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IMMUNOLOGICAL TOLERANCE AND DERMAL EOSINOPHILIA INDUCED IN DINITROCHLOROBENZENE-SENSITIZED GUINEA PIGS

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Abstract. Guinea pigs sensitized to dinitrochlorobenzene (DNCB) were injected intravenously with 200 mg/kg dinitrobenzene sulphonic acid sodium salt (DNCB SO_3). The animals became totally and temporarily unresponsive if they were tested at the same time with 6 × 0.025 ml 0.5% DNCB, but only partially unresponsive if the test dose was 1 × 0.025 ml. Also only partial unresponsiveness was produced if the test dose (0.025 ml) was applied 24 hours after the DNCB SO_3 injection or if DNCB SO_3 was injected in 3 divided doses of 100 mg/kg. Histological examination of the test sites showed an infiltration with eosinophils into both epidermis and dermis, which was most marked in the series where the tolerogenic effect was least pronounced. The eosinophils appeared within 1 day after injection with DNCB SO_3, increased to a maximum between the 4th and 11th day, then decreased, but were still seen at the end of the experiments.

It has been shown that in guinea pigs already sensitized to potassium dichromate a long-lasting tolerogenic effect can be obtained by a high intravenous dose of dichromate followed shortly thereafter by a small epicutaneous dose—"double shot" procedure (5). This double shot effect does not seem to operate in dinitrochlorobenzene (DNCB)-hypersensitive animals when dinitrobenzene sulphonic acid sodium salt (DNCB SO_3) is injected intravenously and DNCB is given intradermally in various amounts (3). According to Frey et al. (3), the intravenous injection of the hapten causes a temporary tolerance by inhibiting the reactivity of all specifically immunologically active cells, and the intradermal application prevents these cells in some way from accumulating in the circulation. The absence of the double shot effect with DNCB–DNCB SO_3 is explained by these authors by the fact that DNCB SO_3, owing to its systemic toxicity, cannot be given in sufficiently high doses as to affect the entire population of immunocompetent cells. According to Polak & Turk (5), on the other hand, there remains after the intravenous injection a small number of immunologically active cells which are only susceptible to conjugates formed between the sensitizer and the skin. This they consider to be reflected in the observed microscopic changes of vasodilatation of the superficial capillaries of the dermis and some slight infiltration of polymorphonuclear leucocytes.

In an earlier paper (7) we have shown that, on injection of DNCB SO_3 into DNCB-hypersensitive guinea pigs, apart from a temporary disappearance of the DNCB reaction, an infiltration of eosinophils in the epidermis and dermis takes place.

To further illustrate the factors which may be active in the development of tolerance, the following variables have been studied:

1. The quantity of DNCB for epicutaneous application: 1 × 0.025 ml and 6 × 0.025 ml 0.5% DNCB respectively.
2. The interval between intravenous and epicutaneous application: less than 15 min and 24 h respectively.
3. DNCB SO_3 intravenously: 200 mg/kg as single injection, 100 mg/kg three days in succession.
4. The number of eosinophils in epidermis and dermis inside and outside area of epicutaneously applied DNCB.

MATERIALS AND METHODS

Animals. Albino guinea pigs of both sexes weighing 400–600 g. They were fed on pellet diet with additional greens and water.

Chemicals. Reagent grade 1.3 dinitro-4-chlorobenzene (DNCB) from E. Merck AG, Darmstadt, Germany, and 2.4 dinitrobenzene sulphonic acid sodium salt (DNCB SO_3) from Eastman Kodak, Rochester, N.Y., U.S.A.

Sensitization. The animals were injected on 10 consecutive days s.c. in the hind-legs with 0.0025 mg DNCB in saline (1).

Table I. *Test with DNCB at different times after DNCB SO_3 injection*

Series no.	Day ...	Before test		1		2		4		9		11		16	
		Mean score	No.of anim.	Mean score	No. of anim.	Mean score	No. of anim.	Mean score	No. of anim.	Mean score	No. of anim.	Mean score	No. of anim.	Mean score	No. of anim.
I.	200 mg DNCB SO_3/kg i.v., 1st test with 0.025 ml 0.5% DNCB on same day	2.8	12	0.8	12			0.8	12	1.9	12	2.0	6	2.0	5
II.	200 mg DNCB SO_3/kg i.v., 1st test with 6 × 0.025 ml 0.5% DNCB on same day	3.0	14	0	14	1.2	14	1.9	14	1.9	14	1.8	14	2.2	10
III.	200 mg DNCB SO_3/kg i.v., 1st test with 0.025 ml 0.5% DNCB on next day	3.0	12			2.0	12	2.0	12	2.2	12	2.3	12	2.2	6
IV.	100 mg DNCB SO_3/kg i.v., 3 days in succession, 1st test with 0.025 ml 0.5% DNCB on last day of injection	2.9	17	1.1	17			1.8	17	2.1	16	2.9	14	2.8	10

Day 0 = Day of injection of DNCB SO_3. 3rd injection in series IV.

Testing. To choose animals for the series of experiments they were tested 1 week after the completion of the sensitization injections and thereafter once weekly. Prior to the test, depilation was performed with an electric razor. Epicutaneous testing was performed on the flank with 0.025 ml of 0.5% and 0.1% DNCB in olive oil and 20%, 6% and 2% DNCB SO_3 in distilled water. Reading after 24 h: the animals selected for the experiments were those which reacted to all test concentrations of DNCB and also to, at the lowest, 6% DNCB SO_3.

Testing of animals in the experimental and central series. Epicutaneous application of 0.025 ml 0.5% DNCB in olive oil on the back. The skin reactions 24 hours after application were graded (arbitrarily) from 0 to 3. 0 = no reaction; 1 = diffuse slight redness; 2 = marked redness and slight swelling; 3 = deep redness and swelling. The average degree of DNCB sensitivity is given by the arithmetical mean of all reactions of an experimental series.

Injection of DNCB SO_3 into jugular vein. The animals were anesthetized with Nembutal®, fixed, the jugular vein was dissected and a silicon-treated polyethylene tube was placed in it. DNCB SO_3 was given in doses of 100 to 200 mg/kg as a 10% aqueous solution.

Histology. The skin specimens were fixed in 10% formalin, embedded in paraffin and stained with haematoxylin and eosin. At least 6 sections from different depths of the specimens were examined. Eosinophilia in epidermis and dermis was determined by counting the number of eosinophils in representative high-power fields. The eosinophilia in Table II indicate experiments in which there was > 1 cosinophil per high-power field. "Pronounced eosinophilia" signifies either eosinophils in the epidermis and/or 5 cosinophils per high-power field in the dermis. In Table II the numerals within parentheses indicate "pronounced eosinophilia".

Experimental series I. 200 mg DNCB SO_3/kg was injected intravenously in 12 animals and, at the same time, 0.025 ml of 0.5% DNCB in olive oil (within 15 minutes after the intravenous injection) was applied on the back. This was the first test and the reading was made on day 1 (Table I). Further testing with 0.025 ml DNCB was then performed at various intervals up to 15 days after the injection of DNCB SO_3. At the time of test readings, skin biopsies were taken from the test area and from an area about 10 mm outside the test reaction.

Experimental series II. 200 mg DNCB SO_3/kg was injected intravenously in 14 animals and at the same time 6 × 0.025 ml of 0.5% DNCB in olive oil was applied close together on the back. This was the first test and the reading was made on day 1. Further tests and biopsies as in series I.

Experimental series III. 200 mg DNCB SO_3/kg was injected intravenously in 12 animals and 24 hours later 0.025 ml 0.5% DNCB in olive oil was applied on the back. This test was read on day 2. Further tests and biopsies as in series I.

Experimental series IV. 100 mg DNCB SO_3/kg was injected intravenously on three consecutive days in 17 animals. Within 15 minutes after the last injection 0.025 ml DNCB

Table II. *Occurrence of eosinophilia*

In series I, day 1, accordingly, 6/12 signifies that in 6 of 12 experiments there was >1 eosinophil per high-power field. Within ( )="pronounced eosinophilia"

Experiment no.	Day ... Before injection (%)	1		2		4		9		11		16	
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
I. 200 mg DNCB SO_3/kg i.v., 1st test with 0.025 ml 0.5% DNCB simultaneously	0	6/12 (2/12)	50			10/12 (1/12)	83	5/10 (3/10)	50	4/5 (0/5)	80	3/5 (0/5)	60
II. 200 mg DNCB SO_3/kg i.v., 1st test with 6×0.025 ml 0.5% DNCB simultaneously	0	3/14 (1/14)	21	3/14 (1/14)	21	7/14 (1/14)	50	6/14 (1/14)	43	8/14 (1/14)	57	3/10 (0/10)	30
III. 200 mg DNCB SO_3/kg i.v., 1st test with 0.025 ml 0.5% DNCB on next day	0			7/14 (4/12)	58	10/12 (3/12)	83	11/12 (3/12)	92	9/12 (2/12)	75	4/6 (3/6)	66
IV. 100 mg DNCB SO_3/kg i.v., 3 days in succession. 1st test with 0.025 ml 0.5% DNCB on last day of injection	0	7/17 (1/17)	41			11/17 (0/17)	65	8/16 (1/16)	50	7/14 (0/14)	50	6/10 (1/10)	30

in olive oil was applied on the back. This test was read on day 1 (Table I). Further tests and biopsies as in series I.

Controls. Six non-sensitized animals were injected with 100 mg DNCB SO_3/kg on 3 consecutive days. Tests and biopsies as in series IV.

Nine other sensitized animals which had grade 2 reactions to 0.025 ml 0.5% DNCB were biopsied 24 hours after application of DNCB.

RESULTS

Table I shows the results of the four experimental series.

Macroscopically a reduction of DNCB hypersensitivity was noted in all series. A return of the DNCB reactions to the same intensity as before the DNCB SO_3 was observed during the experimental period only in series IV. In experiment II, in which 6×0.025 ml DNCB was applied epicutaneously simultaneously with injection of DNCB SO_3, this application of DNCB did not give rise to macroscopic lesions in any animal (Fig. 1). If only 0.025 ml was applied (experiment I), on the other hand, hypersensitivity of degree 0.8 was obtained.

The *microscopic* study of positive reactions revealed more or less pronounced acanthosis, cell infiltration and oedema in the epidermis, largely correlated to the macroscopic grading. In the case of strong reactions, corresponding microscopic features were also seen in the area outside the macroscopically visible reaction.

In the biopsies from the macroscopically negative reactions there were no or only slight lesions. A closer analysis was made of the 14 negative reactions to the single application of 6×0.025 ml DNCB in experiment II (day 1). No difference was observed in the quantity of infiltrated mononuclear cells in the test areas compared with the areas outside them. The infiltration of cells was very slight and localized

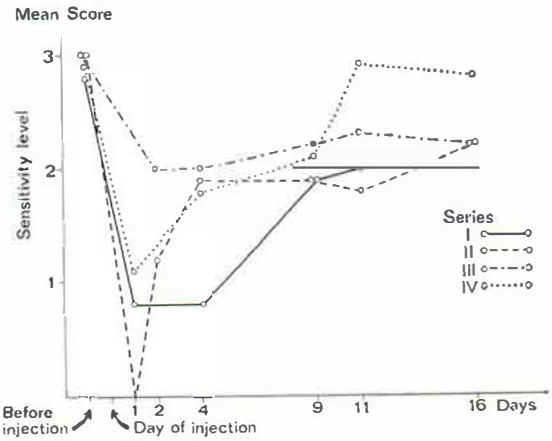

Fig. 1. Result of epicutaneous test with DNCB at various times after DNCB SO_3 injection. See Table I.

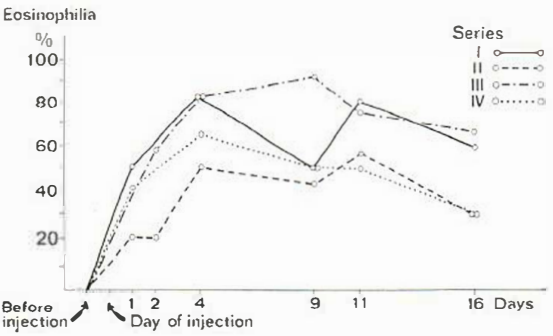

Fig. 2. Occurrence of eosinophilia at various times after DNCB SO_3 injection. See Table II.

to the dermis. In three of these negative reactions there were eosinophilic granulocytes in the test area, and in one reaction outside it. Mild acanthosis and slight oedema in the epidermis occurred in six experiments in the test area and in two of them outside as well. Vasodilatation was seen both inside and outside the test areas.

Eosinophilia was more or less pronounced in all experimental series, especially in the dermis. Table II shows the distribution in the experimental series and the number of experiments in which the quantity of eosinophils clearly exceeded the number appearing in the control experiments. Most experiments with eosinophil infiltration were in series III and the smallest number in series II. Eosinophils occurred throughout the experimental period. They appeared immediately after the DNCB SO_3 injection (experiments I, II, IV) and were most in evidence on the 4th–11th day after the injection. In some experiments a more pronounced eosinophilia was seen, usually in the epidermis, at the same time (Table II and Fig. 2). This was observed especially in series III (Fig. 3).

Control experiments. No reactions occurred and no definite microscopic lesions were observed in the 6 normal guinea pigs injected with DNCB SO_3.

The 9 DNCB-sensitized guinea pigs tested solely with 0.025 ml of 0.5% DNCB exhibited microscopical oedema and acanthosis in the epidermis and mononuclear cell infiltration in the dermis. Eosinophils were never observed in the epidermis; in the dermis an occasional eosinophil appeared in each specimen.

DISCUSSION

Our results, like those of earlier investigators, show that intravenous injection of DNCB SO_3 in DNCB-hypersensitive animals induces a weakening of the DNCB hypersensitivity. The original degree of hypersensitivity had not been attained during the 16-day experimental period. We also found that if a larger quantity of DNCB (6×0.025 ml) was applied simultaneously with the DNCB SO_3 injection, the animals became totally unresponsive to this test dose, in contradistinction to the use of 0.025 ml DNCB alone, when 5 of 12 animals reacted. In earlier studies it has not appeared that the quantity of epicutaneously applied DNCB is of any signif-

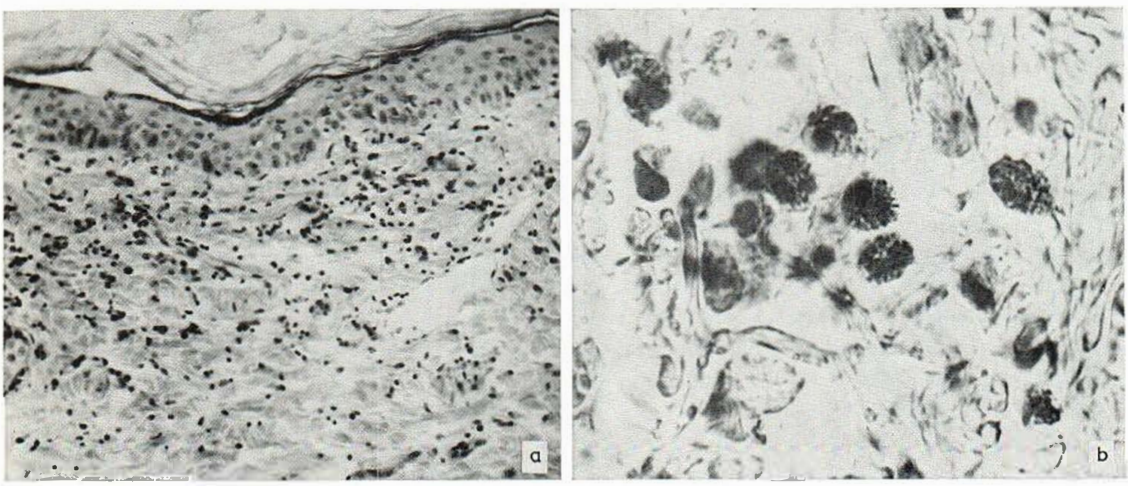

Fig. 3. (*a*) Series III. Test with 0.025 ml DNCB on day 4. Day 5: grade 1 reaction. Microscopically slight oedema in epidermis, moderate infiltration of mononuclear cells and fairly abundant eosinophils diffusely in dermis. Hematoxylin-eosin, × 150. (*b*) Eosinophils in dermis. × 700.

icance for the production of total unresponsiveness. The condition disappeared quickly, however, and did not become permanent as in corresponding tests with potassium dichromate (5) or Neoarsphenamine (2). It has been discussed (3), whether the size of the i.v. dose of DNCB SO_3 is significant for the production of total and, especially, long-lasting unresponsiveness. We used a dose which was one-third of that of Frey et al. (3). If the amount of the i.v. injection is of importance, the epicutaneously applied quantity is equally important. The difference between the Chromium, Neoarsphenamine and DNCB systems is difficult to understand. May differences in the degree of hypersensitivity between the different systems have a significance, i.e. is hypersensitivity to DNCB considerably more pronounced than to the other systems? Another explanation may be that different elimination rates of the substances (8) result in different durations in the organism, and thus in differences of inactivation of specifically immunologically active cells.

According to Polák & Turk (6) the simultaneous epicutaneous administration will have a significance, since conjugates are then formed which eliminate the remaining immunologically active cells which are sensitive only to conjugates between the sensitizer and the skin. This is reflected in a microscopically observable inflammation at the site of application, without visible macroscopic lesions. Polák & Turk (6) recorded a marked infiltration with mononuclear cells, slight infiltration with polymorphonuclear cells, and marked vasodilatation of the superficial capillaries of the dermis. In experiment II we compared the microscopic picture of negative reaction with an area immediately outside it. The lesions described by Polák & Turk (6) were recognized but only slight cell infiltration and vasodilatation were observed, and to roughly equal extent where the sensitizer had been applied and outside this area. Our histological studies, therefore, do not fully support the theory of the significance of the conjugate for the development of tolerance. The significance of the epicutaneous application may lie solely in the fact that a depot of antigen is formed there, which takes charge of the remaining immunologically active cells.

The eosinophilia earlier reported by us (7) was recorded in the present series too. Eosinophilic leucocytes were most numerous where tolerogenic effect was least pronounced (experiment III, Fig. 2), i.e. it was here that the most pronounced DNCB reactions occurred. This accords with what we have observed earlier (7), that the eosinophils were most numerous where the epidermis was most affected. There were eosinophils also in the other test series, and likewise in the macroscopically negative test areas in series II (day 1). It is therefore difficult to evaluate the significance of eosinophilia. The most likely explanation is that several types of immunological reaction have developed, the positive reaction being a type IV reaction—according to Gell & Coombs (4)—and the eosinophilia and the generalized dermatitis being expressions of a type I or type III reaction. This would in such case have its maximum on the 4th–11th day after the DNCB SO_3 injection, as it was then that we observed the largest number of eosinophils.

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