

STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

VII. SKIN SENSITIZATION BY INTRAPERITONEAL INJECTIONS

BY K. LANDSTEINER, M.D., AND M. W. CHASE, PH.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

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From a survey of the literature it would appear that experimental skin sensitization with simple chemicals, unlike common anaphylactic sensitization, has been accomplished with certainty only by application of the incitant on or into the skin. Observations to this effect have been recorded by several authors. Simon *et al.* (1, 2) state that in the course of experiments on sensitizing guinea pigs to poison ivy they had "no results except with the direct application of the extract to the skin." The same point is made by Straus (3) who found only one out of a group of 10 infants injected subcutaneously with poison ivy extract to develop sensitivity, in contrast to the more than 70 per cent positive results following cutaneous application; he remarks that it cannot be excluded that the one case might have been due to contamination of the epidermis. Similarly with neosalvarsan administered to guinea pigs, where the sensitization is not of the contact dermatitis type, Sulzberger (4) failed to obtain skin hypersensitivity by the intramuscular, intraperitoneal, intratesticular, and intracardial routes, using a dosage effective when given intradermally. And in this laboratory preliminary experiments at sensitizing with intravenous or subcutaneous injections of 2:4 dinitrochlorobenzene were indecisive (5, 6). In fact, it is not infrequently held that skin sensitization is the result of a direct action on the epithelial cells, there inducing a specific change, and experiments which support this view have been performed by Straus and Coca (7; *cf.* 8) and more recently by Schreus and his coworkers (9, 10) (*vide* 11). There are some experiments, indeed, with apparently positive outcome after administration by non-cutaneous routes, *e.g.* with primula extract given intravenously (Bloch (12)) and with Japanese lacquer (*Rhus vernicifera*) by subcutaneous injection (Kobayashi (13)). The sensitivities so found were of lesser degree than those secured by dermal treatment, and were open to doubt since no mention is made in these reports of precautions against accidental contamination of the skin, a matter of im-

portance because substances like the active principle of rhus may sensitize when very small quantities come into contact with the epidermis;¹ also, the subcutaneous method by its nature can scarcely be expected to yield decisive results.

In experiments of our own with various substances we likewise have had occasional positive sensitizations but mostly not of satisfactory grade, and then in attempts to repeat the results the outcome would be entirely negative: consequently the significance of the infrequent effects was not clear.

For instance, in a lot of 8 animals which had twice received 1/5 mg. picryl chloride intraperitoneally, with methods designed to avoid skin contamination, one became weakly, one moderately sensitive. Taking now another 6 animals, not one became sensitive when injected in a similar manner; further, none of 4 animals twice given 1 mg. developed sensitiveness. (Again, later experiments, examples being given below, showed several positive results.) In contrast to this, intracutaneous administration of picryl chloride is almost regularly followed by pronounced hypersensitiveness (5, 14).

Then we have conducted experiments analogous to Sulzberger's mentioned above, using larger groups of animals and salvarsan instead of neosalvarsan because of the regular effects obtainable with the former compound (15). Guinea pigs were injected with 0.15 mg. salvarsan freshly dissolved in saline, without neutralizing, the volume being 0.1 cc. for intracutaneous, intratesticular, and intracranial routes, 0.4 cc. for intrajugular injection, and 0.2 cc. for the other series. In the extracutaneous injections special precautions were devised to avoid contact with the dermis, *e.g.*, entry of clean, empty needles through slits in the skin, etc. One month later, 0.15 mg. salvarsan in 0.1 cc. saline was injected intracutaneously into the skin of the belly. Readings were made on the day following, after use of a depilatory. All 14 animals injected in the skin had developed pronounced sensitivities; 2 out of 4 pigs injected subcutaneously appeared to be sensitive, one faintly, the other weakly so; of 8 animals receiving the substance intraperitoneally, one displayed a weak skin reaction and a second showed a trace of sensitivity; following intramuscular injection into 5 guinea pigs, one became moderately, another faintly, sensitive; on the other hand, of the 7 animals treated intravenously, or of the 5 given the substance intratesticularly, none had been sensitized. From these experiments it may be inferred that at least with salvarsan positive results by extracutaneous administration can be achieved, though very irregularly and of rather low degree. It may be remarked that some of the animals injected intraperitoneally or subcutaneously showed moderate or slight anaphylactic symptoms when reinjected intravenously with a mixture of guinea pig serum and neutralized salvarsan, a method giving fatal shock in a large percentage of the animals prepared by intracutaneous injection (15, 16).

In order to establish convincingly that the skin is not essential as the site for application it was desirable to have a method which would yield re-

¹ With 2:4 dinitrochlorobenzene we have seen that guinea pigs may become well sensitized after touching only a few sq. mm. of the skin with a concentrated acetone solution of the substance, a method used in human beings by Haxthausen (11).

producibile results of satisfactory degree. To this end we tried to make use of adjuvants which are known to promote immunization with common antigens; we refer to the improvement achieved in antitoxic immunization by adding substances producing inflammatory reactions such as bacterial materials, alumina, tapioca, etc. (17, 18). Some experiments with mixtures of 2:4 dinitrochlorobenzene and alumina, and of poison ivy extract with tapioca, injected intraperitoneally, were definitely promising, yet the effects were not regular enough. Finally a satisfactory method was devised, suggested by the experiences of Dienes (19, 20), who found that the response to protein antigens is qualitatively altered by injecting the antigen into tuberculous lesions. Moreover, a significant increase in the production of antibodies has been observed by Thompson (21), and by Lewis and Loomis (22, 23), with the help of tuberculin or of live or killed tubercle bacilli. In our experiments killed tubercle bacilli suspended in paraffin oil were used as synergistic agent, along with picryl chloride, a compound which, as has been demonstrated previously (14), is an active sensitizer, producing sensitivities both of the contact dermatitis and anaphylactic types. The method presented itself on account of the observation that high tuberculin sensitivity can readily be induced with the aid of killed tubercle bacilli suspended in oily menstrua, preferably paraffin oil (Dienes and Schoenheit (20), Saenz (25, 26), Freund *et al.* (27)). It was of course necessary to have a technique for administering the incitant so as to avoid any contact with the skin tissues.

EXPERIMENTAL

Injection Materials.—The suspension of killed tubercle bacilli in paraffin oil used in the first experiments was kindly supplied by Dr. Jules Freund; later, we prepared suspensions with the virulent human Jamaica No. 22 strain of *Mycobacterium tuberculosis*.² Pellicle growths cultivated at 37°C. on 5 per cent glycerine bouillon were harvested after 6 weeks and rinsed with saline; the mass growth was ground with saline to break up clumps and the suspension was then heated at 100°C. in the Arnold sterilizer for 30 minutes. The killed bacteria were sedimented by centrifugation, washed once with saline, and in thin layer dried for 48 hours to approximately constant weight *in vacuo* over P₂O₅. The dried cells were at once incorporated in sterile paraffin oil (Seybolt viscosity 175–180 seconds at 100°F.) by grinding in a mortar, the oil being added drop by drop, 1 cc. oil finally containing 1 mg. dry bacilli.

Picryl chloride (Eastman), twice recrystallized from a benzene-alcohol mixture, was injected in aqueous and paraffin oil media. Solutions having 0.2 mg. (or 0.4 mg.) in 1 cc. saline were prepared by adding in droplets to 20 cc. saline, kept swirling, 0.3 cc. absolute alcohol containing 4 mg. (or 8 mg.) of the substance: the solutions were rejected

² For this culture and details of the method of preparation we are indebted to Drs. E. L. Opie, J. Freund, and R. W. Huntington.

if at all turbid. Paraffin oil solutions were prepared by dissolving picryl chloride, with gentle heating, in the proportion of 20 mg. to 30 cc. solvent; this was diluted with paraffin oil or with the suspension of tubercle bacilli to give 0.5 mg. in 1 cc.

Injection Methods.—Because of uncertainty as to the slight effects upon extracutaneous administration, mentioned above, procedures were devised for injecting the materials intraperitoneally so that contamination of the skin was well nigh excluded. One, used with sensitization procedure A, consisted of making a short slit in the skin of the posterior half of the flank and passing slantwise through the muscle an empty, fine gauge needle fitted to a syringe containing enough material to fill the needle and discharge the intended dose; the other precautions taken were the same as in the more detailed description following. In introducing paraffin oil solutions of picryl chloride (procedure B, below), safety was insured even further by making the injection through the thick tissues of the back: on the left half of the dorsum in the lumbar region and over the musculus sacrospinalis the hair was clipped and a disc of skin, 5 to 8 mm. in diameter, was removed along with the underlying panniculus carnosus, the animal being prone and under ether anesthesia. The syringe was filled to contain 0.2 cc. more than the required amount of injection material, residual solution carefully wiped from the tip and a dry, gauge No. 22 needle, 2 inches long, attached. The needle, which remained unfilled, was inserted directly into the exposed muscle, thrust posterolaterally for about a centimeter to increase the intramuscular passage, then downwards into the peritoneal cavity; the final position of the needle could be ascertained by palpation. After discharge of the injection material, the body of the guinea pig was shielded, and the needle was withdrawn slowly with a twisting movement, so as to wipe the shaft, the plunger meanwhile being kept slightly retracted. The base of the defect was at once blotted with gauze, and the defect and adjacent skin sponged in turn several times with ligroin, finally with alcohol, drying with gauze after each application. Thymol iodide was then applied to the area.

Further, special control experiments were made to put to a deliberate test the effects of brief contact with the skin, and the cleaning methods employed: one drop of the picryl chloride-paraffin oil-tubercle bacilli injection suspension was allowed to fall onto fresh skin defects prepared as for injection, followed after a few minutes by washing with organic solvents as described.

Sensitization.—Male albino guinea pigs weighing 450 to 520 gm. were used. The treatment with tubercle bacilli naturally produced peritoneal lesions and had the effect of keeping down the ordinary increase in weight (in certain series there was some loss in weight during the experiment). The effectiveness of the two following methods was on the whole about the same.

Procedure A: A primary intraperitoneal injection of 0.1 to 0.25 mg. tubercle bacilli in paraffin oil was given, and 2 days later 1/5 mg. picryl chloride dissolved in 1 cc. (or 0.5 cc.) saline. On the next day, there was made a second similar injection of tubercle bacilli, followed after another 48 hours by a terminal intraperitoneal injection of 1/5 mg. picryl chloride in 1 cc. (or 0.5 cc.) saline.

Procedure B: An intraperitoneal injection of 0.2 or 0.25 mg. tubercle bacilli in 0.2 cc. paraffin oil was given, and, on the 3rd day after, 0.2 or 0.25 mg. tubercle bacilli in 1 cc. paraffin oil containing 0.5 mg. picryl chloride, was administered intraperitoneally. In control series, groups of animals were treated as above, commonly using first paraffin oil and then picryl chloride in paraffin oil, but without tubercle bacilli, and other lots were

given corresponding volumes of paraffin oil with the given dose of tubercle bacilli but omitting picryl chloride.

Testing.—A period of 3 weeks was allowed after the administration of picryl chloride. The guinea pigs were tested for hypersensitiveness by gently spreading 1 drop of a 1.5 per cent solution of picryl chloride in olive oil on the clipped abdomen over an area of 8 to 10 sq. cm.; the same treatment was used on animals which had received like intraperitoneal treatments with killed tubercle bacilli only, and on normal guinea pigs. The reactions were read on the following day, after use of a depilatory 2 or 3 hours before. Caution should be exercised in the clipping and in application of the depilatory in order to avoid any false reactions. (Incidentally, it should be mentioned that the animals given tubercle bacilli acquired strong tuberculin reactivity as described by the authors quoted.)

The intensity of the reactions is represented by the symbols: + + + +, 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 direct comparison, the animals in an experiment were sorted from random assemblage into four primary classes (negative, up to high reactors) before recording the readings.

A number of experiments were carried out, of which those presented in Tables I and II are examples.

The results furnish definite proof of the possibility of sensitizing animals with a simple chemical compound given in a manner which excludes contamination of the skin. The various experiments were consistent as regards frequency and strength of the sensitizations. In all, with the two procedures using picryl chloride and tubercle bacilli, 76 animals were treated in 11 groups and of these 60 became sensitized, the great majority showing a high degree of skin hypersensitiveness. In some of the experiments (as, Table II*a*) the scarcity of moderate or weak sensitivities was striking: the animals either became well sensitized or failed to develop any sensitivity. The reactions in the guinea pigs treated intraperitoneally were like those observed in animals sensitized by intracutaneous injections, namely sharply delimited pink, at times elevated, areas on the skin following test applications of the incitant, in the former group the color being sometimes even brighter and commonly persisting longer, not seldom with a slight increase on the 2nd day. (A yellowish pink color on the test site was seen in some of the highly sensitive animals, and occasionally the yellow color preceded the typical reaction.) The concentration of the test solution (1.5 per cent) was chosen as one which gave no reactions, or at most insignificant ones in normal controls. Yet the best sensitized animals reacted to a test solution of 0.1 or 0.02 per cent, and even lower.

Guinea pigs which had been treated with the suspension of killed tubercle

bacilli alone were repeatedly included among the controls because of the reports of increased non-specific reactivity of tuberculous animals, but the tests showed that these pigs (19 in all) did not react differently from normal animals. Another sort of control experiment designed to show that acci-

TABLE I

Synergistic effect of killed tubercle bacilli in paraffin oil on the development of skin sensitivity to picryl chloride by intraperitoneal injections of the latter (procedure A, see text). Group A received intraperitoneally 0.1 cc. paraffin oil containing 0.1 mg. tubercle bacilli, preparation 1, followed after 48 hours by intraperitoneal injection of 0.2 mg. picryl chloride in 0.5 cc. saline; the same amount of tubercle bacilli was reinjected on the day following, and the picryl chloride again after a lapse of 48 hours. Concurrently with group A, group B was given the 2 injections of picryl chloride in saline, and group C received the two injections of tubercle bacilli only. 3 weeks after the last injection of the incitant the animals were tested by spreading 1 drop of 1.5 per cent picryl chloride in olive oil on the belly. The reactions were read the next day after application of a depilatory, and also one day later.

Tubercle bacilli and picryl chloride given intraperitoneally (Group A)			Picryl chloride in saline given intraperitoneally (Group B)		
No.	First reading	Second reading	No.	First reading	Second reading
1	++±	+++±	9	—	—
2	++++	++++	10	+	tr.
3	+±	±	11	tr.	—
4	+±	+++	12	±	+
5	++	++++	13	+	+±
6	—	—	14	++	++
7	++	+	15	—	—
8	±	+±±	16	—	—
Tubercle bacilli given intraperitoneally (Group C)			Normal controls (Group D)		
17	—	—	21	tr.	—
18	—	—	22	tr.	—
19	±	tr.	23	tr.	—
20	tr.	—	24	—	tr.

dental contact with the skin at the time of the injection could introduce no error consisted of intentional contamination (see page 240) followed by cleaning in the usual manner. Of 16 such animals, none became sensitive. Some positive results were obtained after injection of paraffin oil solutions of picryl chloride alone (*e.g.* Table II*a*, group B). Likewise solutions in saline have yielded a number of positive sensitizations; the most striking example encountered is that shown in Table II*a*, group C. Despite the

TABLE II a

The synergistic effect of tubercle bacilli on skin sensitization by intraperitoneal injection of picryl chloride (procedure B, see text). Group A received intraperitoneally 0.2 cc. paraffin oil containing 0.2 mg. tubercle bacilli, preparation 2, followed after 72 hours by an intraperitoneal injection of 1 cc. paraffin oil containing 0.5 mg. picryl chloride and 0.2 mg. tubercle bacilli. Group B was given 0.2 cc. paraffin oil intraperitoneally, and 72 hours later 0.5 mg. picryl chloride in 1 cc. paraffin oil. Group C received one intraperitoneal injection of 0.5 mg. picryl chloride in 2.5 cc. saline. The testing was as described in Table I.

In Table II b are shown results from another experiment with animals treated as in groups C and B respectively.

Tubercle bacilli and picryl chloride in paraffin oil given intraperitoneally (Group A)			Picryl chloride in paraffin oil given intraperitoneally (Group B)		
No.	First reading	Second reading	No.	First reading	Second reading
25	++++	++++	35	±	±
26	++++	++++	36	++++	+++
27	++	++++	37	—	f.tr.
28	++++	++++	38	tr.	tr.
29	++++	++++	39	—	—
30	—	—	40	f.tr.	tr.
31	++	+++	41	—	—
32	+++	++++	42	—	—
33	tr.	tr.	43	tr.	tr.
34	++++	++++			
Picryl chloride in saline given intraperitoneally (Group C)			Normal controls (Group D)		
44	±	±	53	tr.	±
45	++++	+++	54	—	tr.
46	tr.	tr.	55	—	—
47	±	±	56	tr.	tr.
48	—	—	57	±	±
49	+++	++	58	—	—
50	—	—			
51	±	++			
52	++++	+++			

TABLE II b

Picryl chloride in saline given intraperitoneally			Picryl chloride in paraffin oil given intraperitoneally		
59	±	tr.	68	+	+
60	+	+++	69	±	tr.
61	—	±	70	±	±
62	+	±	71	f.tr.	tr.
63	—	—	72	tr.	f.tr.
64	++++	++++	73	tr.	—
65	f.tr.	tr.	74	tr.	—
66	f.tr.	—	75	+	—
67	±	tr.	76	+	—

unexplained irregularity of these occurrences, one may conclude that extracutaneous administration of the substance without adjuvant can be effective in some animals.

The question whether the oily medium used has a subsidiary influence along with tubercle bacilli, which may be surmised, has not yet been investigated by employing the bacteria in aqueous suspension. Indeed, as regards the rôle of the paraffin oil in tuberculin sensitization of rabbits with killed tubercle bacilli, Freund and Casals (28) have found that the quantity of paraffin oil injected influences the degree of sensitivity, the amount of tubercle bacilli being kept constant; and Saenz (25), using guinea pigs, reports that paraffin oil is superior to olive oil in tuberculin sensitization by means of killed bacilli. In any event, the outcome of our experiments emphasizes the rôle of the tubercle bacilli in markedly increasing the sensitizing effect of the chemical.

The phenomenon of Dienes—the production of a tuberculin-type sensitivity to egg white induced by injecting the protein into tuberculous foci—could not be reproduced, he found, when other inflammatory agents were substituted for tubercle bacilli. So far we also have failed to duplicate the results obtained with picryl chloride by means of the paraffin oil-tubercle bacilli method when the substances commonly used as adjuvants in immunization were employed: this naturally does not preclude the possibility that such a method could still be developed. The procedures outlined here might be improved upon, for instance by varying such conditions as quantities and intervals, a somewhat laborious task. Furthermore, study of the possibility of sensitizing the skin by extracutaneous administration of other sensitizing chemicals is indicated; to judge from our experiences the methods may well differ for individual compounds.

The mechanism whereby skin sensitivity is brought about with the procedure described is not clear: to ascertain definitely the reasons for the especial influence of tuberculous lesions in improving antigenic effects (22, 23), in modifying the response to antigens (19, 20), and in enhancing sensitization by a simple chemical in the manner we have seen, still leaves room for future work. In a general way, more rapid mobilization and modified activity of macrophages under the influence of tubercle bacilli—there is even greater phagocytic capacity for inert particulate matter (Lurie (24))—have been taken to be responsible for the effects mentioned (*cf.* 21). Also, in the present instance, the peculiarity of the tissue reactions to tubercle bacilli and their influence on antigenic response come first to mind, rather than supposition of the formation of a conjugate having special antigenic properties. An analysis of the cell reactions taking place would merit investigation. Apart from the theoretical implications, the

results secured suffice to show that an effect which had seemed to point to a special function of the skin can be obtained in an entirely different manner than by direct treatment of the dermis.

SUMMARY

A method has been described by which sensitization to a simple chemical, picryl chloride (2:4:6 trinitrochlorobenzene), can be satisfactorily attained by means of intraperitoneal injection of the compound when killed tubercle bacilli suspended in paraffin oil were used as adjuvant. Sensitivity of the contact dermatitis type results therefrom. It follows that although skin sensitization of this type is most easily obtained by dermal application this route of administration is no necessary condition for such sensitivity.

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