and they used Cy and UVB to enhance induction. These three studies show that surprisingly low doses of contact an photocontact sensitizers give strong reactions. After this paper had been submitted, Sullivan et al. (6) reported strong reactions in mice with lower doses of trinitrochlorobenzene (TNCB) at sensitization than at challenge.

Our timing studies showed different patterns of development and waning of reactivity for the three sensitizers. DNFB and DNCB reactivity peaked at 5 days after sensitization, but OX peaked at 3 days and declined considerably by 5 days. Such a remarkably short (3 days) development time is not without precedent. Möller (2) found sensitivity to picryl choloride by 3 days after topical application in mice, and Ljunggren & Wirestrand (7) found sensitivity by 3 days in mice systemically sensitized with the photoallergen quinidine.

Sensitivity is known to persist for years in humans and months in guinea pigs, but usually lasts only 2 or 3 weeks in mice (8). We have previously shown that senitivity to TCSA can be prolonged to 7 weeks in mice by optimizing the dose (9). Möller (2) reported a 16-week duration of reactivity to picryl chloride in mice, and, Ljunggren & Wirestrand (7) found an 8-month duration for photosensitivity to systemically administered quinidine. The present study shows that Cy can increase reactivity and prolong the waning period to varying extents, depending on the antigen. A prolongation of waning by Cy has also been shown by others (8), but waning still takes place, so some mechanism other than Cy-sensitive suppression must be involved, possibly the soluble suppressor factors described by Fairchild & Moorhead (10).

Sy et al. (11) and Schwartz et al. (12) have suggested that supraoptimal doses of sensitizers activate suppressor cells, whereas optimal doses do not. Our results do not support that hypothesis, since there was some degree of enhancement by Cy at all doses of DNFB, although this enhancement was not always statistically significant. Schwartz et al. (12) have proposed an additional regulatory mechanism: that suboptimal doses activate Cy-sensitive regulatory cells which boost the reactions. Our results with DNFB do not support this hypothesis because even doses as low as 10 μg showed Cy-sensitive suppression, instead of boosting. Also, with TCSA, suboptimal doses produced Cy-sensitive suppression rather than boosting (1 and unpublished data). Sullivan et al. (6) also found slight (not significant) increases in reactivity with Cy and optimal and suboptimal doses of TNCB, and significant increases with Cy and supraoptimal doses. These studies are consistent with the hypothesis that optimal and suboptimal doses cause slight Cy-sensitive suppression (not always significant with small numbers of mice), while supraoptimal doses cause significant suppression.

It was surprising to find increased reactions to lower doses of DNFB, down to 10 μg. No doubt even lower doses would eventually produce lower reactions. Sy et al. (11) also showed declining reactions to DNFB doses greater than 250 μg, but 100 μg produced a lower reaction. The more common pattern of reactivity is increasing reactivity with increasing dose, as we have shown with TCSA in mice (1) and Friedmann (13) has shown with DNCB in humans.

Changing the dose by changing the concentration is not necessarily the same as changing the volume. Friedmann (13) has shown that by increasing the concentration (and dose) of DNCB, one caused in-

creased reactions in humans, but when the concentration was constant and the volume and area were reduced, we found little change until very small areas and doses were reached (8 mm^2, 3 μg). With 0.5% DNFB, we found increasing reactivity as we decreased the dose to 10 μg. At 10 μg the area was about 8 mm^2 (3 mm diameter). Thus, it appears that the number of Langerhans cells in 8 mm^2 of mouse skin (approximately 8,000) is suficient to produce a full reaction, but it may be too small an area in human skin.

The reduction in reactivity with increasing doses of DNFB may be due to a greater increase in suppression than helper function. The mechanism of suppression does not depend solely on the proliferation of Cy-sensitive T suppressor cells, since the reactions with Cy also decline as the dose of DNFB is increased. The major component of suppression may involve the stimulation of soluble suppressor factor production. Perhaps different antigens strike a different balance of help and suppression due to the activation of different subsets of lymphocytes that may differ in number or reactivity.

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