TUBERCULAR ALLERGY WITHOUT INFECTION*

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Plates 29 to 31

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For a period of years we have been studying the effects of materials from tubercle bacilli on the tissues of normal and tuberculous rabbits and guinea pigs. The present study is the beginning of the project to use some of the fractions together to see if the presence of two substances in the tissues modifies the effect of either given alone. In the experiments described here it has been found that the addition of tuberculo-phosphatide to tuberculo-protein enhances the sensitization to the protein to a marked extent, producing, after three or four injections of relatively small amounts of protein, a degree of sensitization like that of the disease.

The subject of sensitization in connection with tuberculosis has a long and complex history.

In 1910 and 1911 Baldwin (1) published important studies on the nature of tuberculin sensitivity in which he concluded that the presence of tubercles was necessary in order that tuberculo-protein should elicit skin reactions like those of the disease. He found that after repeated intraperitoneal injections of tuberculo-protein (filtered water-extract) into both rabbits and guinea pigs they exhibited a form of anaphylactic response, that is, they would die if the same extract were injected intravenously or intracerebrally but they did not react locally to intradermal injections. He used accessory materials to induce the skin-sensitizing

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power of the protein, namely, charcoal, lipids extracted from tubercle bacilli with benzol, and beeswax, but with negative results. On the other hand, he noted that when tubercles were formed, even by killed tubercle bacilli, sensitization occurred. Hence he concluded that " . . . if tuberculin reactivity is present in any animal, the presence of tubercles must be predicated" (1 *b*). This concept was confirmed and strengthened by Krause (2).

It is now firmly established that skin sensitivity can be induced by heat-killed tubercle bacilli, through the early work of Borrel (3), of Baldwin (1 *b*) just referred to, and of Bessau (4), and extending to the more detailed and conclusive studies of Petroff (5), Petroff and Stewart (6), and Lange and Freund (7).

Many failures to sensitize animals so that they would give a local reaction to an intradermal injection with tuberculo-protein have been reported, but since Dr. Seibert and Drs. Heidelberger and Menzel have now extracted more highly antigenic tuberculo-proteins the negative evidence need not be reviewed. However, two points have been brought out in this recent work that may help to explain the discrepancies with previous reports. First, both Seibert (8 *a*) and Heidelberger and Menzel (9) have shown that differences in methods of preparation of these proteins give modifications in the products. Second, Seibert (8 *b–e*) has shown that the heating used in the preparation of the so called old tuberculin (OT) changes the size of the active protein molecule, the molecular weight of which is about 32,000, to a molecule the weight of which is about 16,000 and which is not an effective antigen by itself, and yet is able to elicit a skin reaction in the already sensitized animal.[1]

The fact that an accessory substance may enhance the antigenicity of a protein has been reported recently. Some of the materials used have been relatively inert, such as carbon particles or alum to which the protein is probably adsorbed; another group has been lipids which may give rise to more complex cellular changes. In 1926 Gaehtgens (10) reported that the use of a mixture of serum and lipid (alcoholic extracts of tissues) injected into rabbits induced antisera with a higher precipitin titer than the serum alone. This was not confirmed by Dresel and Meissner (11) who reported their observations in 1927. Tytler (12) summed up previous studies on the antigenic power of tuberculo-protein as inconclusive but stated that he could elicit sensitization by incorporating dry bacillary tuberculo-protein in beeswax and injecting them together. In this way he obtained excellent skin sensitization with necrosis and noted that animals with the stronger reactions showed a flare-up of previous reactions on re-injection with the wax-protein.

In 1935 Saenz (13 *a*) showed that dead tubercle bacilli enveloped with petro-

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[1] These are the figures from Dr. Seibert's recent studies with the ultracentrifuge technique in Professor T. Svedberg's laboratory at Upsala. Her results have recently been published (Seibert, F. B., Pedersen, K. O., and Tiselius, A., *J. Exp. Med.*, 1938, **68**, 413).

leum jelly induced allergy sooner, that is, in from 6 to 8 days, and of more intense degree than dead bacilli in saline, or living bacilli. He interpreted this to mean that the oil induces an inflammatory reaction that favors the increase in allergy. In 1937 he showed (13 *b*) that with small doses of bacilli in the presence of oil there was a slight reduction in the dispersion of the bacilli.

In 1935 Seibert (8 *d, e*) showed that a mixture of the purified protein derivative (that is the preparation of the smaller molecules) with aluminum hydroxide or carbon particles produced a complete antigen. She interpreted this result to mean that the antigen is then composed of particles of aluminum hydroxide with adsorbed protein. The cellular reaction to aluminum hydroxide (Willstätter type C), inducing epithelioid cells forming tubercle-like structures, has been followed by Olitsky and Harford (14) and by Jørgensen (15). Jørgensen discusses the immunological significance of this type of cell reaction. The foreign body reaction to carbon particles is well known.

Materials and Methods

We obtained from Dr. Seibert three preparations of tuberculo-protein designated TPA, SOTT, and PPD. The TPA is a purified tuberculo-protein precipitated by ammonium sulfate and separated in large part from free polysaccharide (Seibert, 8 *a, d, e*; and Seibert and Munday, 16). It is the equivalent in potency and purity of Seibert's newer preparation of protein designated TPT which is prepared by precipitation with trichloracetic acid. The SOTT and PPD are also equivalent preparations as regards tuberculin activity. SOTT (the letters signifying synthetic-media; old-tuberculin; trichloracetic-acid-precipitate) was the original designation for the material now called purified protein derivative, PPD. This preparation is made from an old tuberculin obtained from synthetic media after growth of standard strains of human tubercle bacilli for from 6 to 8 weeks and heated for 3 hours in an Arnold sterilizer. The heating reduces the size of the protein molecule. The material is then submitted to ultrafiltration to remove salts, glycerine, etc., and finally is precipitated with trichloracetic acid to remove polysaccharide (Seibert, 8 *c*).

The proteins designated D and G were given to us by Drs. Heidelberger and Menzel (9). They were extracted from human tubercle bacilli, strain H-37. The D fraction was obtained with M/15 phosphate buffer at pH 6.5 after the frozen and dried bacilli had been extracted with acetone and with ether to remove the lipid and with buffer at pH 4 to acidify the proteins and remove free polysaccharide. The D fraction was then precipitated with acetic acid. The G fraction was obtained with water made alkaline to about pH 11.0 after previous extractions with less alkaline buffers. These processes were all carried on in the refrigerator to minimize enzyme action. These preparations are very low in free polysaccharide but contain bound polysaccharide because they elicit the formation of antibodies to the carbohydrate in rabbits, as well as antibodies to the protein.

The protein MA-100 was given to us by Dr. Reichel; it was prepared at the Mulford Laboratories of the Sharp and Dohme Company from synthetic culture media on which human tubercle bacilli, strain H-37, had been grown (Masucci and McAlpine, 17). This protein was obtained by precipitation with ammonium sulfate, as was Seibert's protein M-9 (Seibert and Munday, 16 *a*; and Seibert, 8 *b*).

The tuberculo-phosphatide and the yeast-lecithin used in these experiments were prepared by Dr. R. J. Anderson. The phosphatide was extracted from human tubercle bacilli, strain H-37, in 1932 (18). It was filtered through Chamberland candles; it is predominantly crystalline and has a nitrogen content of 0.4 per cent due to ammonia nitrogen. The methods by which we determined that the enhancement of sensitization was not caused by contaminating tubercle bacilli but by the phosphatide itself are discussed in this paper. The phosphatide gives rise to uniform and stable suspensions when rubbed in water. A suspension of the phosphatide in saline was made and added to the protein in solution just before making the injections. Aluminum hydroxide in the form of Willstätter's gel was furnished us by Dr. Peter K. Olitsky. This material, in the dilution used, contained combined aluminum in the amount of 15 mg. dry weight per cc.

All control materials, which were to be free of tuberculo-protein, were handled in new glassware which had never been in contact with tubercle bacilli nor any of their products. All dilutions of the tuberculo-protein were made just prior to performing the injections in order to insure no loss of potency.

We used guinea pigs in the experiments, whose average weight was about 300 gm. and they were grouped according to weight so that the average weight of each group corresponded to that of the other groups. At the suggestion of Dr. Karl Landsteiner we have used the intradermal route for the sensitizing injections and have limited the injections to the dorsal region where the skin is thickest. The injections were placed about 2 cm. apart and each succeeding one was made on the side opposite to the previous injection. Reactions to the injected materials and the final skin tests were measured with calipers at 24 hours and 48 hours.

RESULTS

These experiments were planned on the basis of two previous studies in sensitization, one by Dr. Seibert (8 *a*) and the other by Smithburn, Sabin, and Geiger (19). In 1932 Seibert reported that local cutaneous sensitization (Arthus phenomenon) could be induced in rabbits and guinea pigs with tuberculo-protein. Seibert used repeated intraperitoneal injections of 10 mg. of the protein. The least amount of the protein required to sensitize any rabbit was 47 mg. and other rabbits took as much as 225 mg. The least amount of protein required to sensitize any of the guinea pigs was 30 mg. In 1934 Smith-

burn, Sabin, and Geiger (19) reported sensitizing both rabbits and guinea pigs by repeated subcutaneous injections of tuberculo-protein (MA-100) over a period of 13 weeks. The total amount of protein used in the rabbits was approximately 27 mg. and in guinea pigs, 5 mg.

In the present experiments we used phosphatide in the hope of reducing the amount of tuberculo-protein necessary to sensitize in order to eliminate the discrepancies between artificial sensitization and that occurring in the disease.

These studies are presented through three experiments, first, a comparison of the reactions of guinea pigs to intradermal injections of the protein MA-100 by itself and as enhanced with tuberculo-phosphatide, lecithin, and aluminum hydroxide. Earlier experiments in sensitization in this laboratory had been made with MA-100 (Smithburn, Sabin, and Geiger, 19). Second, a study of the two more highly antigenic tuberculo-proteins, the TPA of Seibert and the D fraction of Heidelberger and Menzel, used with and without the tuberculo-phosphatide; and third, the effect of the phosphatide mixed with the protein derivatives SOTT and PPD of Seibert.

A general survey of the power of the tuberculo-phosphatide to enhance sensitization is shown on Chart 1.

For this group of animals the quantity of phosphatide was 5 mg. and the protein 0.5 mg. in 0.1 cc. saline for each injection and there were six injections. For the final test of sensitization 0.1 mg. of MA-100 (the standard test dose for guinea pigs) was used for all the animals. These tests were made a week after the last sensitizing injection. The standards for estimating the skin tests were those generally accepted as stated by Hetherington, McPhedran, Landis, and Opie (20) for human reactions. The standard is that a reaction called one plus is an area of edema measuring 10 mm. in diameter; a two plus reaction has more marked edema and measures from 10 to 15 mm.; the three and four plus reactions both have more marked edema and measure more than 15 mm., but the three plus reaction lacks, while the four plus reaction is characterized by necrosis.

The basic control for the use of this phosphatide to enhance sensitization to tuberculo-protein consisted in determining that this phosphatide by itself did not sensitize to the protein. Six guinea pigs received six dorsal intradermal injections of 5 mg. of phosphatide at intervals of 1 week. The mean size of these reactions 24 hours after the injections did not vary appreciably, as is illustrated in the second line of Chart 1, and all six were negative to the final test with the protein MA-100.

The phosphatide was obtained by Dr. Anderson from the bacilli by means of

appropriate solvents, but, though by these means he could separate this lipid from other lipids, such as fatty acids and waxes (alcohols), a few acid-fast bacilli remained in the first preparation of the phosphatide. To remove these residual bacilli, he filtered the material, in a lipoidal solvent, through Chamberland candles.

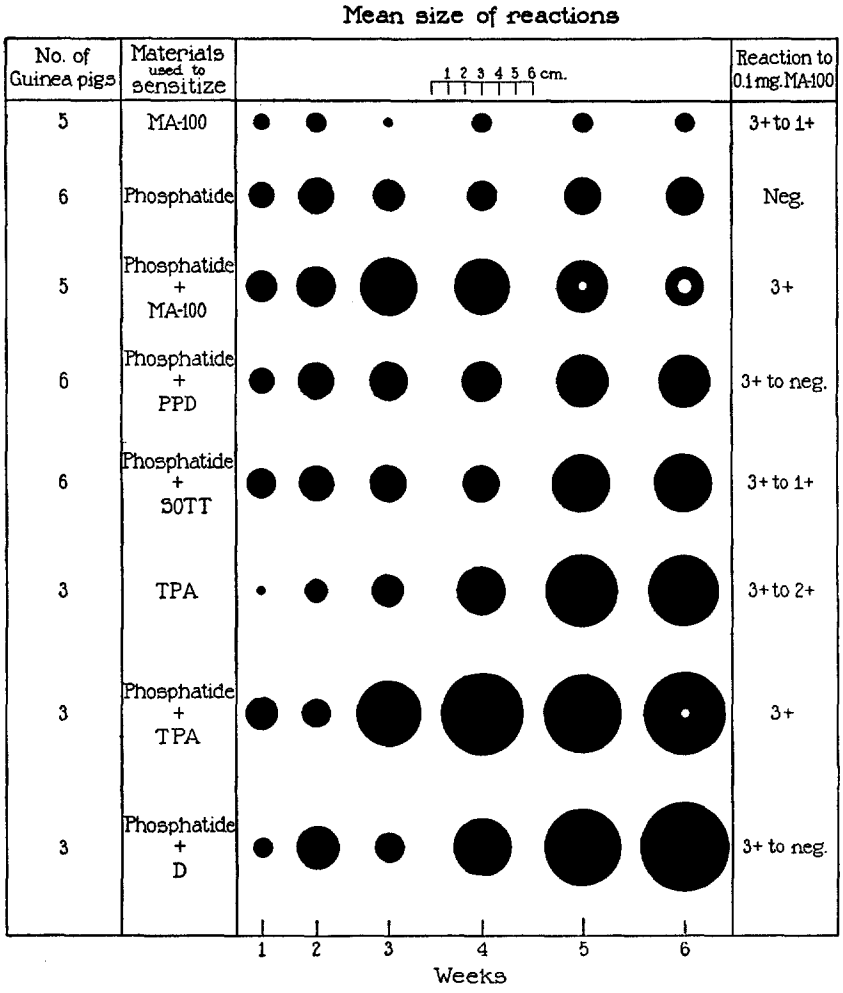


CHART 1

The use of a lipoidal solvent in this procedure alters the conditions of filtration to such an extent that all of the bacilli are not prevented from passing through the candles in every filtration. Repeated attempts to demonstrate acid-fast bacilli in stained preparations of the phosphatide used in these experiments have been

negative. Such negative results, however, cannot be considered as establishing the complete absence of bacilli in the phosphatide. As a further check we dissolved 200 mg. of this phosphatide in a mixture of three parts of chloroform and seven parts of ether, proportions that give a fluid, the specific gravity of which allows sedimentation of tubercle bacilli. This solution was then centrifuged at high speed for 4 hours and in the sediment we were again unable to find acid-fast bacilli. As a further check we devised the following experiment. The amount of protein containing nitrogen in an amount equivalent to the total nitrogen content per milligram of phosphatide was computed and added to the phosphatide. Eight guinea pigs then received ten daily intradermal injections of this mixture, 0.1 mg. of phosphatide with 0.0025 mg. of protein, making a total of 1 mg. of the phosphatide and 0.025 mg. of the protein. 25 days after the last injection, all of the animals were tested with 0.1 mg. MA-100, with the following results. In this experiment four of the guinea pigs received the protein, which was the Heidelberger-Menzel type G in the form of an alum precipitate. When tested with MA-100, one of these was negative and three showed three plus reactions (measuring 741, 966, and 1050 sq. mm.); the other four animals received the same protein in solution and the tuberculin test showed three to be negative while one showed a reaction which measured 156 sq. mm. and was considered two plus. On the basis of these tests, with four out of eight guinea pigs sensitized with the addition of so small an amount of protein, namely, 0.025 mg., we consider it legitimate to have used this preparation of phosphatide as an enhancing agent comparable to the other materials, lecithin and aluminum hydroxide.

Experiment 1.—The mean size of the reactions 24 hours after intradermal injections of protein MA-100 is given on the first line of Chart 1 and the reactions to the same protein plus phosphatide on the third line. Evidences of sensitization, that is, of changed reactions, are indicated in three ways on the chart: first, by a change in the size of reaction to the successive weekly injections of the same antigen; second, by the appearance of necrosis which is signified by the white circles in the center of the black zones; and third, by the effect of the final test injection with 0.1 mg. of protein MA-100, recorded in the last column.

With the MA-100 there was relatively little change in the mean size of the reactions on repeated injections, showing that this preparation is a less potent antigen than protein TPA. This observation confirms the results obtained by Seibert (8 *b*). This is also borne out by the fact that in the final test with the same protein, only one of the five guinea pigs exhibited a three plus reaction, while two of them were two plus and two were one plus. This protein, however, when used with the phosphatide, was a good antigen, showing an increase in the size of the reaction after the second injection and giving well marked necrosis after the fifth and sixth injections. After the six injections of MA-100 plus phosphatide, all of the guinea pigs gave three or four plus reactions on testing with the MA-100 alone. These data are clear evidence of the power of the phosphatide to enhance the sensitizing potency of protein MA-100.

On Table I is summarized the comparison of the power of two other materials

not obtained from tubercle bacilli, namely, lecithin from yeast and aluminum hydroxide, with the power of tuberculo-phosphatide to enhance the sensitizing power of MA-100. None of the three accessory materials given alone induced any significant increase in the size of the reaction to the repeated injections and none of them sensitized to tuberculo-protein, but all three enhanced the sensitizing power of the protein, lecithin, and aluminum hydroxide to a moderate degree and tuberculo-phosphatide to a striking degree. Both lecithin (Sabin, Doan, and Forkner, 21) and aluminum hydroxide (Olitsky and Harford, 14 *a* and *b*) induce the formation of epithelioid cells.

TABLE I

Comparison of Lecithin and Aluminum Hydroxide with Tuberculo-Phosphatide in Power to Enhance Sensitization to Tuberculo-Protein MA-100

Number of animals	Material	Mean size of reactions						Reaction to 0.1 mg. of MA-100
		1st injection	2nd injection	3rd injection	4th injection	5th injection	6th injection	
		sq.mm.	*sq.mm.*	*sq.mm.*	*sq.mm.*	*sq.mm.*	*sq.mm.*	
5	MA-100	87	106	21	124	120	100	3+ to 1+
6	Phosphatide	317	412	329	289	432	460	Negative
5	Phosphatide and MA-100	344	482	1114	1064	913	584	3+
6	Lecithin	150	178	169	226	240	237	Negative
5	Lecithin and MA-100	270	414	599	481	283	530	3+ to negative
6	Aluminum hydroxide	89	74	101	115	109	116	Negative
6	Aluminum hydroxide and MA-100	204	310	460	513	1000	752	3+ to 2+

Experiment 2.—We have also used two highly antigenic proteins in these experiments, Seibert's TPA and the D fraction of Heidelberger and Menzel, as shown on Chart 1. The reactions to the protein TPA steadily increase in size with each weekly injection, indicating that the material is a potent antigen. With the addition of phosphatide, however, there was a more rapid increase in the size of the local reactions. This was but a small part of the difference in reaction to the reinforced protein, for there were qualitative differences of still greater significance. With the protein by itself reactions were elicited which did not go on to necrosis, after the number of injections used in these experiments, and they were faded in 48 hours; while, after the mixed injections, the reactions showed marked necrosis in 24 hours and persisted for the 48 hour period.

These differences were apparent both on inspection of the lesions in the animals and on study of sections of the material. A histological study of the reactions was made in a second series of guinea pigs which received successive injections of the protein and the protein plus phosphatide in the same dosages as previously and at the same intervals. The local reactions in the skin 24 hours after the injections of the protein are shown on Figs. 1 to 4 and the corresponding series for the mixed injections on Figs. 5 to 8. In this series one animal of each group was sacrificed 24 hours after each sensitizing injection for histological studies. Other guinea pigs received only one injection and were sacrificed at varying intervals for study of the residual reaction.

As usual, the first injection of the protein alone gave a negative reaction, as determined by gross inspection; its site, as seen in the animal (indicated by the arrow on Fig. 1) was scarcely more than a needle puncture. Quite different, on the other hand, was the response of the mixture (Fig. 5). In sections the sites of the two injections also showed a marked contrast, as can be seen in Figs. 9 to 11. In Fig. 10 is shown at low magnification the amount of the 24 hour reaction to a single injection of the protein plus phosphatide. In Fig. 9 is shown the densest zone of reaction to the protein alone, in the deepest layers of the dermis and the subcutaneous level, to be compared with a corresponding zone after the mixed injection shown in Fig. 11. After the protein alone there was a moderate infiltration of the tissues with neutrophilic leucocytes, most numerous just beneath the epithelium, and some increase in monocytes, especially in the subcutaneous tissues. As shown in the photograph, there was a perineural and perivascular infiltration with monocytes. After injection of the protein plus phosphatide the same types of cells were involved, but there was a much greater outpouring of neutrophiles and a much greater increase in monocytes (Fig. 10). Moreover, the monocytes had been altered functionally because they had phagocytized the phosphatide. This was shown in sections by a vacuolization of these cells, the lipid having been dissolved out of them in the processes of embedding. Previous studies in this laboratory (Sabin, Doan, and Forkner, 21; and Smithburn and Sabin, 22 *a*) have shown that phagocytosis of phosphatide can be the first step in the formation of epithelioid cells from monocytes. It is this very marked difference in cellular response to the phosphatide plus protein, as compared with the response to the protein by itself, that initiates the speeding up of sensitization to be made out with succeeding injections.

The reaction to a single injection of these two materials was studied in sections after an interval of 9 days. An effect of the protein alone was still demonstrable and readily discriminated, even with low power of the microscope, from the effect of the mixed injection. In both all traces of the neutrophilic lecucocytes had disappeared; the differences were in the number and the qualitative changes in monocytes. After the protein TPA alone there was some increase in monocytes over the number in normal skin and there were a few epithelioid cells. After the less potent protein MA-100 Smithburn and Sabin (22 *b*) did not find

any epithelioid cells. After the protein plus phosphatide there was a much greater residual reaction of monocytes and epithelioid cells. In both series there was an infiltration of the tissues with eosinophiles.

Still more striking differences are to be made out in the tissues after repeated injections of protein and protein plus phosphatide, that is, as the animals become sensitized. In Figs. 12 to 14 are illustrated the residual effect of a first injection of protein (Fig. 12) and of protein plus phosphatide (Figs. 13 and 14) taken 24 hours after a second injection had been made. Thus these reactions are 1 week old. 24 hours after the second injection of the mixture there was a lighting up of the first injection, the site of which could not be noticed in the animal at the time the second injection was made (Fig. 6). This flare-up of the site of the first injection was due to edema and hemorrhage. This phenomenon indicates already a difference in the sensitivity induced by the presence of the phosphatide.

The residual reaction to the protein alone, after 1 week (Fig. 12), shows a considerable increase in monocytes throughout the dermis over the number in the normal skin. They occurred in small foci comparable to the milk spots of the omentum, indicating a local increase in these cells rather than an infiltration from the blood stream. Even with the protein alone this residual injection must be considered in the light of sensitization inasmuch as the second reaction to an injection of the protein was larger than the first (Figs. 1 and 2).

The corresponding residual reaction of 1 week's duration to protein plus phosphatide, taken 24 hours after the second intradermal injection, shows both the direct effects of the materials injected and the effects of sensitization. The dermis and the subcutaneous level were dense with cells (Fig. 13), largely epithelioid cells in small foci or tubercles. Such a tubercle-like mass is to be seen below the center of Fig. 13; at higher magnification its center shows a fresh hemorrhage. The edge of this tubercle is shown in Fig. 14. In the sections of this material there were a few neutrophiles, some monocytes, and a considerable infiltration with eosinophiles, especially just under the epithelium. The epithelium was much thickened, as can be seen by comparing Figs. 10 and 13. This thickening of the epithelium was still more marked in some of the later injections, with an increase in the number of mitotic figures.

The site of the 24 hour reaction to the second injection of phosphatide plus protein was markedly different histologically from the corresponding 24 hour reaction to the first injection. The most striking change was in the presence of edema; the fibers were spread apart and it could be seen that they were swollen and in places had a changed reaction to the stain. The cellular infiltrations, both of neutrophiles and of monocytes, were much greater. It will be noted in Fig. 6 that there was a slight ulcer in the center of the reaction; this was probably due to a bleb of the injected material which had raised and damaged the epithelium. Beneath this ulcer there was a massive infiltration of the tissues with neutrophiles.

The reactions to the third injection were the same in kind but showed increas-

ing discrepancies between the simple and mixed injections. After the protein plus phosphatide there was an increasing formation of monocytes, extending not only throughout the dermis and subcutaneous levels but also beneath the carnosus muscle. Such a mass of young monocytes, with the area infiltrated with eosinophiles, is shown at this level in Fig. 15, 24 hours after a third injection. In this material there was considerable perivascular infiltration. The reactions to the third injections did not differ greatly in size, but the qualitative differences were marked. The lesion after protein alone (Fig. 3) was soft and flat; it was an erythema without much edema, whereas the site of the third injection of the mixture (Fig. 7) was markedly raised and was indurated. The thickness of the lesion is just suggested in the photograph by the relief given by a band of hemorrhage on the left border.

After the fourth injection of protein plus phosphatide, massive necrosis became the predominant difference between the two types of injections. This will be plain if Figs. 4 and 8 are compared. It will be noted that the site of the fourth, necrotic reaction to the mixed injection was decidedly smaller than that of the third injection. This has been a constant phenomenon, that is, as soon as the degree of sensitization has given marked necrosis, there has always been a reduction in the area of the lesion. Three out of four of the guinea pigs of this series receiving the mixed injection showed marked macroscopic necrosis after the fourth injection. In the series shown on Chart 1, necrosis became as marked only after the fifth injection. With massive necrosis the lesions became very complex. There was the increase in cells due to the materials injected; but besides these direct effects there were edema and hemorrhage, clotting of material in lymphatics, and swelling of the endothelium of the veins. The death of cells and the damage to fibers caused new infiltrations with neutrophiles and phagocytosis of debris. Ultimately there was replacement of the necrotic tissue with fibroblasts. These residual reactions were studied after intervals of 14, 22, 27, and 35 days. In 35 days there was little or no cellular reaction to be seen. When necrosis had been present the residual reaction was predominantly of scar tissue.

With a testing injection of 0.1 mg. of MA-100 we did not obtain necrosis, even in the case of animals which had shown necrosis after later injections of the phosphatide plus protein. Thus, as is shown on Chart 1, the groups of guinea pigs which received either MA-100 or TPA with the phosphatide showed only three plus reactions in the final test with MA-100. In another series of guinea pigs, however, the final test was made with 0.5 mg. of MA-100 and in these tests the highly sensitized animals did show four plus reactions, that is, with necrosis.

Dr. Seibert[2] has found that guinea pigs sensitized with tuberculoprotein TPA until they gave three and four plus reactions with the same antigen did not react to the purified protein derivative, PPD.

[2] Personal communication.

This is in agreement with the results of Boquet, Sandor, and Schaefer (23) who sensitized guinea pigs to tuberculo-protein and failed to elicit skin reactions in them with tuberculin. We have confirmed these results. A series of ten guinea pigs were sensitized with five injections of phosphatide (5 mg.) plus tuberculo-protein TPA (0.5 mg. in 0.1 cc. saline) intradermally every 4 days. To the fifth injection all of them showed either three or four plus reactions. The five which were the most highly sensitized were then tested with 0.1 mg. of PPD in saline and all of them were negative. The other five received another injection of the phosphatide plus protein and then all were inoculated subcutaneously with 0.1 mg. of virulent tubercle bacilli, human strain H-37. The entire group failed to show the Koch phenomenon. The animals were watched for $3\frac{1}{2}$ weeks, during which time they reacted like the normal controls; there was no immediate irritation to the injection of the bacilli; the nodules formed and ulcerated as did those of the non-sensitized animals.

DISCUSSION

For the thesis that tuberculo-phosphatide has the power to enhance sensitization to tuberculo-protein, it was necessary to establish the point that this power is due to the phosphatide itself and not to contaminating dead tubercle bacilli containing protein. If bacilli were present in adequate numbers they would certainly be a factor in inducing particular cellular reactions as well as sensitization. In some preparations of phosphatide other than the one used in these experiments, tubercle bacilli have been present and have been readily demonstrated both in films of the phosphatide and in giant cells induced in animals after injection of the material. However, in the preparation of phosphatide used in these experiments no bacilli were found. More conclusive than this negative evidence is the fact that a total of 30 mg. of this phosphatide alone, given in 6 weekly intradermal injections, failed to sensitize any of six guinea pigs to the protein, and the addition of 0.025 mg. of a tuberculo-protein to 1 mg. of the phosphatide produced a mixture that did sensitize four out of eight guinea pigs. This indicates that the preparation of phosphatide used in these experiments did not carry enough protein to be biologically significant and this makes valid the claim that the material

itself has the power to enhance the potency of the protein to sensitize.

It is clear that the active tuberculo-protein sensitizes guinea pigs when given by the intradermal route, as shown in the increasing reactions exhibited on Chart 1. The enhancement of this sensitization by the addition of tuberculo-phosphatide is shown by the greater size of corresponding reactions, by the lighting up of the site of previous injections, by the development of marked induration in the lesions, the early appearance of necrosis, and the fact that the reaction does not fade as quickly as when protein alone is used; in addition, animals so sensitized react positively to tuberculo-protein alone.

Every injection of either protein alone or with phosphatide is followed immediately by an outpouring of neutrophiles from the blood stream as well as by an increase in monocytes. Both of these types of cells appear in greater numbers after the mixed injections. However, it is the amount of the residual reactions of monocytes and epithelioid cells, also greater after the mixed injections, which correlates with the degree of sensitization.

Returning to the concept of Baldwin, that "if tuberculin reactivity is present in any animal, the presence of tubercles in that animal must be predicated," it is our opinion that it is possible to sensitize guinea pigs to active tuberculo-protein by virtue of the cellular reactions which this material induces. The protein itself can induce a new formation of monocytes and some preparations of the protein give rise to a few epithelioid cells. The use of the phosphatide with the protein greatly increases the formation of monocytes which become transformed into tubercle-like masses of epithelioid cells. We consider that it is this much increased cellular reaction due to the use of the phosphatide which, in the presence of the active antigenic agent, is correlated with the more rapid and the more effective sensitization. The use of the skin as the locus of the injection is also a factor since in this tissue the antigen remains concentrated for a longer time around the cells. The phosphatide plus protein used in the skin brings about a sensitization which is much like the sensitization of the disease itself. From these studies it seems justifiable to stress the fact that an increase in monocytes and epithelioid cells accompanies the phenomenon of sensitization and that in some way the phagocytic

mononuclear cells, specifically monocytes and their derivatives, epithelioid cells, play a definite rôle in the mechanism that sets up sensitization.

In the cellular reactions it is important to discriminate between the direct effects of the materials introduced and the indirect effects of the sensitization. The direct effects are the infiltrations with neutrophiles and the new formation of monocytes and epithelioid cells. There is also some infiltration of the lesions with eosinophiles certainly after the initial stages. Since they also occur after injections of phosphatide without sensitization, it is not possible at the present time to analyze their relation to sensitization. They have been noted repeatedly in sensitized tissues (Seibert, 8 *a*). It is interesting to note that we have not found any increase in lymphocytes in these dermal reactions.

The indirect effects of sensitization are edema, hemorrhage, and necrosis. The hemorrhage and necrosis set up new and complicated cellular reactions, new infiltrations with neutrophiles, phagocytosis of debris, and ultimately the new formation of vessels, new fibrous tissue, and bands of fibroblasts.

Our experiments indicate that the skin is a much more effective organ for inducing the type of sensitization that expresses itself in skin reactivity than either the lining of the peritoneal cavity or the subcutaneous tissue. This difference in effectiveness can be expressed by the following comparison. Using the peritoneal route of injection, the minimum amount of protein necessary to sensitize any guinea pig was 30 mg. (Seibert, 8 *a*); by the subcutaneous route, 5 mg. (Smithburn, Sabin, and Geiger, 19); by the intradermal route the amount can be reduced to 0.025 mg. when enhanced with phosphatide. These materials bring about the same cellular reactions whether introduced intraperitoneally, subcutaneously, or intradermally. After injection by the intraperitoneal route the induced cells are widely dispersed throughout the omentum and under the serosal lining of the abdominal viscera and parietal peritoneum. In the dermis, on the other hand, the reaction is limited to a small area, due to the fact that the injections are made into a dense feltwork of fibers which acts as a mechanical barrier to the spread of fluid. It is, of course, true that some of the injected fluid enters the dermal lymphatics immediately

and is carried to the regional lymph nodes, but there is also a drainage of fluid into lymph nodes from every zone of injection. With intradermal injections, however, the amount of protein remaining in a restricted area is much greater so that the effect of the unique structure of the skin as a sensitizing zone may be summed up in the phrase that it provides a greater "dose per cell" of the sensitizing agent.

The question must now be considered of whether the sensitization which can be induced by tuberculo-protein and enhanced by tuberculo-phosphatide, as evidenced by the intradermal test, is identical with the type of sensitization to foreign protein first described by Arthus, or is rather to be regarded as a special type to be known as tubercular allergy. If by Arthus phenomenon is meant a reaction in the skin of sensitized animals that runs somewhat parallel to the precipitin titer of the serum of the animal to the same antigen, and to the phenomenon of passive transfer by antibodies, then neither the sensitization in the disease tuberculosis, nor the sensitization induced by tuberculo-protein can be regarded as identical with the Arthus reaction. For Freund, Laidlaw, and Mansfield (24) have shown that in tuberculous rabbits there is no correlation between complement fixation and the skin test, and Seibert (8 *a*, *d*, *e*) has shown that the same is true of rabbits sensitized to the tuberculo-protein. By means of the use of tuberculo-phosphatide with protein, it is possible to induce in guinea pigs a sensitization manifested by a delayed reaction which appears in 24 hours, lasts for 48 hours, shows induration, hemorrhage, and necrosis, and is like a four plus tuberculin test in a tuberculous animal. When these animals are tested with 0.1 mg. of another preparation of protein (MA-100), they show characteristic three plus tuberculin reactions and four plus with necrosis when 0.5 mg. is used. It is our opinion that in the disease tuberculosis there is a mechanism of which some part, at least, is like an Arthus phenomenon; that in the disease the mechanism may be much more complex than is the reaction to a single protein, but that some of the difference may be due to a variation in the amount of antibody free in the circulation and in the amount and kind of change in the cells. If it be not legitimate to consider the change in the sensitized cells of the tuberculous animal as due to antibodies, it is, nevertheless, due to some change in the cells themselves, probably induced by proteins

set free from the infecting bacilli. That the cells themselves are sensitized to the protein was shown by Rich and Lewis (25) by the method of tissue culture. It was later shown by Moen (26) that the cells from a tuberculous animal remained sensitive after several generations in cultures.

Inasmuch as the animals highly sensitized to tuberculo-protein did not react to tuberculin, PPD, and did not show the Koch phenomenon when they were inoculated with living tubercle bacilli, it is clear that the changes in tuberculous animals are much more complex than in simple sensitization. The suggestion is made that the processes of immunization in tuberculosis are not identical with sensitization. Such highly sensitized animals offer valuable material for the further study of this relationship.

CONCLUSIONS

1. Guinea pigs can be rendered hypersensitive to tuberculo-protein by small, repeated, intradermal injections of active tuberculo-protein.

2. The addition of tuberculo-phosphatide to the protein speeds up the process of sensitization and enhances it so that the reactions become indurated and necrotic, closely simulating those of the disease.

3. Active tuberculo-proteins induce a new formation of monocytes and some epithelioid cells. The addition of phosphatide to the protein brings about a massive formation of epithelioid cells.

4. With the increased cellular reaction to the mixed injections may be correlated the increase in the speed and intensity of the sensitization.

5. The intradermal route is the best for these sensitizations, probably because it provides the greatest dose per cell of the sensitizing agent.

6. The degree of sensitization artificially obtainable by the synergistic action of tuberculo-phosphatide and tuberculo-protein is quite comparable to the degree of sensitization naturally occurring in tuberculous animals; moreover, this degree of sensitization may be induced with amounts of the materials from the bacilli which could conceivably be present in the tissues of an infected host.

BIBLIOGRAPHY

1. Baldwin, E. R., (*a*) *J. Med. Research*, 1910, **17**, 189; (*b*) *Nat. Assn. Stud. and Prev. Tuberc., Tr. 7th Ann. Meeting*, 1911, 351.
2. Krause, A. K., *J. Med. Research*, 1916–17, **30**, 1.
3. Borrel, A., *Bull. Soc. path. exot.*, 1908, **1**, 420.
4. Bessau, G., *Berl. klin. Woch.*, 1916, **53**, 801.
5. Petroff, S. A., *Am. Rev. Tuberc.*, 1923, **7**, 412.
6. Petroff, S. A., and Stewart, F. W., *J. Immunol.*, 1925, **10**, 677.
7. Lange, B., and Freund, R., *Z. Hyg. u. Infektionskrankh.*, 1925–26, **105**, 571.
8. Seibert, F. B., (*a*) *J. Infect. Dis.*, 1932, **51**, 383; (*b*) *Tr. Nat. Tuberc. Assn.*, 1933, 29th Annual Meeting, 165; (*c*) *Am. Rev. Tuberc.*, 1934, **30**, 713; (*d*) *J. Bact.*, 1935, **29**, 31; (*e*) *J. Immunol.*, 1935, **28**, 425.
9. Heidelberger, M., and Menzel, A. E. O., *J. Biol. Chem.*, 1934, **104**, 655.
10. Gaehtgens, W., *Deutsch. med. Woch.*, 1926, **52**, 1076.
11. Dresel, E. G., and Meissner, G., *Z. Immunitätsforsch.*, 1927, **53**, 332.
12. Tytler, W. H., Allergy and immunity in tuberculosis, in A system of bacteriology in relation to medicine, Great Britain Medical Research Council, London, His Majesty's Stationery Office, 1930, **5**, 228.
13. Saenz, A., *Compt. rend. Soc. biol.*, (*a*) 1935, **120**, 1050; (*b*) 1937, **125**, 714.
14. Olitsky, P. K., and Harford, C. G., (*a*) *Proc. Soc. Exp. Biol. and Med.*, 1938, **38**, 92; (*b*) *Am. J. Path.*, 1937, **13**, 729.
15. Jørgensen, J. V., *Acta path. et microbiol. Scand.*, 1938, **15**, 1.
16. Seibert, F. B., and Munday, B., *Am. Rev. Tuberc.*, (*a*) 1931, **23**, 23; (*b*) 1932, **25**, 724.
17. Masucci, P., and McAlpine, K. L., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 661.
18. Anderson, R. J., (*a*) *J. Biol. Chem.*, 1927, **74**, 537; (*b*) *Physiol. Rev.*, 1932, **12**, 166.
19. Smithburn, K. C., Sabin, F. R., and Geiger, J. T., *Am. Rev. Tuberc.*, 1934, **29**, 562.
20. Hetherington, H. W., McPhedran, F. M., Landis, H. R. M., and Opie, E. L., *Am. Rev. Tuberc.*, 1929, **20**, 421.
21. Sabin, F. R., Doan, C. A., and Forkner, C. E., *J. Exp. Med.*, 1930, **52**, suppl. 3, 1–152.
22. Smithburn, K. C., and Sabin, F. R., *J. Exp. Med.*, (*a*) 1932, **56**, 867; (*b*) 1938, **68**, 641.
23. Boquet, A., Sandor, G., and Schaefer, W., *Ann. Inst. Pasteur*, 1937, **59**, 577.
24. Freund, J., Laidlaw, E. H., and Mansfield, J. S., *J. Exp. Med.*, 1936, **64**, 573.
25. Rich, A. R., and Lewis, M. R., *Bull. Johns Hopkins Hosp.*, 1932, **50**, 115.
26. Moen, J. K., *J. Exp. Med.*, 1936, **64**, 943.

EXPLANATION OF PLATES

PLATE 29

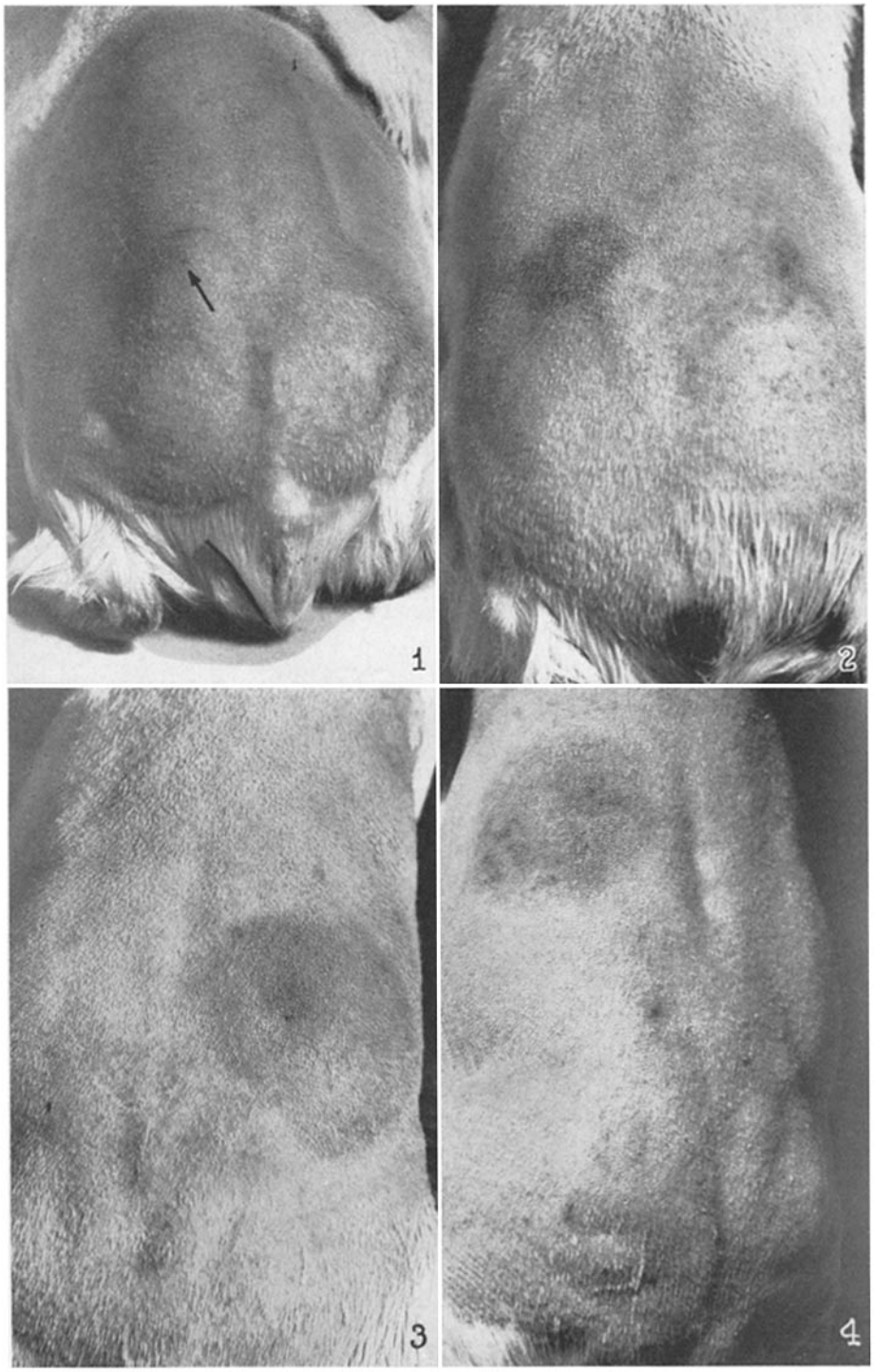
FIG. 1. Site of the reaction 24 hours after an intradermal injection of 0.5 mg. of tuberculo-protein TPA (Seibert) in 0.1 cc. saline in a normal guinea pig (R 6292[3]). Figs. 1 to 8 are ¾ natural size.

FIG. 2. Site of the reaction 24 hours after the second injection of the same protein in guinea pig R 6200.

FIG. 3. Site of the reaction 24 hours after the third injection of the same protein in guinea pig R 6196.

FIG. 4. Site of the reaction 24 hours after the fourth injection of the same protein in guinea pig R 6198.

[3] These numbers are serial numbers covering the work of the laboratory for a term of years.



Photographed by Joseph B. Haulenbeek

(Sabin and Joyner: Tubercular allergy without infection)

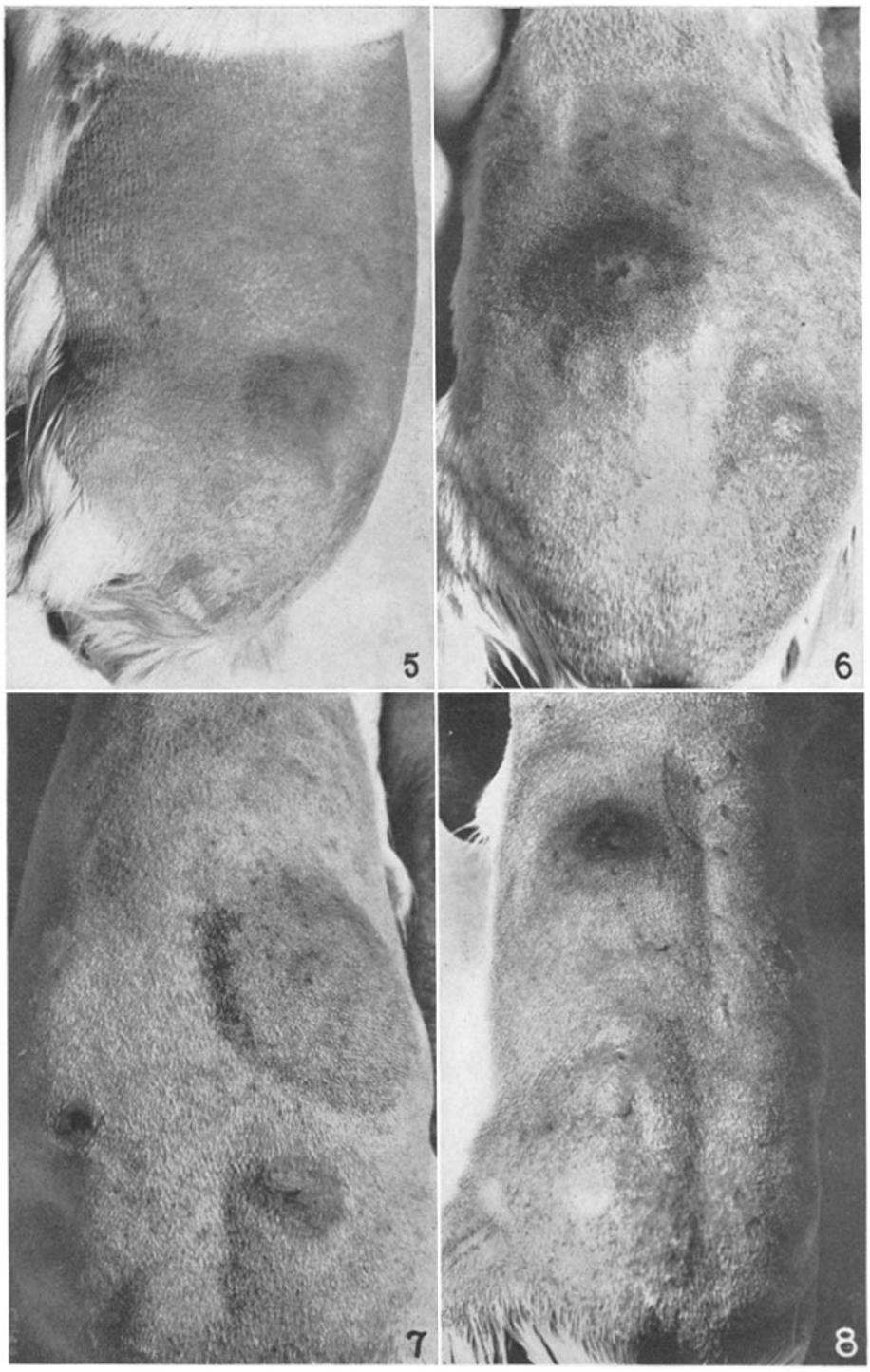
PLATE 30

FIG. 5. Site of the reaction 24 hours after an intradermal injection of 0.5 mg. of tuberculo-protein TPA (Seibert) plus 5 mg. of tuberculo-phosphatide (Anderson) in 0.1 cc. saline in a normal guinea pig (R 6291).

FIG. 6. Site of the reaction 24 hours after the second injection of the same mixture, left side, with a flare-up of the first injection, right side, in guinea pig R 6192.

FIG. 7. Site of the reaction 24 hours after the third injection of the same mixture, upper right side, with a flare-up of the second, lower right, and of the first, left side, in guinea pig R 6188.

FIG. 8. Site of the reaction 24 hours after the fourth injection of the same mixture, showing massive necrosis, in guinea pig R 6191. The other three sites showed the flare-up but the photograph does not include them.



Photographed by Joseph B. Haulenbeek

(Sabin and Joyner: Tubercular allergy without infection)

PLATE 31

FIG. 9. Section of the skin from the back of guinea pig R 6201, 24 hours after a first injection of 0.5 mg. of protein TPA (Seibert) in 0.1 cc. saline to show the increase in monocytes and neutrophiles in the lower part of the dermis and the subcutaneous tissue. Stained in Giemsa. × 375.

FIG. 10. Section of the skin from the back of guinea pig R 5790, 24 hours after a first injection of 0.5 mg. of protein TPA (Seibert) plus 5 mg. of phosphatide (Anderson) in 0.1 cc. saline, to show the increased cellularity. Stained in Giemsa. × 45.

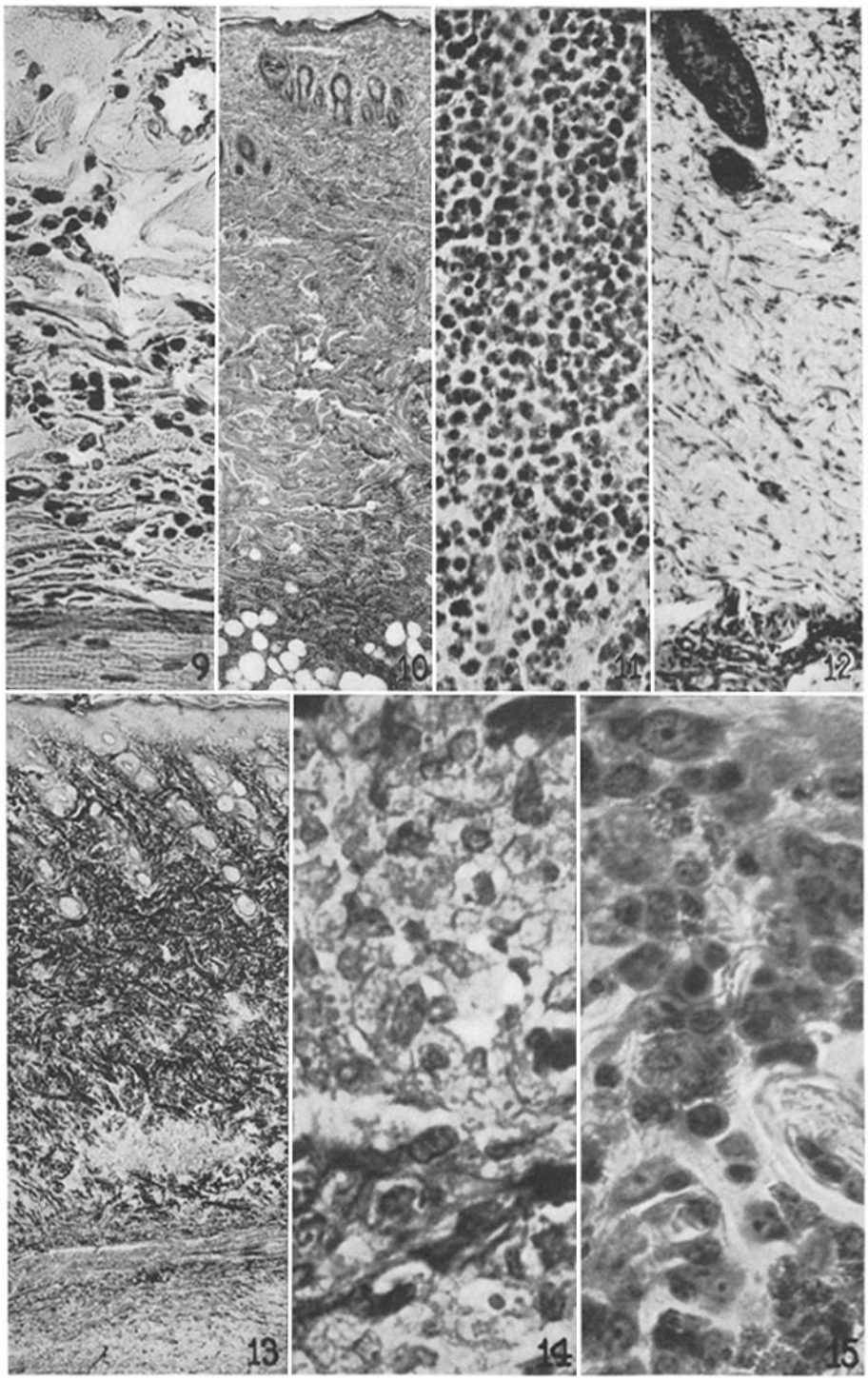
FIG. 11. Section of the area of densest cellularity of Fig. 10, showing a mixture of neutrophiles and monocytes in the deepest layers of the dermis. × 375.

FIG. 12. Section of the skin of the back of guinea pig R 6200-1, showing the site of a first injection of 0.5 mg. of tuberculo-protein in 0.1 cc. saline, taken 24 hours after a second injection had been made. This reaction is 7 days old. It shows an increase of monocytes in the dermis, especially marked in the perivascular areas. Stained in Giemsa. × 105.

FIG. 13. Section of the skin of the back of a guinea pig (R 6193-1), showing the site of a first injection of 0.5 mg. of tuberculo-protein TPA (Seibert) plus 5 mg. of phosphatide (Anderson) in 0.1 cc. saline, taken 24 hours after the second injection had been made. This reaction is 7 days old. It shows tubercle-like masses of epithelioid cells in the dermis and in the subcutaneous level. Stained with Foot's modification of the Masson method. × 37.

FIG. 14. Section of a part of the tubercle shown in Fig. 5, showing the character of the epithelioid cells. × 700.

FIG. 15. Section from the skin of the back of a guinea pig (R 6192-3), 24 hours after a third injection of 0.5 mg. of protein TPA (Seibert) plus 5 mg. of phosphatide (Anderson) in 0.1 cc. saline. The photograph shows young monocytes and eosinophiles beneath the carnosus muscle to which level the reaction had spread. Stained in Giemsa. × 700.



Photographed by Joseph B. Haulenbeek

(Sabin and Joyner: Tubercular allergy without infection)