THE RÔLE OF THE "WAX" OF THE TUBERCLE BACILLUS IN ESTABLISHING DELAYED HYPERSENSITIVITY

I. Hypersensitivity to a Simple Chemical Substance, Picryl Chloride*

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One highly puzzling aspect of the phenomenon of the delayed hypersensitivity of infection, namely, the necessity for the presence of the entire organism in the tissues in order that the hypersensitive state be induced, has been dealt with in previous publications in relation to tuberculosis (1, 2). Although it is well known in the case of the tubercle bacillus that the protein of the organism is the antigenic component responsible for the hypersensitive state, this substance in isolated form is powerless to bring about the typical hypersensitivity which follows either infection or the injection of killed bacterial cells, and this despite the well established antigenic properties of such protein. It was demonstrated that the protein could become effective if at the same time the animal received another component of the bacillus; *i.e.*, the waxy lipid extractable with chloroform (3). A series of criteria established that the hypersensitive state so induced fulfills in all particulars that which follows on the heels of tuberculous infection.

In work of this nature, a point of confusion may arise from the failure to distinguish—in the mind of the investigator as well as in the inductive procedures employed—the nature of delayed hypersensitivity and the basic differences segregating it from hypersensitivity of the immediate type, including anaphylaxis, Arthus reactivity, and the human "atopic" states. Briefly stated, the differences are these:

1. Immediate hypersensitive reactions follow exposure to antigen within seconds or minutes. Delayed reactions require hours and progress relatively slowly.

2. In immediate hypersensitivity a demonstrable humoral antibody is involved, since this will transfer the state to normal recipients. In delayed reactivity no antibody has ever been demonstrated; the only successful attempts to transfer the state have been those employing *cells* obtained, for example, from an induced peritoneal exudate (4–8).

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3. In immediate hypersensitivity certain types of tissue, preeminently smooth muscle and vascular endothelium, are susceptible to the effects of antigen-antibody union. This susceptibility is revealed by contraction in the first instance, and by increased vascular permeability or even damage extending to thrombosis and rupture of vessels in the second. In delayed hypersensitivity all varieties of cells become directly susceptible to the action of antigen, presumably because of their content of some immunologically induced change analogous to antibody, although the nature of this change has never been directly revealed. Such cells may undergo damage extending to necrosis on contact with antigen even when growing in culture in a medium containing no antibody.

As a consequence of the observations on tuberculous hypersensitivity, thoughts of a more general nature suggested themselves, and with one we are concerned here. If a particular lipid of the tubercle bacillus can exert its effect with an antigen—the tuberculoprotein—from the same organism, it might also cause a similar effect with other, non-related antigens. The present work comprised such a study with a simple antigenic substance, picryl chloride.

Picryl chloride was chosen because it serves as a good example of a simple chemical hapten capable of spontaneous combination with protein and conversion to complete antigenic form after injection into the tissues (9–11). In addition, Landsteiner and Chase (12, 13), Gell and coworkers (11), and Chase (14) have provided an extensive immunological characterization of this substance, including the serological and hypersensitive manifestations to it under different conditions. The immunological facts established have been these: that picryl chloride is antigenic when administered by any parenteral route, inducing antibodies and anaphylactic (immediate) hypersensitivity. If, however, it gains entrance into the body via the *skin*, either by applications to the surface or by intracutaneous injection, there is established in addition a state of delayed reactivity to the substance so that future application to the skin results in a typical contact dermatitis, developing slowly and with no resemblance to an Arthus phenomenon. This state has been shown to be independent of the simultaneous existence of humoral antibodies and anaphylactic reactivity, for Landsteiner and Chase (12) have desensitized to the latter and demonstrated that the delayed contact reactivity remains. It is essential for the induction of the contact reactivity that the drug gain access to the body by way of the skin.

Now this description of events under ordinary conditions of sensitization with picryl chloride becomes altered when special circumstances are introduced. Thus, Landsteiner and Chase (15) showed several years ago that if killed tubercle bacilli were injected along with picryl chloride in an oil menstruum *intraperitoneally*, guinea pigs developed delayed reactivity to the chemical. This work was in fact a specialized extension of earlier observations concerning

a change in the type of hypersensitive response following injection of various antigenic substances into tuberculous lesions (Dienes and Schoenheit (16–19), Hanks (20)), as well as lesions induced with killed tubercle bacilli (19, 21). Later similar observations were made with killed bacilli mixed with antigens (19, 21–26).

A summary of these various observations and their mutual relationships is in essence as follows:—

(*a*) The isolated protein antigen of the tubercle bacillus cannot induce tuberculin hypersensitivity as can the entire organism.

(*b*) The wax component of the bacillus when administered with the protein causes the delayed tuberculin response.

(*c*) Picryl chloride induces delayed hypersensitivity only when administered through the skin, but if tubercle bacilli are injected along with picryl chloride by another route (intraperitoneally) a delayed hypersensitive responsiveness to the chemical ensues.

From these facts the consequence appears probable that the wax of the tubercle bacillus may determine the delayed type of response to picryl chloride equally as well as it determines this kind of response to tuberculoprotein. Such a demonstration may provide the beginning of a chemical basis for generalization concerning the factors determining the occurrence of delayed hypersensitivity.

EXPERIMENTAL

Picryl chloride (Eastman Kodak Company) was purified by several recrystallizations from a benzene-alcohol mixture. Picrylated serum antigen was prepared from guinea pig serum by the method of Landsteiner and Chase (12). Purified wax of the tubercle bacillus was obtained from H37Rv organisms, employing the chemical procedures of Anderson (3). The repeated ultrafiltrations and ultracentrifugations carried out in order to free the lipid of residual bacillary bodies are described in detail in a preceding report (2).

Preparations of Picryl Chloride for Injections.—When injections were made in saline as the vehicle, a solution of 15 mg. of picryl chloride per ml. in absolute alcohol was added drop by drop to the saline to a final concentration of 0.5 mg. per ml.

For intracutaneous injections, 0.10 ml. of a solution containing 0.0025 mg. of picryl chloride was employed.

For subcutaneous injections, 0.4 ml. containing 0.20 mg. of picryl chloride was used.

For intraperitoneal injections, 1.0 ml. containing 0.50 mg. of picryl chloride was used.

Picryl chloride in water-in-oil emulsion was prepared by the method of Freund (22). To 0.4 ml. of melted aquaphor, 0.4 ml. of warm saline was added drop by drop, mixing continuously with a pestle. To this mixture was added 4.0 mg. of solid picryl chloride. Then 1.2 ml. of warm paraffin oil was mixed in and the whole thoroughly emulsified in a mortar. The intraperitoneal dose consisted of 0.25 ml. of emulsion containing 0.5 mg. of picryl chloride.

For skin applications, a 1.5 per cent solution of picryl chloride in olive oil was employed. One drop was applied to the shaved skin and spread over an area of about 2.5 cm. with a glass rod. Each application thus consisted of about 1.0 mg. of the chemical.

The intraperitoneal injection of picryl chloride and wax was carried out as follows:—5 mg. of wax in emulsion in 0.5 ml. of distilled water was first injected. Following this, an injection was made of 0.5 mg. of picryl chloride in 1.0 ml. of saline.

In all injections made beyond the integument, precautions were observed to guard against contamination of the skin by the picryl chloride (15). A 1.0 cm. slit was made in the skin of the flank. The skin edges were retracted, and the picryl chloride preparation was introduced subcutaneously or intraperitoneally through a clean 26 gauge needle. After withdrawal, the wound was blotted with petroleum ether followed by alcohol, and 10 per cent thymol iodide in paraffin oil was applied.

Cutaneous Tests.—Contact tests were carried out by application to the shaved skin of the flank or the abdomen of the same 1.5 per cent solution of picryl chloride in olive oil used for the sensitizing inunctions.

Intracutaneous testing consisted of the injection of 0.05 mg. of picryl chloride in 0.1 ml. of saline. This substance induced some irritation, but not sufficient to interfere with readings. Later, picryl serum was employed for this purpose with avoidance of much of the local toxicity.

RESULTS

1. Induction of Delayed Cutaneous Hypersensitivity to Picryl Chloride by Concomitant Use of the Wax of the Tubercle Bacillus.—Groups of guinea pigs have been treated with picryl chloride under various circumstances in order to establish a comparative basis for assessing the rôle of the wax of the tubercle bacillus in modifying the allergic response.

In order to reproduce the observation that inunction or injection of the chemical into the skin may eventuate in delayed contact hypersensitivity, the following groups were run:

Group 1.—Picryl chloride in olive oil applied to the skin.

Group 2.—Picryl chloride in saline injected intradermally.

In addition, in order to confirm the observation that administration of the substance by non-dermal routes will not result in contact hypersensitivity, two other groups of guinea pigs were employed:

Group 3.—Picryl chloride in saline subcutaneously.

Group 4.—Picryl chloride in saline intraperitoneally.

Further, it was desirable to determine whether an immunologic adjuvant might be capable of causing the drug to induce delayed hypersensitivity following non-dermal administration:

Group 5.—Picryl chloride in water-in-oil emulsion intraperitoneally.

The last group served to test the ability of the purified wax of the human tubercle bacillus to modify the response to picryl chloride in the direction of delayed contact hypersensitivity:

Group 6.—Picryl chloride plus tubercle bacillary wax intraperitoneally.

In the latter four groups of animals where a non-dermal injection route was employed, precautions to avoid skin contamination by the chemical were followed, as described earlier.

The results of subsequent contact and intradermal tests in these six groups of animals were entirely unequivocal. Representative data are shown in Table I. Among the first five groups indications of very moderate hypersensitivity to contact and intradermal application of picryl chloride were seen only in groups 1 and 2, those which had been treated by the dermal route and for prolonged periods of time. Only three of fourteen animals responded; the

TABLE I

Delayed Contact and Intracutaneous Responses to Picryl Chloride in Guinea Pigs Sensitized by Various Methods

Group	Sensitizing treatment	No. of animals	No. of sensitizing treatments	Time after last treatment	No. of animals reactive	Average results of skin tests			
						Contact*		Intracutaneous‡	
						24 hrs.	48 hrs.	24 hrs.	48 hrs.
				days					
1	PCl applied to skin	8	31–38	10	2	0.15 +	0.04 +	11.8 1.5	9.3 1.2
2	PCl intracutaneously	6	26	10	1	0.10 ±	0	13.8 1.6	10.8 1.4
3	PCl subcutaneously	5	4	13	0	0	0	10.6 1.3	8.2 1.0
4	PCl intraperitoneally	5	4	17	0	0	0	11.5 1.5	9.5 1.0
5	PCl + water-in-oil intraperitoneally	8	2–4	17	0	0	0	10.6 1.0	8.6 1.0
6	PCl + T.B. wax intraperitoneally	15	1	13	13	2.5 4+	2.0 4+	21.7 2.1	18.0 1.8
	Controls	12	0	0	0	0	0	9.7 1.4	8.0 1.2

All groups except 6 received additional picryl chloride through periodic skin testing by application and intracutaneous injection. Thus, even groups 3 and 4 received some dermal stimulus (four contact and three intracutaneous tests) before the results recorded above were observed. This amount of skin application did not suffice to induce cutaneous reactivity.

* Contact test readings: The first figure indicates the thickness in millimeters of the indurated skin above the level of surrounding normal skin. The second symbol indicates degree of erythema, 4+ being maximum.

‡ Intracutaneous test readings: The first figure is average diameter, the second estimated height of the reaction, both in millimeters.

reactions were weak and irregular, but discernible.[1] In groups 3, 4, and 5, in which subcutaneous and intraperitoneal administration had been employed,

[1] These results have been much more moderate than those described in similarly treated guinea pigs by Landsteiner and Chase, and in communications from Dr. Chase. We can only infer a difference in guinea pig stocks. For our present purpose the very modest results of sensitization by the cutaneous route have by contrast emphasized the marked sensitivity established by the addition of wax with another avenue of injection.

in the last instance with admixture of an oily adjuvant, there was at no time any indication of the development of such responsiveness in a total of eighteen guinea pigs. In sharp contrast to these results it was found that thirteen of fifteen animals of group 6, after only one injection of picryl chloride with tubercle bacillary wax intraperitoneally, developed marked delayed sensitivity to subsequent contact or intradermal tests with the chemical. The contact sites were intensely erythematous and in most instances the skin was elevated by induration to 3 mm. Intracutaneous test sites showed the slow development and general characteristics of tuberculin reactions, and in six of the instances listed in the table these were accompanied by central necrosis. Two of these animals failed to react (these negative results are included in the averages given in the table), and one of these likewise failed after a second injection.

The figures listed in the table fail to convey adequately the differences among the groups described. Wax-treated animals 24 hours after testing could be recognized at a glance. These reactions are illustrated in color in Figs. 1 and 2.

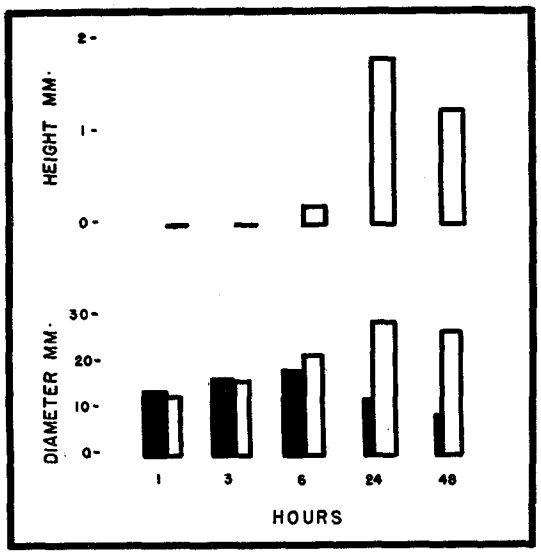
It was found that the level of reactivity of the wax-treated animals was not intensified by more injections, to a total of three. The hypersensitive state is persistent once established, for five reactive animals tested at 164 days after a single sensitizing injection showed contact and intracutaneous reactions of the same degree as those seen at 13 days after the sensitizing treatment.

2. Characterization of the Delayed Hypersensitivity Induced by Picryl Chloride Plus Wax.—The delayed contact reactivity established by the use of tubercle bacillary wax with picryl chloride is of high degree and appears to depend upon a specific qualitative effect of the wax rather than a simple adjuvant activity, as indicated by the failure of water-in-oil emulsion to cause the same responsiveness to the antigen.

It was of interest now to characterize the delayed hypersensitivity *per se* and also in relation to other immunologic responses of the animal, particularly antibody production and anaphylactic reactivity. The delayed hypersensitive reaction should bear no relationship either to humoral antibody occurrence or level, or to immediate hypersensitivity of an anaphylactic or Arthus nature.

(*a*) *Chronological Development of the Delayed Cutaneous Reaction.*—It should be said at the outset that we have never observed a cutaneous reaction of the Arthus type in any of the animals listed in Table I, even though repeated tests were made at intervals after succeeding injections or applications. These animals either showed delayed responses to contact and intracutaneous injection of the picryl chloride, or no response at all to either test. When the reactions were positive, they showed the chronological development characteristic of the tuberculin type of reaction. This is portrayed in Text-fig. 1, in which the upper row of blocks respresents the average development of contact responses in five guinea pigs sensitized by one intraperitoneal injection of picryl

chloride with wax 90 days earlier. At 6 hours, two of the five animals showed a faint beginning erythema and induration. Full development of the reaction was seen in all guinea pigs at 24 hours. The lower blocks depict simultaneous intracutaneous tests carried out on the opposite flanks of the same animals. Since picryl chloride is moderately irritating to normal tissues, the average reactions of three control animals similarly tested are shown also. In the wax-treated animals the intracutaneous responses parallel the contact tests



TEXT-FIG. 1. Timed readings of contact and intracutaneous tests with picryl chloride.

Upper. Contact tests in guinea pigs sensitized with picryl chloride plus tubercle bacillary wax. Height of block indicates thickness of induration in millimeters.

Lower. Intracutaneous tests in the same group of animals. Height of block represents diameter of reaction in millimeters, the width represents thickness of induration.

Black boxes indicate results seen in normal animals due to the irritative activity of picryl chloride.

in their development; at 6 hours there is hardly a significant indication of incipient reaction, while at 24 hours this is marked, at a time when the traumatic response in control animals has subsided. In both contact and intracutaneous responses there is seen to be some diminution at 48 hours. This was a rather regular but not invariable occurrence, and has been noted by us also in tuberculin tests in guinea pigs.

The absence of significant reactivity in the animals of the groups other than those treated with wax could be related to a failure of the picryl chloride to act as an effective antigen. That this was not the case is illustrated in Table II and discussed in the following section.

TABLE II

Correlation of Serological Responses with Delayed Cutaneous Reactivity to Picryl Chloride

Group	Sensitizing treatment	Guinea pig No.	No. of treatments	Time since last treatment	Complement fixation titer	24 hr. contact test
				days		
1	PCl applied to skin	C-60	31	10	0	0
		C-64	31	10	16	0
		C-65	31	10	16	0
		D-51	24	9	8	0
		D-55	24	10	0	0.3 ±
		D-58	24	9	1	0
2	PCl intracutaneously	E-86	20	15	16	0
		E-87	20	15	8	0
		E-91	20	15	16	0
3	PCl subcutaneously	C-66	3	14	4	0
		C-68	3	14	8	0
		C-70	3	14	0	0
		C-71	3	14	32	0
		C-73	3	14	16	0
4	PCl intraperitoneally	C-26	2	46	16	0
		C-27	3	14	4	0
		C-28	3	14	16	0
5	PCl + water-in-oil intraperitoneally	C-88	3	13	4	0
		C-58	3	13	16	0
		C-89	3	13	2	0
		C-90	3	13	16	0
		D-49	2	13	1	0
		D-53	2	13	0	0
		D-56	2	13	0	0
6	PCl + T.B. wax intraperitoneally	C-29	2	39	8	3.0 +
		C-33	3	14	64	1.5 +
		D-48	2	14	16	1.0 +
		D-68	1	26	4	2.5 +
		F- 5	1	14	1	2.5 +
		F- 6	1	14	1	1.0 ±
		F- 7	1	13	8	4.0 +
		F- 8	1	13	32	1.0 ±
		F- 9	1	13	8	0
		F-10	1	13	32	4.0 +
	Controls	N- 1	0		0	0
		N- 2	0		0	0
		N- 3	0		0	0
		N- 4	0		0	0
		N- 5	0		0	0
		N- 6	0		0	0

(*b*) *Lack of Relationship of Delayed Cutaneous Reactivity to Humoral Antibody.*—It is recognized that Arthus reactivity in its occurrence and intensity is directly related to the level of circulating antibody (27–31), while delayed hypersensitive responses are entirely independent of humoral antibody. It was therefore desirable to determine whether the cutaneous reactions described here could be dissociated from humoral antibody in the picryl chloride–wax-treated animals. In addition, it was necessary to know that animals in those groups without skin reactivity had, nevertheless, received an immunologic stimulus from the picryl chloride applied or injected. Sera from animals of all groups were titrated against picrylated serum by the complement fixation method. On the day previous to bleeding all animals received contact skin tests. The correlated results of these antibody and skin tests are shown in Table II. Most of the animals in all groups responded to picryl chloride with the elaboration of antibody, but at the time of these tests only the animals of group 6, sensitized with pircyl chloride plus wax, showed significant contact reactivity of the skin. Yet this group of guinea pigs did not produce significantly greater amounts of measurable antibody than did those which failed to develop skin reactivity. Furthermore, within this group there is no correlation of intensity of skin response with titer in any individual instance. It may be concluded, therefore, that the cutaneous reactivity induced by picryl chloride plus wax is independent of humoral antibody, that the failure of picryl chloride, in other combinations and by other routes, to induce such sensitivity was not due to a lack of antigenic activity of this substance, and finally that the wax, whatever its effect may be, does not act as an immunologic adjuvant in intensifying immunologic responses as judged by antibody titers. The last conclusion was arrived at from experiments with tuberculoprotein antigen (2) and with egg albumin (32) also.

(*c*) *Lack of Relationship of Delayed Cutaneous Reactivity to Anaphylactic Reactivity.*—In order further to demonstrate that the reactivity observed in the picryl chloride–wax-treated animals is delayed in character, an experiment was carried out to dissociate this reactivity from anaphylaxis. Landsteiner and Chase (12) have shown that guinea pigs with simultaneous reactivities can be desensitized more readily to the anaphylactic type, so that the delayed cutaneous form remains intact.

A desensitization experiment was carried out as follows:—

Guinea pigs of all groups were skin tested by the contact and intradermal methods. Twenty-four hours later, part of each group received 0.25 ml. (containing 4.6 mg. dry residue weight) of picrylated serum subcutaneously in the dorsal nuchal area, a second injection 4 hours later into the dorsal lumbar area, a third 3 hours later into the sacral area, and a fourth the next morning in the dorsal thoracic region. The four injections, totaling 18.4 mg. of material, were completed in a period of 24 hours. Several hours after completion of the desensitizing injections all animals were again skin tested by the contact and intracutaneous methods. On the following day the 24 hour skin reactions were read and intravenous tests (saphenous

vein) for anaphylaxis were carried out by the injection of 1 ml. (18.5 mg.) of picrylated serum. The results are shown in Table III.

The experiment was carried out sufficiently long after the last sensitizing injections so that spontaneous loss of anaphylactic sensitivity had probably occurred in some of the animals. Previous observers (12) have indicated that anaphylactic sensitization to picryl chloride may diminish considerably by the 5th week after a sensitizing injection. Whether desensitization was spontaneous or induced by the procedure employed is of no consequence to the essential points which emerge from this experiment, however. Spontaneously or experimentally desensitized animals exhibited well developed cutaneous reactivity in the absence of any indication of anaphylactic responsiveness. Conversely undesensitized guinea pigs without cutaneous reactivity showed anaphylactic shock. The independence of the two types of hypersensitive response to the same antigen is thus evident.

(*d*) *Failure of Serum to Transfer the Delayed Cutaneous Reactivity Passively.*—The well known inability of serum to convey delayed hypersensitivity to normal recipient animals is one of the basic features distinguishing this from the hypersensitivities of the immediate type. Chase (14) has recently indicated the most favorable circumstances for the transfer of the immediate form of reactivity. In the present experiment the various factors were so arranged as to provide such circumstances.

Well nourished albino guinea pigs weighing between 350 and 400 gm. were employed as recipients. Into the skin of one flank of each animal 0.2 ml. of a test serum was injected. The same quantity of another test serum was injected in the opposite flank. Each serum was given to two guinea pigs. Thus, each animal received two different sera, and each serum was tested in two different animals. Forty-eight hours later all animals received 2 mg. of picryl chloride in 0.5 ml. of olive oil subcutaneously in the ventral abdominal region. Readings of the serum injection sites were made at 15 minutes and at 1, 2, 3, 6, and 24 hours after injection of antigen. The summarized results of these tests are shown in Table IV along with the cutaneous reactivities of the donor animals at the time of serum collection and the titers of these sera in complement fixation tests.

As has been mentioned earlier, no clear cut evidence of Arthus reactivity has been obtained in any of our groups of animals, and it is therefore not surprising that such reactivity was only an occasional occurrence in the transfer test guinea pigs. The figures set forth in the table indicate the infrequency of such reactions, and furthermore show that:

(*a*) All passive reactions occurred early; none persisted to 24 hours, a time when the delayed cutaneous reaction reaches its apex.

(*b*) The transfer of the immediate form of reactivity bears no relationship to the occurrence of delayed cutaneous reactivity in the donor animals, either with regard to groups or in individual cases.

(*c*) There was no relationship between the complement fixation titer of a

TABLE III

Dissociation of Delayed Cutaneous from Anaphylactic Hypersensitivity by Desensitization

Group	Guinea pig No.	No. of injections or applications	Time since last treatment	Contact test* before desensitization	Contact test* after desensitization	Anaphylactic‡ reaction
Desensitized						
			days			
6. PCl plus wax intraperitoneally	F- 7	1	162	1.0 +	2.0 +	0
" " " "	F- 8	1	162	0.5 +	0.3 +	0
" " " "	F-10	1	162	3.0 +	2.0 +	0
2. PCl intracutaneously	E-87	26	82	0.5 +	0.3 ±	0
" "	E-91	26	82	0	0	0
5. PCl in water-oil intraperitoneally	C-58	4	89	0	0	0
PCl in water-oil intraperitoneally	C-88	4	89	0	0	0
PCl in water-oil intraperitoneally	C-90	4	89	0	0	0
Not desensitized						
6. PCl plus wax intraperitoneally	F- 5	1	162	2.0 +	2.0 +	4+
2. PCl intracutaneously	E-90	26	82	0.3 +	0.5 ±	4+
1. PCl on skin	E-62	25	82	0 ±	0 ±	0
" " "	E-63	25	82	0 ±	0 ±	0
" " "	D-58	31	82	0	0	0
" " "	E-61	25	82	0	0	0
4. PCl intraperitoneally	C-28	4	89	0	0	3+
5. PCl in water-oil intraperitoneally	D-49	4	89	0	0	4+
PCl in water-oil intraperitoneally	D-53	3	89	0	0	0
2. PCl intracutaneously	E-93	26	82	0	0	2+
3. PCl subcutaneously	C-73	4	89	0	0	0
6. PCl plus wax intraperitoneally	F- 6	1	162	0	0	0
" " " "	F- 9	1	162	0	0	0
Normal controls						
	N-11§	—	—	0	0	0
	N-12§	—	—	0	0	0
	N-13§	—	—	0	0	0
	C-14	—	—	0	0	0
	B-85	—	—	0	0	0

* 24 hour readings.

‡ 4+ indicates acute anaphylactic death.

§ These normal animals were subjected to the desensitization procedure.

serum and the transfer of immediate hypersensitivity in any individual case.

The central point is the failure of animals with well developed delayed cutaneous reactivity to picryl chloride to transfer this passively *via* serum.

3. Conditions Governing the Activity of Tubercle Bacillary Wax in Its "Directive" Rôle in Hypersensitivity.—It would be of much interest to define the circumstances under which wax may act in the capacity described, and the mechanism of its activity. Dienes in his earlier observations on the injection of

TABLE IV

Passive Transfer Tests

Group	No. of guinea pigs in group	No. of injections or applications	Time since last treatment	Positive skin reactions at time of bleeding	Positive complement-fixing antibodies	Positive transfer sites at*			
						1 hr.	3 hrs.	6 hrs.	24 hrs.
			days						
1 PCl applied to skin	15	18–31	10–15	0/15	13/15	5/30	3/30	2/30	0/30
2 PCl intracutaneously	3	20	15	0/3	3/3	0/6	1/6	0/6	0/6
3 PCl subcutaneously	3	3	14	0/3	2/3	0/6	0/6	0/6	0/6
4 PCl intraperitoneally	3	3	14	0/3	3/3	0/6	0/6	0/6	0/6
5 PCl in water-oil intraperitoneally	7	3	13	0/7	5/7	2/14	2/14	1/14	0/14
6 PCl plus wax intraperitoneally	9	1–3	14	8/9	9/9	1/18	1/18	2/18	0/18
Normal animals	3	—	—	0/3	0/3	0/6	0/6	0/6	0/6

* Numerator indicates positive reactions, denominator total tests (two tests for each serum).

various antigenic substances into tuberculous guinea pigs (18) found that administration of the antigens directly into foci of infection was most favorable if not essential for the development of altered hypersensitive reactions. It seems dubious that the tuberculous cellular response in such areas was the essential determinant, since Hanks (20) found that if infection were initiated in the testicle, egg albumin injected into the area within 18 hours, and the testicle removed 6 to 12 hours later, altered reactivity to the antigen eventuated.

In the work reported here, picryl chloride and wax were injected into the peritoneal cavity in succession, so that these were in intimate contact within the limits permitted by a cavity of this surface area, and by the volumes of fluid injected (wax in 0.5 ml., picryl chloride in 1.0 ml.). We have thus far

made only tentative attempts to delimit the conditions under which the rôle of wax can be exercised, and so far as these go they indicate that this substance and antigen must be in rather close proximity in the tissues in order for the effect to become apparent. Preliminary experiments in this connection are the following:

(*a*) Six guinea pigs were treated by four daily applications of picryl chloride in olive oil to the skin. The day following the last application, 5 mg. of wax was given intraperitoneally to each animal, and on subsequent days fourteen more applications of picryl chloride were made to the skin. After a pause of 10 weeks, a series of seven more applications was made. The results of this treatment were negative. This contrasts sharply with the result of the same single dose of wax along with picryl chloride, both into the peritoneal cavity.

(*b*) Six guinea pigs were treated by daily application of picryl chloride and wax in olive oil to the skin. The picryl chloride was present in a concentration of 15 mg. per ml., the wax 10 mg. per ml. The one drop employed for each application contained approximately 0.75 mg. of picryl chloride and 0.5 mg. of wax. After twenty-four daily inunctions, these animals showed in two instances very mild contact reactions, a result similar to that following treatment of the skin with picryl chloride alone. Since application of wax to the skin produced no visible response in this tissue, it might be inferred that a cellular response to this material is of some importance in determining the kind of hypersensitive reactivity set up in the body. On the other hand, the failure may be due to impermeability of guinea pig skin to the wax molecules.

(*c*) Six guinea pigs which had been infected with virulent human bacilli by the subcutaneous and intracutaneous routes in the left inguinal area 4 weeks earlier, and which showed only beginning dissemination of the disease as judged by autopsies of similarly infected animals, were treated by eight daily applications of picryl chloride to the skin. The last application resulted in the development of good contact responses in all animals. On retest 2 weeks later the responses were much diminished, but again unanimous. This suggests that the distribution of bacilli, and consequently of wax, more widely through the body as the result of generalized infection may serve as a conditioner for the development of altered reactivity to antigen no matter where it gains access to the tissues.

This experiment, as well as the early experiences of Dienes and others (16–21) with injections of antigens into tuberculous animals, leads to a consideration of "heteroallergic" phenomena in tuberculosis. Weissfeiler (33), Higginbotham (34), and others have observed the reactions of tuberculous guinea pigs to cutaneous injections of *E. coli*, *Staphylococcus aureus*, *Brucella suis*, diphtheroids, *Actinomyces*, and *Sarcinas*. Such responses appear to have the characteristics of the Koch phenomenon. Rich (35) has described a patient dying of staphylococcic septicemia; at autopsy, about the periphery of a fibro-

caseous tuberculous pulmonary lesion, a hemorrhagic reaction to the staphylococcus was found. It seems a possibility that such phenomena may be explicable not as "heteroallergic" responses on an immunologically non-specific basis, but as the result of the marked tendency of the wax portion of the tubercle bacillus to cause tissues to respond with tuberculin-type reactions to various antigenic substances. In the present paper this is exemplified by the use of picryl chloride.

DISCUSSION

Picryl chloride is a substance which, although antigenic to the animal body by any parenteral route, induces delayed hypersensitivity only when it gains entrance through the skin either by application or intracutaneous injection. Under these specialized conditions a moderate level of delayed contact reactivity develops in a proportion of guinea pigs treated.

Because in previous work (1, 2) we had observed that the wax fraction of the tubercle bacillus possesses the property of causing the animal body to respond to tuberculoprotein with typical tuberculin allergy, it seemed reasonable to propose that this lipid might have similar "directive" properties in altering the type of hypersensitivity to other antigenic substances. It is demonstrated in the present report that such an effect is markedly evident when picryl chloride is employed as antigen, injections of this and the wax being given by a route (intraperitoneal) which does not result in delayed hypersensitivity when the picryl chloride alone is employed. Responses in picryl chloride–wax-treated animals are much more regular and far more intense than those which follow sensitization with picryl chloride *via* the only "natural" route available, the skin. Previous observations of a similar effect of killed tubercle bacilli (15) in inducing this same altered hypersensitivity are thus referable to an isolated lipoidal constituent of the organism which, if injected with antigenic substances, possesses the biological property of causing delayed allergic responses to the antigens themselves.

The delayed nature of the reactivity induced in this manner is evidenced by the chronological and morphological character of the cutaneous response, its lack of relationship to humoral antibody level, its failure to be passively transferred to normal recipients by serum, and its independence of the anaphylactic state.

The specific activity of the wax is indicated, as in previous experiments on tuberculosis (2) and with egg albumin (32), by the failure of an ordinary immunologic adjuvant, represented by water-in-oil emulsion, to effect a similar change in hypersensitive responsiveness. Nor could other workers (13, 15) effect such a change by intraperitoneal injections of other adjuvants including alumina, tapioca, and charcoal, along with picryl chloride. Furthermore, the wax has given no evidence of being an immunologic adjuvant, since with three

antigens in our own experience it has in no instance caused increased antibody production over that seen in animals receiving the antigens alone.

The mechanism of activity of this bacterial wax is not known. The marked histologic response occasioned by the substance would seem to be unrelated to this activity, for similar cellular responses are occasioned by another lipid—the phosphatide—of the same organism, and this does not influence the hypersensitive response. Furthermore, as Rich (36) has pointed out, the bacterial type of delayed allergy occurs in diseases in which no lesion comparable to that of tuberculosis occurs at all.

So far as available evidence is concerned, it seems necessary that antigen and the lipid must be in rather intimate contact within the body in order for the alteration in hypersensitive response to occur. Observations derived from work with another antigen (32) indicate that several hours may intervene between injection of wax and antigen into the same site. It is of interest, however, that the tuberculous animal, in which the bacillus and its lipids are disseminated through the body, may provide conditions for the establishment of the marked delayed type of hypersensitivity as the result of applications of picryl chloride to the skin. It is pertinent to speculate whether in the human being with quantitatively sufficient infection there may be a tendency to development of delayed hypersensitivity to any antigenic substance which, in the uninfected individual, would cause a form of immediate allergic reactivity. Involved in this consideration also is the question of so called "heteroallergic" reactivity described in tuberculous animals (33, 34) and the human being (35), wherein bacteria unrelated to the tubercle bacillus may provoke reactions analogous to the Koch phenomenon. It is entirely conceivable that under the influence of the tubercle bacillary wax responses to the antigens of these bacteria would develop as forms of delayed hypersensitivity.

It may be questioned how the demonstrated activity of tubercle bacillary wax bears any relationship to the spontaneous occurrence of delayed contact reactivity to picryl chloride or other simple chemical substances. In the absence of the wax described, these must act through the skin in order to sensitize effectively. This special circumstance may have an analogy in the case of tuberculin reactivity, for here it is necessary that the entire bacillus be present in the tissues for sensitization to eventuate. A common factor may be involved in both of these cases, for we find that the same component of the tubercle bacillus which causes delayed hypersensitivity to isolated tuberculoprotein to become established also permits delayed hypersensitivity to picryl chloride without the intermediation of the skin. Perhaps then a lipid with activities similar to those of tubercle bacillary wax is present as a cellular component of skin. When released by injury (most substances inducing contact dermal hypersensitivity are primarily irritating) such lipids may function in the same manner as does the bacillary wax. This possibility is being investigated.

SUMMARY

The purified wax fraction of the tubercle bacillus, which has been previously demonstrated as an essential element in causing delayed tuberculin hypersensitivity in response to the protein of the tubercle bacillus, is now found to have the same activity with regard to a simple chemical antigen, picryl chloride. One injection of this compound with wax intraperitoneally into guinea pigs results in a marked delayed cutaneous hypersensitivity, demonstrable by contact and intracutaneous test, and of long duration. The effect is not related to an adjuvant activity of the wax as defined by ordinary standards.

The relationship of these observations to the occurrence of "heteroallergic" phenomena in tuberculosis is discussed.

The possibility that the occurrence of spontaneous contact hypersensitivities may depend upon the presence of similarly active lipoidal components of the skin is commented upon.

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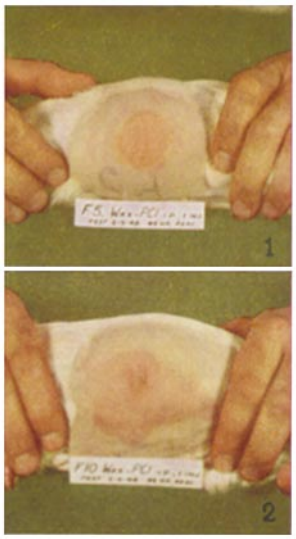
EXPLANATION OF PLATE 23

FIG. 1. Contact test 90 days following one injection of picryl chloride plus wax intraperitoneally. 48 hour reaction.

FIG. 2. Intracutaneous test under the same conditions as above. 48 hour reaction.



(Raffel and Forney: Tubercle bacillus wax and delayed hypersensitivity)