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Interference of simultaneous skin tests in delayed hypersensitivity

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Summary. The interference of two simultaneous skin test reactions of intermediate strength has been studied in the guinea-pig, using four different antigens, i.e. ovalbumin, horse cytochrome c, PPD and oxazolone.

Skin test reactions were evaluated at 4, 24 and 48 h by measuring three parameters: increase in skin thickness, diameter of erythema and intensity of erythema.

When an Arthus reaction was elicited simultaneously with a delayed hypersensitivity (DH) reaction, no effect on the DH reaction was observed. When two simultaneous DH reactions were elicited with different antigens, the risk of interference appeared to be rather small. When, however, the same antigen was used for both skin tests, suppression of at least one parameter of a DH-reaction was found in almost all experiments. Suppression of one skin test by another one could not be reduced by introducing a larger distance between the two skin tests.

As complete inhibition of either of the parameters never occurred, multiple skin testing may allow one to obtain a qualitative impression of the state of delayed hypersensitivity; when, however, reliable quantitative data are needed, the performance of more than one skin test at a time should be avoided.

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INTRODUCTION

Skin testing represents the most commonly used *in vivo* method to evaluate the presence of delayed hypersensitivity (DH) in both clinical and experimental immunology. With respect to contact allergens, the performance and reading of skin tests in guinea-pigs have been studied extensively in the past (Magnusson & Kligman, 1970; Turk, 1975). The evaluation of skin test reactions to intradermally injected protein antigens has recently been analysed by Scheper, Noble, Parker & Turk (1977). Their observations emphasize the importance of measuring all three main parameters, i.e. increase in skin thickness, intensity and diameter of erythema, at several times after skin testing.

Confusion however still exists about whether it is permitted to perform more than one skin test simultaneously in the same animal. The number of skin tests commonly performed at one time in an animal appears to be determined by the site of skin testing and the available surface area. It may vary from one in mice (Robinson & Naysmith, 1976) to over twenty in man (Malten, Nater & Van Ketel, 1976). As guinea-pigs are very appropriate for the demonstration of different types of hypersensitivity states and the available surface area for skin testing is relatively large, one is easily tempted to do several tests at one time in these animals.

Suppression of tuberculin skin reactivity by a second tuberculin skin test has been reported both

in man (Thestrup-Pedersen, 1975) and guinea-pigs (Baer & Kolb, 1967). Using guinea-pigs, Jokipii & Jokipii (1973) found suppression of delayed reactions to both PPD and ovalbumin, when a second strong reaction to ovalbumin was induced simultaneously. In these studies however only one or two parameters of the skin reaction were measured at one time (24 or 48 h) after skin testing.

We decided to study the interference of simultaneous skin tests in delayed hypersensitivity in more detail by measuring all three parameters at several times after skin testing. Furthermore we investigated a possible relationship between the interference and the distance between simultaneous skin tests. Four different antigens were used in this study, i.e. the contact allergen oxazolone, the protein antigen ovalbumin and two protein antigens, which are known to elicit relatively pure DH-reactions, i.e. horse cytochrome c (Reichlin & Turk, 1974) and PPD. The interference of an Arthus reaction on simultaneously induced DH-reactions to these antigens was studied using a reverse passive Arthus reaction to ovalbumin.

MATERIALS AND METHODS

Animals

Outbred albino female guinea-pigs (TNO, Holland), weighing 300–400 g were used.

Antigens

Horse cytochrome c (HCy) was purchased from Serva (Heidelberg, Germany), 4-ethoxymethylene-2-phenyl oxazolone (OXA) from BDH (Poole, England), Freund's complete adjuvant $H_{37}Ra$ (FCA) from Difco (Detroit, U.S.A.), purified protein derivative (PPD) from R.I.V., Holland and ovalbumin (OVA), grade V from Sigma (St Louis, U.S.A.).

Immunization

HCy was dissolved in physiological saline, emulsified with an equal volume of FCA, and 0·1 ml was injected into each footpad to give a total dose of 500 μg HCy per animal. At the same time these animals were contact-sensitized with OXA, by painting each ear with 100 μl of a 4% OXA solution in ethanol.

Some groups of guinea-pigs received a booster with 0·2 ml FCA subcutaneously into the neck, 3 weeks after the first immunization. In a few experiments guinea-pigs were sensitized with 10 μg OVA in FCA.

Skin testing

Unless otherwise stated, all guinea-pigs were skin tested on the caudal side of the right flank 10–12 days after immunization. Some groups of guinea-pigs received an extra skin test more cranially on the same flank (at a distance of 5 cm) or just opposite this latter site on the left flank. The skin reactivity to protein antigens was assessed by intradermal injection of 1–100 μg protein in 0·1 ml of saline into the shaved flank; contact skin reactions to OXA were elicited by epicutaneous application of 25 μl of a 4% OXA solution in ethanol. Reverse passive Arthus reactions were performed as described by Scheper *et al.* (1977), by intravenous injection of 5 mg OVA, followed by an intradermal injection of a high-titred guinea-pig anti-OVA antiserum after 30 min.

Skin tests were read after 4, 24 and 48 h; with respect to protein antigens three parameters were measured: (1) intensity of erythema: numerical values 0–3 (Turk & Polak, 1968); (2) diameter of erythema: in mm; (3) increase in double skin thickness: in mm $\times 10^{-1}$.

Contact reactions were evaluated by scoring the intensity of erythema as described above.

Statistics

Groups of at least five animals, tested on the same day were compared, using the Wilcoxon test.

RESULTS

The effect of a very strong skin test reaction on a weaker one

At the beginning of this study a few pilot experiments were done in which we tried to confirm the findings of Jokipii & Jokipii (1973). At 4, 24 and 48 h after intradermal injection of 1 μg of ovalbumin three skin test parameters were compared with the same parameters in another group of guinea-pigs in which an extra skin test with 100 μg ovalbumin was performed simultaneously on the same flank (Table 1). A significant inhibition of the delayed reaction up to 60% was seen both in diameter of erythema and induration.

Two more pilot experiments were done to assess the effect of a strong skin test reaction on a weaker one using different antigens. It was found that a

Table 1. The effect of a strong skin test reaction on a weaker one

Extra‡ skin test→Skin test (doses in μg)	Extra skin test reaction 24 h			Skin test reaction without/with extra skin test††								
				4 h			24 h			48 h		
	ery§	diam¶	ind**	ery	diam	ind	ery	diam	ind	ery	diam	ind
OVA→OVA*												
100 1	1·1	26	24	0·2/0·1	—	5/5	0·6/0·4	24/13	18/3	0·3/0·2	16/10	13/4
PPD →OVA*												
20 1	1·4	17	21	0·2/0·2	—	5/3	0·6/0·5	24/22	18/15	0·3/0·5	16/13	13/14
PPD →HCy†												
20 20	1·8	20	29	0·1/0·1	—	8/5	0·7/0·5	16/13	12/6	0·6/0·6	15/13	11/5

* Immunized with OVA in FCA 3 weeks before sking testing.
† Immunized with HCy in FCA, boosted after 3 weeks with FCA, and skin tested after another 3 weeks.
‡ The extra skin test was located on the same flank.
§ Intensity of erythema.
¶ Diameter of erythema (mm).
** Increase in double skin thickness ($\times 10^{-1}$ mm).
†† Each value represents the mean reactivity calculated from groups of at least five guinea-pigs. Significant differences (Wilcoxon $P < 0{\cdot}05$) between test and control groups are underlined.

strong PPD-reaction did not significantly alter a skin test reaction to ovalbumin, whereas a DH-reaction to HCy was severely suppressed by a concomitant strong PPD-reaction.

It was concluded from these experiments that strong skin test reactions might indeed interfere with weaker ones, and we decided to study a possible interference of DH-reactions of intermediate strength.

The interference of two DH-reactions of intermediate strength

To evaluate more systematically the risk of suppression (or potentiation) of delayed hypersensitivity reactions by an extra skin test reaction, we used three different antigens, i.e. the contact allergen oxazolone and two protein antigens, PPD and horse cytochrome c, which are known to induce a virtually pure cell mediated response. The time course of

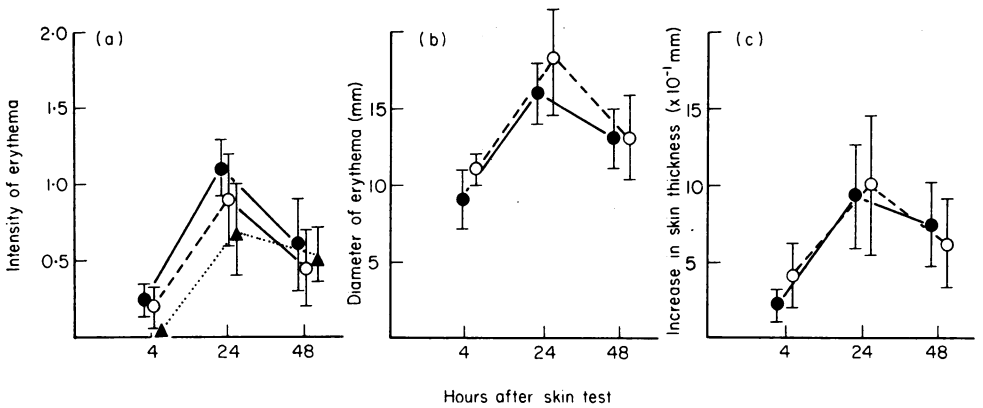

Figure 1. Skin test reactivity as measured by intensity (a) and diameter (b) of erythema, and increase in double skin thickness (c) 10 days after immunization with HCy in FCA, and oxazolone. Groups of at least five guinea-pigs were skin tested with either 5 μg PPD (●), 20 μg HCy (○) or 1000 μg oxazolone (▲). Vertical bars show standard deviations for each point.

Table 2. The interference of two DH-reactions elicited by the same antigen

Extra* skin test→skin test (doses in μg)		Extra skin test reaction 24 h			Skin test reaction† without/with extra skin test					
					24 h			48 h		
		ery†	diam	ind	ery	diam	ind	ery	diam	ind
PPD →PPD										
20	5	1·1	18	14	1·1/0·9	15/15	8/4	0·4/0·4	—	6/4
20	5	1·0	18	16	1·1/0·9	19/16	12/6	0·9/0·9	15/13	11/7
20	1	0·9	20	17	0·7/0·6	14/11	8/4	0·6/0·4	9/9	8/3
HCy →HCy										
100	20	1·0	17	12	0·9/1·0	20/15	9/5	0·4/0·4	15/9	6/4
100	5	1·2	21	13	0·9/0·6	17/10	6/3	0·2/0·1	—	4/1
OXA→OXA										
1000	1000	0·8			0·7/0·3			0·4/0·2		
1000	1000	0·8			0·6/0·6			0·6/0·3		

* The extra skin test was located on the same flank.
† For details see Table 1.

three parameters of the skin test reactions to commonly used test doses of antigen (5 μg PPD, 20 μg HCy, 1000 μg OXA) is given in Fig. 1. All three antigens elicit a prominent DH-reaction, although both protein antigens show some Arthus-reactivity. The discrepancy between this finding and the results of Reichlin & Turk (1974), who did not detect any antibody-mediated activity upon HCy-sensitization, is probably due to different sources of the antigen. In our hands sensitization with HCy (Sigma) and HCy (Serva) resulted at day eleven in haemagglutination titres of 1 : 1 and 1 : 4 respectively (unpublished results). Extra skin tests were elicited with 20 μg PPD, 100 μg HCy and 1000 μg OXA.

Using the same antigens for two skin tests of intermediate strength on the same flank, we ob-

Table 3. The interference of two DH-reactions elicited by different antigens

Extra* skin test→Skin test (doses in μg)		Extra skin test reaction 24 h			Skin test reaction† without/with extra skin test					
					24 h			48 h		
		ery†	diam	ind	ery	diam	ind	ery	diam	ind
HCy →PPD										
100	5	1·1	21	13	0·9/1·0	13/14	8/7	0·8/0·5	11/11	6/6
OXA→PPD										
1000	5	1·0			1·4/1·1	18/17	9/7	1·1/0·7	13/12	6/5
PPD →HCy										
20	20	0·8	14	10	0·9/0·6	20/16	16/8	0·5/0·2		8/6
20	20	1·2	17	9	1·1/1·2	17/16	9/9	0·4/0·6	10/10	8/5
OXA→HCy										
1000	20	0·7			1·5/1·4	26/22	13/14	0·6/0·4	22/11	8/6
PPD →OXA										
20	1000	1·3	17	11	1·0/0·7			0·6/0·3		
20	1000	0·9	15	7	0·7/0·7			0·5/0·5		
HCy →OXA										
100	1000	1·4	22	14	0·5/0·7			0·5/0·5		

* The extra skin test was located on the same flank.
† For details see Table 1.

served suppression of a DH-reaction in almost all experiments (Table 2). The suppression of skin test reactivity at both 24 and 48 h was most pronounced for the diameter of erythema and the increase in skin thickness. The intensity of erythema was found to be significantly suppressed in one experiment on the interference between contact skin reactions.

When we used different antigens for two skin tests of intermediate strength on the same flank, an inhibition of a DH-reaction did occur occasionally (Table 3).

A contact hypersensitivity reaction to oxazolone suppressed the erythema of a simultaneous PPD reaction, but failed to suppress a HCy reaction. A positive PPD skin test reaction significantly inhibited a concomitant stronger DH-reaction to HCy. This inhibition could not be reproduced in a second experiment. HCy reactions did not exert any effect on simultaneous skin test reactions to either PPD or OXA. Furthermore contact hypersensitivity to OXA was not affected by an extra skin test reaction elicited by PPD or HCy.

Significant effects could not be detected in the 4 h reactions in any of the experiments.

The relationship between the location of two skin tests and their interference

We thought it of importance to know whether contra-lateral skin test reactions would interfere to the same extent as skin tests located on the same flank. Therefore in five different experiments from Tables 1, 2 and 3 not only homo-lateral but also contra-lateral interference was assessed by including a third group of guinea-pigs with each skin test on a flank. In all of these experiments skin test reactions were similarly affected by simultaneous skin tests on either flank (Table 4).

The interference of Arthus reactions with DH skin reactions

A strong reverse passive Arthus reaction was induced by a high-titred anti-ovalbumin antiserum (4 h induration 2·6 mm). DH-reactions of intermediate strength were elicited in different groups of guinea-pigs with 5 μg PPD (24 h induration 1·0 mm), 20 μg HCy (24 h induration 0·6 mm) or 1000 μg OXA (24 h erythema 0·7). No significant effect of the Arthus reactions could be detected on any of the DH-reactions studied.

DISCUSSION

These experiments show that, as reported by Jokipii & Jokipii (1973), strong delayed hypersensitivity reactions can suppress each other. We found that

Table 4. The relationship between the location of two skin tests and their interference

Extra skin test→Skin test	Location	Skin test reaction§ without/with extra skin test					
		24 h			48 h		
		ery§	diam	ind	ery	diam	ind
OVA→OVA*	homo-lateral	0·6/0·4	24/13	18/3	0·3/0·2	16/10	13/4
	contra-lateral	0·6/0·4	24/14	18/4	0·3/0·2	—	13/4
PPD →HCy*	homo-lateral	0·7/0·5	16/13	12/6	0·6/0·6	15/13	11/5
	contra-lateral	0·6/0·9	18/13	13/8	0·6/0·8	17/14	13/11
PPD →PPD†	homo-lateral	1·1/0·9	15/15	8/4	0·4/0·4	—	6/4
	contra-lateral	1·1/1·2	15/15	8/5	0·4/0·4	—	6/5
OXA→PPD‡	homo-lateral	1·4/1·1	18/17	9/7	1·1/0·7	13/12	6/5
	contra-lateral	1·4/1·1	18/17	9/6	1·1/0·9	13/10	6/4
PPD →HCy‡	homo-lateral	0·9/0·6	20/16	16/8	0·5/0·2	—	8/6
	contra-lateral	0·9/0·8	20/14	16/9	0·5/0·3	14/11	8/5

* See also Table 1.
† See also Table 2.
‡ See also Table 3.
§ For details see Table 1.

suppression of DH-reactions was marked even by simultaneously induced DH-reactions of intermediate strength. Complete suppression, however, was never observed, which is probably because we avoided introducing the extreme differences in antigen doses for skin testing as used by Jokipii & Jokipii (1973).

Suppression was detectable in most cases at both 24 and 48 h, and was revealed particularly by induration and diameter of erythema measurements. The fact that in fewer cases significant suppression of the intensity of erythema was observed, may be related to the fact that these measurements tend to give larger standard deviations. On the other hand, our present results clearly show that suppression of one parameter of a skin test reaction may occur without any effect on other parameters. This conclusion confirms our earlier findings that the optimal parameter for detection of a certain effect is usually unpredictable (Scheper *et al.*, 1977).

Whereas from the work of Jokipii & Jokipii (1973) it was not clear if the interference between two skin test reactions was caused by a DH-reaction or an earlier occurring Arthus reaction, our experiments show that the latter reactivity does not significantly interfere with a simultaneously induced DH-reaction.

A remarkable difference was found between the interference of two DH-reactions to the same antigen and to different antigens. When we used one antigen for both skin tests almost all experiments showed suppression, while suppression could only be detected in a few experiments when we used different antigens. At this stage several mechanisms have to be taken into account as possibly causing antigen-specific suppression. It is unlikely that a direct effect of one skin test reaction on the other by an extravascular route is involved, as no difference was observed between the interference of two skin test reactions located on one or both flanks. Nor are antigen-antibody complexes needed for the occurrence of interference in our experiments, as FCA is known to be a very poor antibody inducer to PPD (Thestrup-Pedersen, 1975). Suppression of skin test reactivity by a simultaneously induced DH-reaction to the same antigen is most likely to be caused by intervention of antigen with antigen-specific T-effector cells, either locally or systemically following the drainage of antigen from the site of skin testing. Furthermore non-specific mechanisms may play a role, as have been suggested to account for the desensitization phenomenon (Poulter & Turk, 1976). Such mechanisms may vary from the systemic blockage of macrophage function by lymphokines released into the circulation, to immuno-suppressive mediators released upon induction of DH-reactions, e.g. alpha-2-globulins (Burger, Lilley & Vetto, 1974).

Although the theoretical basis of the phenomenon observed in this study is still unclear, our results do have practical consequences. When interpreting two simultaneous skin test reactions with different antigens in one animal, inhibition of each reaction by the other has to be seriously considered. Such inhibition is even very likely to occur when both skin test reactions are elicited by the same antigen. This probably also applies to skin tests made with different antigens containing similar antigenic determinants, e.g. conjugates with varying hapten-carrier ratios.

In conclusion, when *in vivo* data are needed concerning the state of delayed hypersensitivity to more than one antigen, one has to choose carefully between multiple skin testing with possible interference, the use of different groups of animals for each antigen to be tested, or to establish safe time intervals between each skin test to be made.

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