AN EXPERIMENTAL ANALYSIS OF BACTERIAL ALLERGY.

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Plates 29 and 30.

(Received for publication, July 15, 1926.)

I.

In the study of hypersusceptibilities a differentiation has been gradually developed between the phenomena concerned in true anaphylaxis to the coagulable proteins, in which it is generally assumed that a true antigen-antibody union upon the cells of the body underlies the responsible mechanism, and the many other manifestations of hypersusceptibility in which no such specific antigen-antibody relationship has been demonstrated. In the case of the bacterial hypersusceptibilities it is quite generally accepted that true protein anaphylaxis plays a relatively unimportant rôle in the occurrences of spontaneous infection, but there are other manifestations, spoken of as bacterial allergy, which are recognized both by systemic and local reactions, and which are of considerable importance diagnostically and perhaps pathologically and prognostically. The mechanism of these conditions has not so far been clearly understood.

In our own studies we have occupied ourselves for a number of years chiefly with the tuberculin reaction, since this phenomenon represents the most extreme example of bacterial allergy and is easily amenable to experimental study. Since, however, we encountered considerable experimental difficulties in elucidating the mechanism of the tuberculin reaction by direct attack, we were forced to extend our studies to experiments with many different bacteria, choosing, in addition to tubercle bacilli and their products, such organisms as the staphylococcus, streptococcus, typhoid bacillus, *Bacillus abortus bovis* and pneumococcus, thus including organisms of many degrees of solubility

and of far greater potency for free antibody production than *Bacillus tuberculosis*.

The premises from which the work reported in the present paper takes its departure, some of them elucidated by our associates and ourselves, many of them either suggested or independently worked out by others, may be briefly stated as follows:

1. The tuberculin reaction is independent of general anaphylaxis to tuberculoprotein. (This was quite clear from our early paper (1), which confirmed by other methods the suggestions made long before by Baldwin (2) and by Krause (3), and confirmed also by Selter (4) and by Bessau (5).)

2. Tuberculin hypersensitiveness in its typical and extreme form cannot be induced by dissolved extracts of the tubercle bacillus provided that these are filtered through Berkefeld filters to remove formed particles, but it can be characteristically induced not only by infection with the living bacilli, but by treatment with dead organisms, even when these are boiled (6). These facts confirm the importance of typical tissue reactions spoken of as tubercles in the mechanism of tuberculin sensitization.

3. The mechanism of tuberculin reactions is independent of the presence in the animal body of those precipitating or agglutinating antibodies which act either upon the whole bacilli or on the precipitable carbohydrate "residue" antigen *in vitro*.

4. Conversely, the substance in tuberculin which is responsible for reaction in the sensitized animal is chemically separable from the precipitable residue carbohydrate fraction which reacts with antibodies *in vitro*, and is probably a nitrogenous substance, perhaps a form of protein (Mueller (7); Laidlaw and Dudley (8)).

There are many other minor facts which we have emphasized in preceding papers and which might be included in the premises from which we have worked, but these have been discussed by us in other places and will appear, where important, in other parts of this communication.

The difficulties of working with bacterial allergy have been manifold, chiefly because the bacterial cell is chemically complex, often possessing primary toxicity which produces reactions that are not easily distinguished from allergy in the sensitized.

Also, there have been difficulties in the choice of the proper animals for experimental purposes in that, here even more than in protein anaphylaxis, fundamental differences exist in the mechanism by which different species of animals respond to sensitization. The primary toxicity of many of the bacterial materials, and the necessity of often working with suspensions rather than with true solutions, render unreliable intravenous injection, with constitutional allergic responses, and we have preferred to confine our studies chiefly to skin reactions. This is, of course, not absolutely dependable, since responses of the skin need not necessarily run parallel to general hypersusceptibility. But accumulating clinical evidence seems to indicate that such a parallelism is fairly close, and if we remember possible divergences under special circumstances, this method need not lead us into error.

In choosing the proper animals for the experiments, the primary purpose of elucidating bacterial allergies as they occur in man naturally inclines one to choose that animal in which skin allergy is apt to be similar to that occurring in man. We have worked with guinea pigs as fulfilling this purpose more closely than most other animals, combining with this great convenience of experiment. But it is important to remember that with allergy as with true anaphylaxis, things which are true of guinea pigs are often quite inapplicable to other animals, notably rabbits. Reactions such as delayed bacterial allergy and the typical tuberculin reaction follow in guinea pigs very much the same rules, as to time and appearance, which are observed in man, though probably with considerably less quantitative delicacy. In favour of rabbits, of course, is the fact that the Arthus phenomenon which cannot easily or typically be elicited in guinea pigs occurs in rabbits, and we believe that in man the hastened reaction occurring upon subcutaneous injection of diphtheria antitoxin and the often disturbing reactions following the later injections of rabies vaccine are closely analogous to the Arthus phenomenon. On the whole, however, we believe that observations made upon guinea pigs by intracutaneous reactions will be most directly useful; and after this, it may perhaps be of some value to "cross-index" these facts with other animals.

In the observation of skin reactions of guinea pigs, there are many pitfalls which have led to a good deal of confusion to us and, we believe, to others, and before describing the experimental results it will pay to consider these, since no adequate observation can be made without taking them into consideration.

In the first place, it is necessary to evaluate the primary toxicity of the bacterial materials worked with, since thereby allergic skin effect becomes merely comparative by reason of the inflammatory reactions produced by the substance on the normal animal. In the second place, there are variations in individual guinea pigs in their reaction to bacterial materials, sometimes because of pregnancy, at other times perhaps because of infectious processes previously sustained. The most serious source of error is a non-specific hypersusceptibility which is not always, but usually developed in guinea pigs 8 to 14 days after the injection of massive amounts of any foreign protein. Such injections seem to induce a non-specific change of reaction capacity which gives rise to moderate but confusing inflammatory reactions when bacterial or other substances are subsequently

injected. We believe that many erroneous interpretations have resulted from this. Moreover, we believe that in itself this non-specific irritability of the skin, whatever its mechanism, following large parenteral injections of protein material may have considerable significance in skin reactions in general. Incidentally, the recent papers of E. F. Müller (9), on the profound influence of any intracutaneous injection upon the involuntary nervous system and the capillary mechanism, furnish a clue to the explanation of these occurrences.

For all these reasons we feel that it is of great importance to define the different types of skin responses to the injection of bacterial substances, although these have often been looked upon merely as quantitative differences in the intensity of one and the same mechanism. The following types of reaction may occur:

1. The purely toxic reaction which has no direct connection with allergy, and which is due to the primary toxicity of the bacterial materials. Examples of this are the Schick and Dick tests which may be indistinguishable from true allergic reactions.

2. The immediate urticarial skin reaction which appears within a short time is chiefly characterized by edema with little inflammation and fades promptly. This is the reaction which, in man, may be regarded as indicating protein sensitization, and which plays a minor rôle in some of the phenomena of bacterial allergy in guinea pigs.

3. The delayed skin reaction which usually does not become manifest in less than 5 or 6 hours, reaches its highest development within 18 to 48 hours, is characterized not only by edema, but by inflammatory changes, and which, by slowness of complete fading, indicates that more has occurred than a simple edema. These reactions are usually well demarked, like an erysipelas, but do not go on to central necrosis or the central hemorrhages. This is the common type of reaction obtained both in man and guinea pigs with bacterial products and, as we shall see, sometimes occurs with protein sensitization.

The classical tuberculin and abortin reactions probably consist, in part, of reactions of the preceding class but, superadded upon these, there is tissue injury with hemorrhage and sometimes necrosis. Although these reactions may be regarded as quantitatively intensified examples of the preceding, the frequency of severe central cell injury and necrotic changes in relatively small reactions of this type, as contrasted with the absence of these even in large reactions of the pre-

ceding class, suggests that there is in addition a factor involved in the hemorrhagic-necrotic ones which is absent in the former.

This classification is a tentative one, of course, since much about the mechanism of skin reactions is not yet clear, and we submit it as a working basis.

II.

Specificity of Bacterial Allergy.

It is, of course, of the greatest importance to determine from the beginning the degree to which the bacterial allergic phenomena with which we are dealing are specific. Specificity, apart from being a biological phenomenon of the greatest practical importance, furnishes a definite clue to mechanism, and much of the clinical experience of those engaged in doing skin reactions upon patients, as well as investigations in animals, has left much uncertainty regarding this question.

The following experiments, carried out with tubercle bacilli and various strains of *Bacillus abortus bovis* kindly furnished us by Dr. Theobald Smith, serve to define these relations.

The animals listed below were sensitized with living *Bacillus abortus* and subsequently tested by intracutaneous inoculations with O.T. and abortin produced from Bacillus 1211 by the method used to produce O.T.

1.	Living		*Bacillus abortus bovis*		Feb.	17.
2.	"	1211	"	"	Apr.	6.
3.	"	1207	"	"	"	19.
4.	"	1202	"	"	"	19.

Tested May 18.

	O.T. 1–20	Abortin 1–5
1.	+	++++ (slight necrosis)
2.	+	++++
3.	+	+++
4.	±	++

Since the O.T. in these tests had been recently made and had not been tested for potency, and the mild reactions with O.T. obtained above indicated a possibility of an insufficient dosage, these tests were repeated on the following day with an old lot of O.T. which had proved highly potent in 1–10 dilutions on many guinea pig tests.

On May 20 the animals were then again tested with this O.T. 1–10 and the same abortin 1–5 with the following results:

	O.T.	Abortin
1.	−	Severe ++++ with central white spot
2.	+	+++
3.	++	Strong +++
4.	+	+++

In these experiments it would appear that the specificity is a fairly strict one. As allergy becomes more extreme, however, the overlapping becomes more marked, as the following protocol of another set of animals done at a time when necrotic centres and hemorrhagic reactions were more violent illustrates.

	Abortin	O.T.
1. *B. abortus*-sensitized	+++	±
2. " " "	+	−
3. " " "	++++	+++
4. " " "	++	+++
5. Tuberculin-sensitized	±	+++
6. " "	−	++

In general it may be stated that *abortus* animals must remain in fairly good condition and resist infection without too rapid an emaciation. Otherwise they remain negative in the same way as a tuberculous animal in the prelethal stages. Given favourable conditions, however, they become positive about 8 days after infection.

The following experiment is added because it illustrates the same relative specificity with *Bacillus abortus* and staphylococcus animals, and further indicates the interesting fact which we consider of some importance, that with sufficient treatment animals may be rendered abortin-sensitive by injections of dead *Bacillus abortus* suspensions. This is again in contradiction of former opinions that only living bacilli can render such animals allergic, and in this way parallels the observations referred to above on analogous conditions with tuberculin sensitization. In this case the animals were prepared by 6 to 9 injections each of suspensions of *abortus* bacilli killed at 65° in the water bath. The tests here reported were done 3 weeks after the last injection.

Method of guinea pig preparation	Tested with abortin	Tested with staphylococcin
1. Dead *B. abortus*	Slight reaction	0
2. " " "	Large, hemorrhagic, severe	0
3. " " "	" not necrotic	0
4. " " "	Definite, not severe	0
5. Dead *Staphylococcus pyogenes aureus*	1 cm., redness, not raised	Slightly less than the abortin reaction on same pig
6. Dead *Staphylococcus pyogenes aureus*	0	0

Overlapping is further illustrated by a typhoid animal which had received 4 injections of formalinized typhoid bacilli intraperitoneally and 5 weeks after the last injection gave about equal reactions to typhoidin and 1–10 O.T. dilution, when before this it had given merely a weak typhoidin reaction and a negligible O.T. reaction.

It will be seen from these experiments, therefore, that there is a well defined specificity in bacterial allergy sufficiently definite to suggest an antigen-antibody mechanism; but that at the same time there is also a considerable amount of overlapping in animals that are highly sensitive, an overlapping not very different from that encountered in precipitation reactions with various types of bacterial nucleoproteins.

Further examples of specificity will be found recorded below in connection with skin tests on pneumococcus-sensitive animals.

III.

In preceding papers (10, 11) we have reported upon the production of allergic skin reactivity in guinea pigs treated with the formed cells and cell extracts of typhoid bacilli, staphylococci and streptococci. In all of these experiments the skin reactions obtained were of the delayed variety, appearing from 12 to 24 hours after intracutaneous injection, and manifested by well outlined reddened areas, slightly edematous and swollen—never, however, even in the most marked cases, showing the central necrosis and hemorrhage characterizing the most severe forms of tuberculin and abortin reactions.

Our experiments with Grinnell upon streptococci which, in harmony with the observations of Dochez and Sherman (12), showed that it is

relatively easy to sensitize to streptococci by the repeated intraperitoneal administration of dead or of living bacteria; that the skin reactions, best elicited with the Dick filtrates, become positive 2 to 4 weeks after the last injection; and that continued immunization causes this hypersensitiveness to fade. This point, again, is corroborative of Dochez and Sherman's observation on the neutralization *in vitro* of the allergic antigen by antistreptococcus serum, and of the observations of Mackenzie and Woo (13) on a similar fading of analogous sensitiveness induced with pneumococcus extracts.

In later work we extended our observations to allergy induced with a number of bacteria other than the tubercle bacillus, including the pneumococcus, staphylococcus, typhoid bacillus and *B. abortus bovis*. With all of these bacteria it was possible to render animals allergic in the same manner in which this was accomplished with streptococci. In considering the mechanism of these reactions, we were led to undertake investigations concerning the differences in the types of antibodies developed in animals by the injection of the several fractions of the bacterial cell, the results of which we published (14) simultaneously with similar studies by Avery and Heidelberger (15).

This work revealed relations which could not fail to have important bearing upon allergic reactions, if these were in any way dependent upon antigen-antibody combinations. Summarized briefly, it was shown that there was a fundamental difference in the nature of antibody production determined by the form in which the bacterial antigen was injected and upon the particular fraction of the bacterial extracts employed. To illustrate with the pneumococcus, which is the easiest organism to work with in this respect, the facts are as follows:

If a rabbit is immunized with whole pneumococci, the antibodies formed react with whole bacteria, agglutinating them in the usual way, and the same serum will precipitate the carbohydrate fraction or residue (soluble substance of Avery and Heidelberger). Since it is practically impossible to prevent some pneumococci from going into solution, even when formalin is applied directly to the young growths, such serum will also react with nucleoprotein.

If a rabbit is immunized either with pneumococci dissolved in bile and filtered,

or with the so called "nucleoprotein fraction," the antibodies formed react with such nucleoprotein, but hardly at all with the residue material mentioned above, and contain little or none of the ordinary antibodies that act upon the whole formed cells. Immunization with the residue fraction alone of course produces no antibodies whatever. The ordinary antipneumococcus sera put out for therapeutic and diagnostic purposes contain antibodies for both types. This must be borne in mind in subsequent considerations.

In taking account of those relations, then, it became necessary to sensitize in such a manner that both types of antibody would be likely to be formed and to do subsequent tests upon the skin with the materials specifically reacting with the respective antibodies. The pneumococcus offered the most convenient material for this purpose, largely because it has been found possible to destroy the complex antigen responsible for the formation of the anti-residue (anti-"whole" pneumococcus) antibodies by dissolving the bacteria in bile.

We proceeded first, therefore, to endeavour to define the relations of the carbohydrate residue antigen to the allergic reaction, as follows:

Active and Passive Sensitization to the Carbohydrate Residue (Soluble Specific Substance).

Our attempts to produce typical delayed skin reactions in animals with the carbohydrate, type-specific residue antigens were so uniformly negative that they may be very briefly summarized.

Whatever the method of active sensitization, subsequent skin tests with the homologous type-specific carbohydrate residue never produced typical delayed allergic reactions. Occasionally the residue injection gave rise to moderate immediate edematous swellings which disappeared within a short time and were usually gone completely on the following day, when typical reactions are most pronounced.

The same is true of animals treated with homologous sera potent with antibodies which specifically precipitated the residue *in vitro.* A great many attempts were made to sensitize passively in this manner and large amounts of serum were given in single and multiple injections; reactions were attempted at intervals ranging from a few hours after the injection of the serum up to several weeks. But the results remained negative and as described above.

The intracutaneous injection of formed precipitates resulting from the incubation of mixtures of residue and antiserum never produced typical skin reactions. The supernatant fluids of such mixtures were likewise negative.

From many experiments of this nature, together with the corroborative evidence of our other work, we feel confident in asserting that the reaction which is represented by the specific union of the type-specific residue or soluble specific substance of the bacteria (the haptophore group of the bacterial antigen) and their homologous antibodies (representing the agglutinins, precipitins, etc., of antibacterial sera) has absolutely no relationship to that form of bacterial allergy which is manifested by the delayed skin reaction. This, incidentally, is consistent with the results of Mackenzie and Woo, who found that no relationship existed between allergy and that protective mechanism which is represented by such antibodies.

The Relationship of the Bacterial Nucleoprotein and Its Antibodies to Allergic Reactions.

Having failed to demonstrate any relationship between the isolated residue antigen and the bacterial antibodies to the allergic reaction, we next proceeded to carry out skin reactions with bacterial substances so prepared that either the total suspended materials or their nucleoprotein constituents were represented in the test material. The test on page 763 is an experiment of this kind with pneumococcus.

If the following protocol is examined from the point of view of possible relationship between allergy and antinucleoprotein antibodies, it will be seen that skin reactions were obtained with bile solutions of the pneumococcus in animals in which residue reactions were negative, and that such reactions were best developed in animals treated with these same bile solutions. It stands to reason that the cited experiments represent only a few of a considerably larger number and the results such as those reported tend to indicate a possible relationship between nucleoprotein and its antibodies in the allergic phenomena.

Guinea Pig 1 (845 gm.). Received 2 cc. of filtered bile solution of pneumococcus
Guinea Pig 2 (670 gm.). on Mar. 23, 24, 25, 26 and 27, intraperitoneally.

Guinea Pig 3 (670 gm.). Received 2 cc. of 70°C. killed fresh suspensions of whole
Guinea Pig 4 (620 gm.). pneumococci on Mar. 23, 24, 25, 26 and 27. Considerable loss of weight.

Intracutaneous Tests.

	Apr. 6		Apr. 10	Apr. 12		Apr. 13		Apr. 20	
	Skin test with bile solution	Skin test with whole pneumococci		Residue	Bile pneumococci	Pure bile 1–10*	Bile pneumococci	Residue	Bile pneumococci
1. Treated with bile solution	±	+	Reinjected intraperitoneally with 2 cc. bile solution	−†	+++	±	+++	−	−
2. Treated with bile solution	+++	−		−	+++	±	++	−	++
3. Treated with whole pneumococci	+	−	Reinjected with whole, dead pneumococci	−	+	−	+	−	−
4. Treated with whole pneumococci	±	−		−	+	±	++	−	−

* These tests are a few of many that have been done with each preparation to check up the non-toxic action of remnants of bile. Our bile solutions of pneumococcus were all made by adding a minimum amount of ox bile for complete solution which ranged from concentrations of 1–15 to 1–10 with the suspensions used. It was impossible to separate the bile from the nucleoprotein by precipitation because such precipitation brings down the bile salts. These were gotten rid of in most of the preparations used, if not completely at least to a large extent, by 48 hours' dialyzing against salt solution.

† These residue reactions check up information previously obtained that the residue antigen gives no skin reaction in guinea pigs that are sensitized to the nucleoprotein, as shown by the bile solution reaction obtained on the same day. Again, there is no residue skin reaction in Animals 3 and 4, although, being treated with whole pneumococci, there must have been a formation of antibodies capable of reacting with the residue.

An experiment with tubercle bacillus nucleoprotein is illustrated in the following:

The nucleoprotein extract was filtered through Berkefeld candles in order to remove possibility of injecting dead bacilli, or fragments.

The injections were made as follows:

Guinea Pig 1 received 6 injections ranging from 1 to 5 cc. at 4 day intervals between Jan. 13 and Feb. 3.

Guinea Pig 2 received 5 similar injections ranging from 5 to 7 cc. between Jan. 21 and Feb. 7.

Intracutaneous tests with nucleoprotein solutions and O.T. 1–5 were begun on these animals on Feb. 5 and carried out as follows:

Guinea pig	Feb. 5		Feb. 8		Feb. 10		Feb. 18		Feb. 19		Feb. 25	
	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.	O.T.	N.P.
1	−	−	−	−	+	−	++	Not	++	+	−	−
2	−	−	±	−	±	±	++	done	+	+	+	+

Reactions in such an experiment are always better with O.T. than with pure nucleoprotein, a fact which we attribute to a possible denaturization of the nucleoprotein in production, since not only in these experiments but in all experiments such relatively purified nucleoprotein is a very poor antigen and must be injected in large quantities and in many doses.

In all experiments of this nature there is an eventual tendency to desensitization, that is, a fading of the reactions upon continuous treatment, an observation which is in agreement with those of Mackenzie and Woo. Apart from the practical significance of this, it is an immunological fact which tends further to arouse suspicion that such sensitizations may, to some extent, be dependent upon an antigen-antibody mechanism.

The fact that guinea pigs sensitized with tubercle bacillus nucleoprotein reacted well with O.T. suggested another attempt to sensitize with O.T. direct. This has not been noticed either by others or ourselves in the past as a result of repeated skin reaction, but when, in analogy with nucleoprotein sensitization, we injected guinea pigs with relatively large amounts of O.T.—that is, 3 to 6 injections of about 0.3 cc. of concentrated O.T. in proper dilution, intraperitoneally,

filtered through Berkefeld filters to prevent the introduction of bacilli—we found that a sensitization could be obtained which was analogous to that with nucleoprotein; indeed, the animals reacted with large, flat, reddened areas, usually of considerably greater dimensions than those sensitized with nucleoprotein.

Experiments on Passive Sensitization to the Bacterial Nucleoprotein Fraction.

We will not go into detail concerning these experiments because the technique is simple and the results inconclusive. However, when a rabbit was treated with filtered nucleoproteins from the tubercle bacillus for a long time and with large quantities and the serum so obtained was found to precipitate tubercle bacillus nucleoprotein, normal guinea pigs were intraperitoneally injected with amounts ranging from 5 to 8 cc. and skin tests performed on them both with O.T. and nucleoprotein solutions every other day for some time after the serum administration.

It was found that in many of these pigs a definite allergic response was obtained both to O.T. 1–10 and to nucleoprotein solutions. These reactions did not, however, appear sooner than 6, 7 or 8 days after the serum administration, and usually faded within 3 weeks.

Similar experiments were done rather more extensively with pneumococcus substances in which we injected guinea pigs not only with the sera of rabbits immunized with bile extracts, but with the ordinary antipneumococcus Type I therapeutic sera which possessed a not inconsiderable capacity for precipitating nucleoproteins and bile solutions.

We do not cite protocols of these experiments because, carefully analyzed, they did not furnish conclusive evidence of passive sensitization. Although indicating the likelihood of such a process in many instances the results were complicated by the occasional development of similar sensitiveness in control animals prepared with normal horse serum. Moreover the consistent lateness of the appearance of sensitiveness whenever it appeared, indicated that the mere introduction of the antibodies could not be regarded as explaining the phenomenon as a whole.

We are confronted with the curious contradiction, therefore, that

while the facts of active sensitization, specificity and desensitization, can be explained only on the basis of some mechanism analogous to that of antibodies, the passive experiment remains unconvincing. This may be interpreted as meaning either that we are dealing with a type of reaction in which antibodies, in the ordinary sense of the word, play no rôle whatever; or that such antibodies are indeed significant but represent only that part of the mechanism which determines specificity and desensitization, another additional factor being required to complete the reaction. It is this latter interpretation which seems to us the more likely one for reasons that will be elucidated in the following section in which we deal with the tuberculin reaction itself.[1]

IV.

The Tuberculin Reaction Itself.

The preceding experiments may be regarded purely as preliminary to a study of the tuberculin reaction itself in that we believe that they have completely eliminated the possibility of a residue antibody mechanism in the tuberculin reaction, but have shown that a certain amount of sensitization can be obtained by active treatment with nucleoproteins. They have also again emphasized the existence of a specific element in these reactions, facts which to some extent clear up the underbrush but still leave us in the dark concerning the complete mechanism of the reactions.

Another series of preliminary experiments carried out during the past year too voluminous to be reported in detail, but necessary because of the many contradictions in the literature, may be briefly summarized as yielding the following information: (1) that contact *in vitro* of O.T. with the serum of animals immunized with living or dead tubercle bacilli does not produce anything which will give tuberculin

[1] We have so far failed also in obtaining any *in vitro* or *in vivo* neutralization of the allergic substance either by mixing with various antisera and incubating or by preceding skin tests by intravenous serum injections. To this we attribute less importance, however, since we bear in mind the great difficulty that Weil (16) and others have experienced in similar attempts with protein anaphylaxis where the antigen-antibody relations are far more clear. It seems that in all forms of sensitization it is not easy to divert antigen from sensitive cells, even with high concentrations of circulating antibodies.

reactions in the normal animal, whether or not complement be present; (2) that the same is true when the serum of animals immunized with tubercle bacillus nucleoprotein is employed; (3) that precipitates formed in both types of reaction fail to produce tuberculin reactions in normal guinea pigs, even when treated with complement; and (4) that none of the sera mentioned above will neutralize the action of O.T. upon tuberculous guinea pigs.

These facts in general correspond quite closely with those ascertained for bacterial allergy with other organisms. A direction of experimentation which was now indicated was that which dealt particularly with the meaning of the inflammatory tissue reactions, the tubercles, which may be recognized as possessing considerable significance in the development of tuberculin hypersensitiveness. In a former paper we have already shown that a certain amount of passive sensitization can be obtained by the injection into guinea pigs of sera from rabbits in which a large amount of tuberculous inflammatory reaction has been incited by the establishment of multiple tubercles. We were also able at that time to confirm the observation of Lange (17) that some sensitization can be incited by repeated injection of tuberculous tissue filtrates. In none of this work, however, did the sensitization amount to very much more than that which we have more recently obtained with nucleoproteins. Never was there any indication of hemorrhage or necrosis. These experiments have been repeated with the same results.

We therefore investigated the McJunkin (18) experiment in an effort to procure from it a possible clue, since we have been able in the past to confirm this procedure by the method originally reported by him. In attempting to analyze this method we determined the following facts:

1. A normal guinea pig injected with a filtered exudate from the peritoneum of a tuberculous guinea pig killed by intraperitoneal injection of O.T., gives an excellent skin reaction 11 to 12 days after the administration of the filtrate.

2. Similar exudates obtained by injecting a tuberculous guinea pig with broth sensitized definitely but less strongly than in the first case, also in about 11 days.

3. Exudates obtained from normal guinea pigs with broth gave a very slight and probably non-specific sensitization to O.T. in 11 days to 2 weeks.

These experiments confirmed McJunkin's original claims, but did not shed any particular light on the mechanism of the reaction. Indeed, while it seems, as McJunkin supposed in his first report, that we are confronted with an active sensitization of some kind, this is not at all certain.

We now proceeded, therefore, to investigate the possibility that in the reaction between the tuberculous tissues and products of the tubercle bacillus there might be formed a toxic substance responsible for the reaction. The hope of obtaining some light in this direction was encouraged by two observations. One of these was an observation made with pneumococcus at about the same time that Julianelle and Reimann (19) published their observations upon the purpura-producing, autolytic substance derived from pneumococci. The observation was as follows:

> If a freshly prepared pneumococcus suspension is divided into three parts, one of them left undisturbed except for the addition of a minute amount of thymol, the second immediately heated to 65° for 15 minutes and the third dissolved in bile, a similar amount of thymol added to the last two to equalize conditions and all three set into the incubator and allowed to stay there for 48 to 72 hours, and skin reactions carried out with 0.2 cc. of each of these suspensions in a normal guinea pig, there will be very feeble, or practically no reactions over the bile solution area or that carried out with the pneumococci immediately heated, but over the area into which the autolyzed pneumococci were injected there will be formed a violent delayed reaction which in all its morphological and pathological features resembles a violent, severe tuberculin reaction in a tuberculous guinea pig injected with O.T. These relations are shown in the accompanying figure.
>
> Similar experiments carried out with meningococcus show comparable conditions, except that in meningococcus the immediately heated organisms often showed a reaction similar to but milder than that of the autolyzed ones.
>
> With *Bacillus abortus bovis* an analogous experiment can be performed, except that here autolysis seems to be extremely slow and reactions comparable to the allergic ones do not develop until the unheated cultures have been kept in the incubator for 8 or 9 days and then they are not as violent as those produced with pneumococcus.

These experiments indicated the possibility that bacterial products may yield, upon cleavage, a substance toxic for the normal animal as O.T. is for the tuberculous animal.[2]

It was obviously suggested, therefore, again to investigate the results of the incubation of mixtures of O.T. with the extracts of tuberculous tissues.

This thought was further encouraged by observations that had been made in connection with earlier attempts to neutralize O.T. for tuberculous animals by similar incubation. An experiment of this type is as follows:

A mixture was made of one part of O.T. with five parts of a clear, filtered extract made by macerating and shaking the skin of a tuberculous animal in slightly alkaline salt solution. The mixture was allowed to stand for several hours in an incubator and overnight in the ice box.

0.1 cc. of this was then injected into a tuberculous animal, and in another spot a fresh 1–5 solution of the same tuberculin in salt solution. As shown in the figure, in this particular experiment the reaction over the point of injection of the mixture was markedly larger than that which developed where the fresh O.T. dilution had been injected.

Conditions similar to the above were encountered on a number of occasions, usually when extracts of lung or skin were used, less frequently when the tuberculous tissue extract was furnished by spleen or liver tissue.

From these two types of experiment it seems quite likely that in addition to the ordinary antigen-antibody reaction, tuberculin allergy was in some way related to a direct reaction between the inflammatory tissue reactions and the tuberculin, a thought which, incidentally, is suggested by all the past history of studies of tuberculin reactions.

Experiments on the Action of Tuberculous Tissue upon O.T.

This line of investigation was followed for a very long time with tantalizingly encouraging, but inconclusive results. We were never

[2] We assume that this toxic substance is the same as the purpuric poison described by Julianelle and Reimann, since it is produced in more or less the same way and since it possesses a considerable heat stability. The powerful toxicity of this material for guinea pigs, as shown in the figure, opens the question of its relationship to other described pneumococcus poisons and necessitates the further investigation of its possible antigenic properties, a matter that is being worked upon.

satisfied that we were obtaining anything more than definite suggestions in a positive direction until we abandoned the efforts with tissue extracts and began to work with macerated cellular materials, unfiltered. The observation which most encouraged us to continue in this direction was the fact that we again and again noticed that mixtures of O.T. with tissue extracts, even though they had relatively little action upon a normal animal, were considerably more toxic than O.T. alone for the tuberculous animal.

We do not cite a large number of unsuccessful experiments, but restrict ourselves to typical examples of the final experiments which convinced us that the interaction between O.T. and tuberculous tissue played an important rôle in the mechanism of tuberculin reactions. The following is such an example.

Mixtures were made as follows: Lung tissue of a tuberculous guinea pig was macerated in a mortar with sterile sand, and small amounts of salt solution added. A similar maceration of normal lung was made at the same time. The following preparations were then set up in test-tubes.

1. Macerated bits of lung in salt solution, 5 cc., + 0.1 cc. of concentrated O.T.
2. Similar tuberculous lung without O.T.
3. Salt solution, 5 cc., + concentrated tuberculin, 0.1 cc.
4. Normal lung, 5 cc., + O.T., 0.1 cc.

To each one of these tubes a small bit of thymol was added in order to prevent putrefaction, and the tubes were incubated. After 24 hours small amounts of fluid were taken from each of these tubes, centrifuged at high speed and 0.2 cc. respectively injected into a large white normal guinea pig.

The results are shown in Fig. 5 and are self-explanatory. It will be seen that while the normal lung macerate and the O.T. alone produced slight reactions, the incubated tuberculous lung macerate with O.T. gave a strong and extensive reaction.

Another experiment of the same type done with human lung is the following:

Normal human lung and lung thickly studded with miliary tubercles were obtained through the kindness of our hospital associates. Pieces of each were cut up with a pair of scissors and macerated with sand in a mortar, salt solution being added. Finally the sand was allowed to settle and the supernatant suspension containing bits of lung was taken up into tubes as follows:

1. Normal human lung suspension, 10 cc.
2. Normal human lung suspension, 10 cc., + O.T., 0.3 cc.
3. Tuberculous lung suspension, 10 cc.
4. Tuberculous lung suspension, 10 cc., + O.T., 0.3 cc.

Small, approximately equal pieces of thymol were added to each tube. After 48 hours' incubation portions of each tube were taken out, centrifugalized at high speed and 0.2 cc. injected intracutaneously into a normal, large, white guinea pig.

The results are shown in Fig. 6. Again the area into which supernatant fluid from Tube 4, containing the tuberculous lung suspension with O.T., had been injected gave a reaction larger than that given by the material from Tube 3, and both of these were much more extensive than those from the two controls.

We assume that the results in Tube 3 of both experiments were due to the presence of a certain amount of tubercle bacillus material in the infected tissue.

SUMMARY.

Our experiments have confirmed the fact that the so called bacterial allergies are dependent upon a mechanism which differs materially from that determining true protein anaphylaxis. Anaphylaxis to protein substances of the bacteria probably occurs but plays a relatively unimportant rôle in the phenomena of infection. The bacterial allergies, however, are of great importance since they develop rapidly and render the infected animal highly vulnerable to products of the bacterial growth which are relatively innocuous for the normal animal.

Neither the type-specific carbohydrate "residue antigens" (the "soluble specific substances" of Avery and Heidelberger) nor the antibodies reacting with them play any part whatever in bacterial allergy, and since these type-specific substances represent the haptophore groups of the whole bacteria by which they react with the agglutinins, precipitins, sensitizers, etc., of immune serum, allergy, as previously determined by Mackenzie and Woo, is in no way related to that phase of resistance which is determined by these antibodies. This does not, however, preclude the possibility that allergic hypersusceptibility may not in some way be related to other factors of resistance more definitely associated with cellular rather than with intravascular reactions. Our previous studies with Jennings and Ward in tuberculosis point in this direction (20).

Guinea pigs can be actively sensitized with all the bacteria with which we have worked when repeated injections of whole bacteria or of the protein (nucleoprotein) fraction are administered. Large amounts of the latter are necessary since these materials are indifferent antigens, possibly because of the severe manipulations necessary in their production.

Sensitiveness develops usually within 10 days after the first dose and increases with continued treatment for 3 or 4 weeks.

Sensitiveness is relatively specific, by which we mean that there is a definite specificity which, however, in highly sensitive animals is not absolute and shows considerable overlapping.

Continued treatment with considerable quantities of the above substances leads to gradual desensitization in animals in which there are no chronic foci present, which, as in tuberculosis, tends to continue the sensitization.

Attempts at passive sensitization have been irregular and inconclusive. When any degree of sensitiveness has developed after the injection of immune sera, it has appeared late and has been of doubtful specificity. Conversely we have failed in any case to neutralize the activity of the active allergic constituents of bacterial extracts by incubation with any type of immune serum.

We have failed so far to show any increased fixation of tuberculin material on the part of tuberculous tissues or on that of living tuberculous animals. These failures, however, seem to us of relatively slight importance since quantitative experiments of this nature are extremely difficult in the case of a substance as delicately potent for the tuberculous animal.

On the other hand we have obtained definite, though irregular evidence that the incubation of O.T. with fragments of tuberculous lung tissue (less clearly with other tissues) leads to the formation of a substance that produces allergy-like lesions in the skin of normal guinea pigs. With somewhat greater regularity, similar treatment of O.T. has enhanced the potency of the tuberculin for tuberculous animals. And, in these experiments there was evidence that the factor responsible for this action was not easily separable from the cells themselves.[3]

When these experimental data are analytically considered they appear in many respects confusing and contradictory. There has been so much work done on the tuberculin reaction, moreover, that, in the face of experimental inconsistencies it would seem foolhardy to formulate more than tentative suggestions to explain the mechanism

[3] We are permitted by Dr. Petroff of Saranac to state that he has obtained results similar in principle with our own, but also irregular and not repeatable at will

of these reactions. Nevertheless there are a few outstanding and sufficiently reliable facts which compel a limited number of definite deductions.

In the first place there is no question of the complete independence of the true allergic phenomena from the ordinary bacterial antigen-antibody reactions. We know, moreover, that the allergic substance is chemically separable from the carbohydrate "residue" or haptophore group of the bacteria (Mueller, Laidlaw and Dudley). Indeed it has been shown by Long and Seibert (21) that the active allergic substance is either a protein in itself, or at any rate closely associated with the bacterial protein.

Furthermore, the distinct, though limited, specificity of the allergic sensitiveness compels the conclusion that we are dealing with an immunological process in which the tissue cells acquire an increased specific capacity to react with this nitrogenous material, a capacity which, in principle, is not far removed from the supposed "sessile receptor" apparatus which is conventionally held responsible for protein anaphylaxis; and this analogy is further amplified by the apparent desensitization which continued treatment produced in many of our own experiments as well as in those of Mackenzie and Woo.

Here, however, the analogy with protein anaphylaxis ends. Passive sensitization with any form of immune serum or with the sera of highly sensitized animals is either feeble or entirely unsuccessful and indicates quite convincingly that, whatever the receptor apparatus of the cells may be, it is not easily given up to the blood stream as are ordinary antibodies. Further than this, our tissue-tuberculin experiments, irregular and occasional as they were, nevertheless convinced us that:

1. The contact with the tissues of tuberculous animals results in the production of a toxic factor, not unlike the autolytic toxic materials of some bacteria.

2. The active cell constituent by which this action is wrought, is not easily separated from the cells, even by energetic methods of extraction.

This close association of the entire process with the cells themselves is particularly significant in view of the obvious cell injury in which these delayed allergic effects differ from the ordinary urticarial, evanescent reactions associated with protein anaphylaxis.

The process of allergy, as far as we can approach it then, may be conceived as follows:

A nitrogenous, probably protein, constituent of the bacterial growth or of its body substance stimulates a specific reaction in the tissue cell by which its specific capacity to establish contact with this constituent is enhanced.

The cell is thereby enabled to exert a, probably, enzyme-like effect upon this material in consequence of which a toxic substance is liberated, largely upon or possibly within the cell itself.

Both processes may be dependent upon one and the same reaction body. But it seems more likely that increased contact and the increased cell activity are separately developed, an assumption which is rendered probable by the association of the highest degrees of allergy with inflammatory cell reactions, and by the fact that moderate and less specific allergic sensitiveness follows 10 or more days after the administration of considerable amounts of indifferent protein substances to guinea pigs. We interpret this as signifying that such injections may non-specifically increase cellular activity, a change which many earlier workers have spoken of as "cell irritability."

Both processes are closely associated with the altered cell itself and the factors by which the reaction is brought about are not easily given up to the blood stream as are the antibodies formed in response to injections of proteins or whole bacteria.

We are confronted, therefore, with an immunological mechanism which has some close analogies to those others in which circulating antibodies are formed, but which differs from these mainly in the intimacy with which the entire reacting system is associated with the cells themselves.

It is difficult to conceive that a functional cell alteration, as profound as this, should be entirely unrelated to the phenomena of susceptibility or resistance.

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EXPLANATION OF PLATES.

PLATE 29.

FIG. 1. Severe type of skin reaction given in guinea pigs sensitized by active immunization with nucleoprotein. This particular guinea pig happens to have been one sensitized by the McJunkin method with the exudate produced in a tuberculous guinea pig with O.T., but the type of the reaction represents the average severity of the non-necrotic delayed skin reactions referred to in the text.

FIG. 2. Comparative reactions in an *abortus*-sensitized pig during the early stages of severe reaction with (1) 1–10 tuberculin and (2) 1–5 abortin.

FIG. 3. Typical severe skin reaction on a normal guinea pig obtained by the injection of 0.2 cc. of pneumococci autolyzed in salt solution for 48 hours.

PLATE 30.

FIG. 4. Tuberculous guinea pig tested over Area 1 with O.T. 1–5, over Area 2 with O.T. diluted to the same extent with concentrated extract of the skin of a tuberculous guinea pig and kept in the incubator for 4 hours before the two tests were done. Note the much larger extent of the area over 2.

FIG. 5. The figures in this illustration show the resulting inflammatory areas on the skin of a normal guinea pig injected intracutaneously as follows:

1. Supernatant fluid from a 2 days' incubation of fragments of normal lung tissue with O.T. in a concentration of 1–33.
2. O.T. in solution 1–33, similarly incubated.
3. Tuberculous human lung fragments in salt solution.

4. Tuberculous human lung fragments + O.T. in a concentration similar to the above.

Small pieces of thymol had been added to all the preparations to prevent infection with bacteria.

Small amounts of fluid were removed with a pipette and centrifuga'ized at high speed, the clear supernatant fluid being used for intracutaneous injection.

FIG. 6. Comparative areas of inflammatory edema on the skin of a normal guinea pig resulting from intracutaneous injections with the following materials:

1. Normal lung fragments + O.T., total concentration 1–10.
2. O.T. 1–10 in salt solution.
3. Tuberculous lung without O.T.
4. Tuberculous lung with O.T.

FIG. 1.

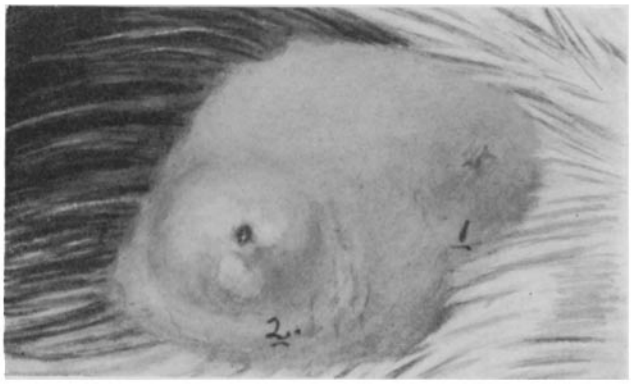

FIG. 2.

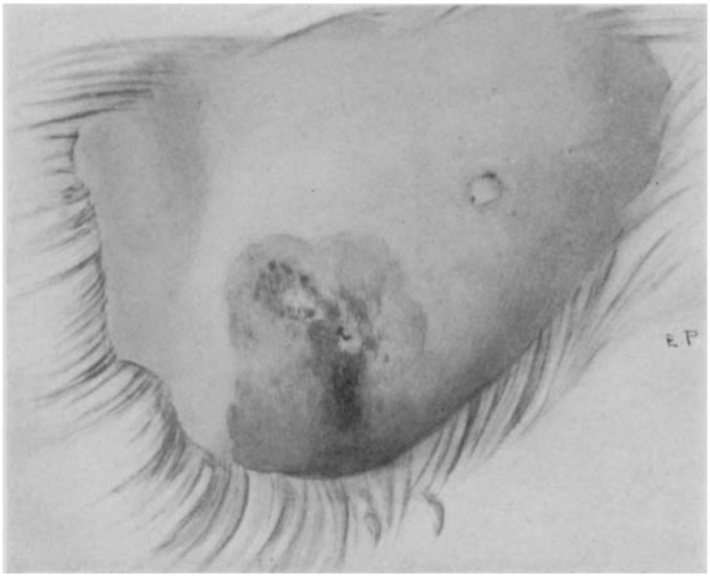

FIG. 3.

(Zinsser and Tamiya: Analysis of bacterial allergy.)

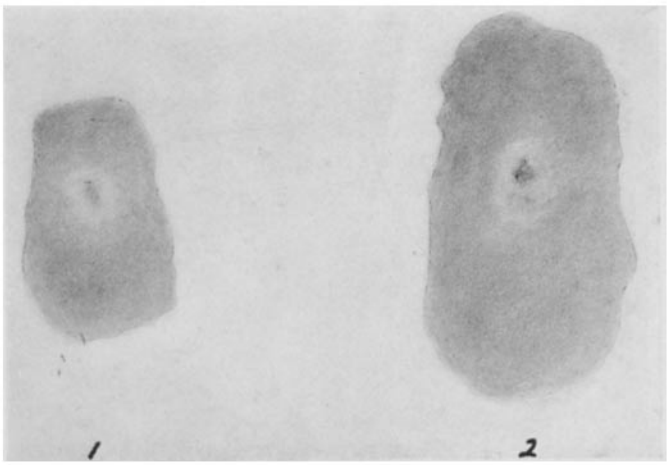

FIG. 4.

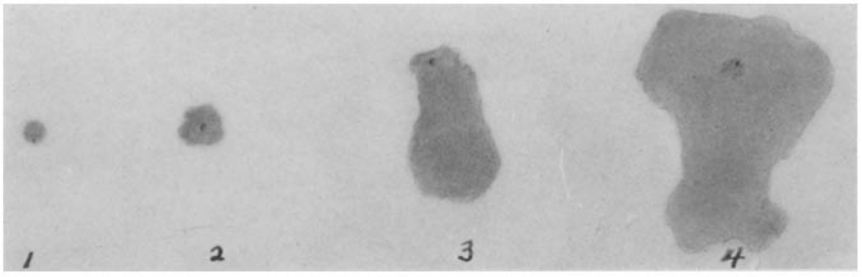

FIG. 5.

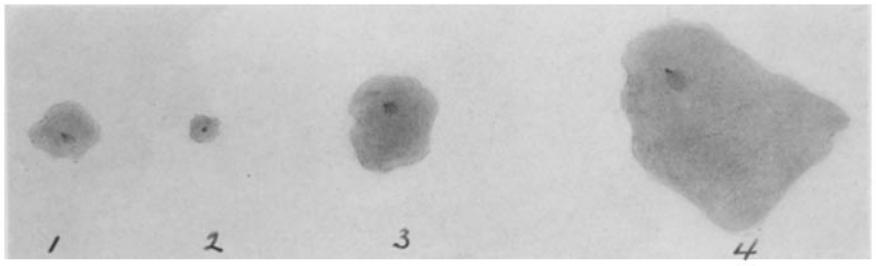

FIG. 6.

(Zinsser and Tamiya: Analysis of bacterial allergy.)