STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

IX. Skin Sensitization Induced by Injection of Conjugates

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(Received for publication, October 10, 1940)

In experimental work on sensitization with a group of simple chemical substances, strong evidence was obtained for the view that the production of hypersensitivity depended upon the formation of antigenic combinations in the body (1). As a corollary, the direct administration of *in vitro* conjugates in these cases should lead to skin sensitivity towards the corresponding low molecular substance. Yet in our previous attempts, the injection of protein compounds (even into the skin), analogous to older experiments with azoproteins in which an anaphylactic state was induced, was not satisfactory in eliciting skin sensitization. However, by employing a technique patterned upon the method used for inducing skin sensitivity by intraperitoneal injection of the simple substance (2), namely concomitant administration of killed tubercle bacilli, positive results were secured, as reported briefly (3).

The complex antigen used was a conjugate made from the erythrocyte stromata of guinea pigs, which were coupled with picryl chloride or 2,4-dinitrofluorobenzene.

A recent paper by Haxthausen (4) which concerns this subject is discussed below.

EXPERIMENTAL

Preparation of Stromata.—The stromata were prepared essentially by the method of Sachs (*cf.* 5): washed erythrocytes suspended in 3 volumes of saline were heated at 56–58°C. for 40 minutes and dialyzed against water overnight (in the case of guinea pig stromata 1 to 2 volumes of distilled water were first added). If crystals of guinea pig hemoglobin formed, these were centrifuged off. After isotonicity was restored by adding 10 per cent NaCl solution, the stromata were collected by sedimentation in an angle centrifuge and washed repeatedly with saline until the supernatant fluid was colorless; small amounts of a gummy sediment were discarded. The washed stromata were kept in the ice box in saline containing 0.25 per cent phenol. Quantities are given in terms of dry weight.

Preparation of Antigen.—1.0 gm. stromata of guinea pig erythrocytes was twice

sedimented, using an angle centrifuge, from 300 to 400 cc. portions of saline, once from a mixture of 4 parts saline solution and 1 part of alcohol, and then was suspended in 100 cc. of this fluid. To this was added a filtered clear solution of 50 mg. picryl chloride in 400 cc. of the above 20 per cent alcoholic saline, prepared by slowly adding 4 volumes of saline to a filtered dilute alcoholic solution of the substance (100 mg. in 160 cc. absolute alcohol). The coupling was conducted by adding 18 cc. N Na_2CO_3 and shaking the colored suspension for 10 minutes. To remove residual picryl chloride, if present, a large excess (2.5 gm.) of glycine was dissolved in the reaction mixture and the alkalinity readjusted with Na_2CO_3 solution to pH 8.5–9.0; the suspension was shaken occasionally during half an hour. The fluid was made neutral by addition of N HCl, and the stromata were collected by centrifugation. The coupled stromata were suspended in 400 cc. 20 per cent alcoholic saline, and another treatment with glycine and Na_2CO_3 given for 1 hour. N HCl was added to neutrality, the stromata were sedimented, and as a further precaution, probably unnecessary, the product was washed by sedimentation from 50 per cent aqueous alcohol and then treated at length with this solvent, *viz.*, continued extraction using six 300 to 400 cc. portions during 40 hours at room temperature. (The preparation employed in the experiment shown in Table I was given a final rapid washing with 70 per cent aqueous alcohol at this point.) The "picryl stromata" were then freed from alcohol by being twice sedimented from saline suspension, and the product was kept as a concentrated suspension in phenolized (0.25 per cent) saline in the ice box. The yield is about 700 mg.

In the case of "dinitrophenyl stromata" 1 gm. stromata in 100 cc. of 20 per cent alcoholic saline was treated at approximately pH 9.0 with a solution of 70 mg. 2,4-dinitrofluorobenzene (used in preference to dinitrochlorobenzene because of its greater reactivity) in 280 cc. of 20 per cent alcoholic saline, the latter prepared by adding 4 volumes of saline to an 0.025 per cent solution in alcohol; the procedure was otherwise as described above.

Stromata of other animals were treated in the same manner.

As antigens for testing anaphylaxis and cutaneous reactivity, conjugates of the simple substances with serum proteins were prepared as described previously (6); further modifications—treatment with alkali and glycine, repeated precipitation from alkaline solution by alcohol—were introduced when the serum conjugates were injected intraperitoneally in attempts to sensitize the skin.

Injection Procedures.—Male albino guinea pigs weighing 450 to 550 gm. were employed. Two preliminary injections of dead tubercle bacilli suspended in paraffin oil, described previously (2), were made intraperitoneally, one of 0.2 mg. tubercle bacilli in 0.2 cc. paraffin oil, then 72 hours later another like dose in 0.5 cc. paraffin oil. A few hours after the latter, 20 mg. stromata antigen in 2 cc. saline was injected intraperitoneally with measures to avoid the skin—entry of clean, dry needles directly through the belly muscles exposed by means of a short slit made in the skin on the posterior half of the flank (see (2) page 240)—and the administration of stromata was repeated on alternate days until 60 mg. had been given. The routine of the injections (quantities of paraffin oil, etc.) was adapted from previous experiments and does not necessarily represent optimal conditions.

Testing.—3 weeks after the last injection of the conjugated stromata, tests for hypersensitivity were made by spreading 1 drop of a 1 per cent solution of picryl chloride or

TABLE I

The first group of animals was injected with erythrocyte stromata conjugated with picryl chloride, and killed tubercle bacilli in paraffin oil (see text). The second group was given "picryl guinea pig stromata" only. 3 weeks after the last injection the animals were tested by spreading 1 drop of 1 per cent picryl chloride in olive oil on the belly. The reactions were read on the following day, after application of a depilatory; the observations at 48 hours are also given (within parentheses) when there was an increase over the earlier reading. Finally, within 3 days of the skin test application, the response to intracutaneous and intravenous injections of picryl guinea pig serum was ascertained, anaphylactic death with characteristic autopsy findings being shown by the symbol †. Amounts given refer to dry weight.

No.	Skin sensitivity to superficial application of picryl chloride	Intravenous injection of picryl guinea pig serum	
		Amount injected	Anaphylactic symptoms
		mg.	
Picryl guinea pig stromata and tubercle bacilli given intraperitoneally			
1	++++	5	† 3 min.
2	+++	5	† 3 "
3	+± (++++)	2	† 3½ "
4	++	2	† 4 "
5	+++ (++++)	1	† 2½ "
6	++++	1	† 4½ "
7	++++	½	† 3 "
8	++++	½	Very slight
9	+++	¼	Moderate to severe
10	++++	¼	None
11	+ (++)		
Picryl guinea pig stromata given intraperitoneally			
12	−	5	Severe
13	±	5	† 4 min.
14	±	2	† 4 "
15	+±	2	† 3 "
16	±	2	† 6 "
17	+±	1	† 5 "
18	−	1	† 2½ "
19	±		
Normal controls			
20		5	None
21	−		
22	− (tr.)		
23	−		
24	−		

After the skin testing with picryl chloride, four animals were injected intracutaneously on the flank with 0.1 mg. picryl guinea pig serum in 0.05 cc. saline; the reactions on the next day were as follows: No. 1, 44 × 44 mm., pale pink; No. 11, 31 × 25 mm., pinkish, slightly elevated, with dark pink center; No. 14, 21 × 20 mm., pale pink, slightly elevated; No. 19, 33 × 33 mm., pale pink, slightly elevated; control animals gave no reaction.

dinitrofluorobenzene in olive oil on the belly, and the reactions were read the following day, after use of a depilatory, and also at 48 hours.

The strength of the reactions is designated as follows: ++++, pink or bright pink, usually slightly elevated; +++, pink, but either somewhat pale or macular; ++, pale pink; +, faint pink; ±, pale or faint pink small spots; tr., trace; f. tr., faint trace; −, negative. For better comparison, the animals in an experiment were sorted from random assemblage into four classes (negative, up to high reactors) before recording the readings.

Other details have been given before (2).

While the procedures devised to eliminate free picryl chloride or dinitrofluorobenzene may seem too elaborate, they were deemed necessary on account of the possible activity of very small quantities of the compounds and because of the basic importance of the issue. Actually, as the precautions in the preparation of the antigen were increased during the work, the sensitization response was in no way impaired.

An experiment showing sensitization following injection of a conjugate made with picryl chloride and guinea pig stromata is shown in Table I: guinea pigs receiving injections of dead tubercle bacilli in paraffin oil became highly skin-sensitive towards picryl chloride, whereas the same treatment except for this adjuvant produced only a few instances of low grade sensitization. This outcome parallels the findings when free picryl chloride was given intraperitoneally (2). On the other hand, animals of both groups had acquired a high degree of anaphylactic sensitivity, dying in acute shock after the intravenous administration of "picryl guinea pig serum" or "picryl horse serum" (6). Furthermore, animals of both sorts gave to intracutaneous injections of these serum conjugates skin reactions which were fully developed on the next day; whether the degree of reactivity was equal was not established.

Other experiments with dead tubercle bacilli gave concordant results, ordinarily as good or nearly so as those presented in Table I, and it may be mentioned that highly sensitive animals react to lower concentrations of picryl chloride than the solution used as routine.

When in experiments on a smaller scale horse or rabbit stromata or guinea pig serum was used instead of guinea pig stromata, the outcome was still positive but only a few animals became highly sensitized. Picryl horse serum, in the single experiment tried, failed to induce skin sensitivity. The reason for the superiority of the homologous stromata is as yet unexplained. Other experiments with mixtures of picryl guinea pig stromata and alumina or tapioca, given subcutaneously, or charcoal, injected intraperitoneally, employed in place of killed tubercle bacilli did not engender sensitivity of the skin to contact with picryl chloride, although here again the anaphylactic state was induced.

Parallel experiments were carried out using guinea pig stromata coupled with 2,4-dinitrofluorobenzene (Table II). Sensitization could be clearly demonstrated by smearing with an oil solution of dinitrofluorobenzene, the reactions being much less pronounced upon testing with the less reactive compound dinitrochlorobenzene. The animals were anaphylactic, to a

TABLE II

Skin sensitization to 2,4-dinitrofluorobenzene elicited by a conjugate made from dinitrofluorobenzene and guinea pig erythrocyte stromata. Procedures are the same as used in Table I; 1 drop of 1 per cent dinitrofluorobenzene in olive oil was applied to the belly 3 weeks after the last injection and the reactions at 24 hours are tabulated. A conjugate made with serum was employed for skin testing and anaphylaxis, these tests being concluded within 3 days of the skin application of the simple substance.

Dinitrophenyl guinea pig stromata and tubercle bacilli given intraperitoneally			
No.	Skin sensitivity to superficial application of dinitrofluorobenzene	Intravenous injection of dinitrophenyl guinea pig serum	
		Amount injected	Anaphylactic symptoms
		mg.	
25	++±	5	† 3 min.
26	++	5	† 13 "
27	tr.	5	† 8 "
28	++++	2	Slight
29	+++	2	"
30	+++±		
31	f. tr.		
32	+++ (+++±)		
Normal controls			
33		5	None
34	tr.		
35	−		
36	−		

Following the testing with the simple substance, injections of 0.1 mg. dinitrophenyl guinea pig serum were made intracutaneously in three animals and the reactions read at 24 hours: No. 30, 23 × 23 mm., pale pink, elevated; No. 31, 24 × 20 mm., pink, elevated; No. 32, 21 × 21 mm., pale pink; control animals gave no reaction.

higher degree than in former experiments where sensitization was induced by 2,4-dinitrochlorobenzene, and gave, as well, reactions to the intracutaneous injection of a conjugate made with guinea pig serum.

The recent report of Haxthausen (4) mentioned above tends to show that human beings can be sensitized to 2,4-dinitrochlorobenzene by injection, also through extracutaneous routes, of the substance mixed with foreign or human serum, a result which the author believes is probably due to the effect of a conjugate. While this interpretation is possible, there remain

doubts whether sensitization was not produced by free dinitrochlorobenzene still present in the solution or in some cases, perhaps, by prior skin testing of the same individuals. Actually when we made mixtures according to the prescription given by the author, we could demonstrate the presence of free dinitrochlorobenzene in considerable yield.

Mixtures of 100 cc. freshly drawn horse serum and 5.25 cc. 1 per cent dinitrochlorobenzene in alcohol were left in a 20°C. chest for periods up to 7 days. The proteins were then precipitated by the addition of acetone, and from the filtrate and washings acetone was removed by evaporation *in vacuo*. The residual solution was extracted several times with ligroin, the filtered extracts being evaporated to dryness. Finally the deposit was extracted with a small amount of absolute alcohol, and the solution briefly warmed with a small amount of aniline. Upon evaporation of the alcohol in a desiccator, fine red needles appeared which were identified as dinitrophenylaniline by melting point, 153–155°C. The yield of dinitrochlorobenzene, after keeping the solution for 3 days, was about one-third of the quantity originally added, also in an experiment when the mixture had been kept for 7 days (32.3 per cent recovered).

COMMENT

Features which seem to separate skin sensitivity to simple substances from most other phenomena of hypersensitiveness are the failure to find circulating antibodies capable of sensitizing the skin passively and the fact that hypersensitivity has been achieved mainly by application to the dermis. In the latter respect, however, it has recently been found possible to obtain the effect using the intraperitoneal route (2). This result has been secured again in the present experiments, but this time the sensitizing agents were conjugates made *in vitro* containing none of the simple active substance. The observation is significant as regards the question of antibodies, showing that sensitivity of the contact dermatitis type can be produced with a material which is an antigen in the common sense and which ought to incite the formation of antibodies. The antigenic activity of the conjugates is clearly evidenced by the demonstration of the anaphylactic state, and it is difficult not to attribute the skin effect likewise to some sort of antibody formation. Theoretical considerations arising from the nature of chemical compounds of simple composition as inciting agents, in contrast to high molecular antigens, are, naturally, inadequate to explain the present experiments. That for positive results special conditions are or may be requisite opens new questions but does not alter the essential findings. It may be in place to recall once more the striking differences obtained in immunization with adjuvants, for instance in antitoxin production, and the alteration of antigenic responses when animals are subjected to treatment with tubercle

bacilli (Dienes (7); *cf.* (2)). A case, possibly pertinent, is the enhancement of sensitization to serum proteins found by Schultz and Swift (8) following intracutaneous injection of streptococci, this being referred to the establishment of a hyperreactivity of the reticulo-endothelial system.

When the stromata conjugate was injected alone, some slight sensitization effects were apparently seen. While it may be assumed that the use of tubercle bacilli merely enhances the antigenic response, this becomes debatable and a qualitative difference seems rather probable because the anaphylactic sensitization induced by the stromata complex was not found to be much different whether or not tubercle bacilli were injected.

A possible explanation of the results presented would be that the picryl conjugates, for instance, are split in the body with the formation of picric acid.[1] This is improbable because of the weak sensitizing capacity of picric acid (9) (likewise of dinitrophenol (10)) and because of the difference in the character of the skin reactions produced by picric acid (9) and picryl chloride in hypersensitive animals. A direct experiment showed, in fact, that the guinea pigs sensitized intraperitoneally by picryl stromata with the method described were not sensitive to picric acid and not (or perhaps slightly so in the case of a few individuals) to 1,3,5-trinitrobenzene. Further, animals treated intraperitoneally with picric acid and tubercle bacilli did not become sensitive to picryl chloride.

SUMMARY

Experiments with guinea pigs are described which show that under special experimental conditions the intraperitoneal injection of conjugates made with homologous erythrocyte stromata leads to typical skin sensitization of the contact type towards the respective simple chemicals, namely picryl chloride or 2,4-dinitrofluorobenzene. Therefore such sensitivity can be brought about not only by low molecular chemical compounds but by a material which must be regarded as a typical antigen.

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[1] Guinea pigs sensitized with picric acid (9) give cross reactions with picryl chloride and animals intensely treated on the skin with large quantities of picryl chloride showed some sensitivity against picric acid, perhaps by liberation of the latter substance in the skin.

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