SENSITIZATION AND ANTIBODY FORMATION WITH INCREASED RESISTANCE TO TUBERCULOUS INFECTION INDUCED BY HEAT KILLED TUBERCLE BACILLI*

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In a previous publication (1) the immunizing effect of killed tubercle bacilli alone or in combination with other antigens or irritants has been compared with that of an avirulent living tubercle bacillus (BCG) by measuring the resistance of immunized rabbits to infection with highly virulent bovine tubercle bacilli. The evidence presented shows that rabbits immunized with heat killed tubercle bacilli acquire effective protection against infection fatal to unprepared rabbits. A few animals are no more resistant than control rabbits, whereas others with scant lesions live much longer and a considerable number recover from the infection completely. Protection by killed tubercle bacilli affords a new opportunity to study the relationship of sensitization and antibodies to immunity against tuberculosis because the progress of immunization is not modified by a living microorganism which may multiply in the body. One purpose of these experiments has been to determine if heat killed tubercle bacilli can be used to protect human beings against tuberculosis. Hence we have attempted to determine what are the conditions that modify sensitization and antibody production and how these changes affect the local lesion produced by killed tubercle bacilli. Furthermore we have attempted to determine the relation of sensitization and antibody formation to resistance produced by protective inoculation.

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Sensitization with Allergic Inflammation of the Skin Induced by Heat Killed Tubercle Bacilli and by BCG and Measured by These Agents or by Tuberculin

The plan of experiment has been to inject a constant amount of heat killed tubercle bacilli into the skin at weekly intervals and observe the skin reactions at the site of injection 2 days later. These intracutaneous injections have served both to sensitize animals and to measure the progress of sensitization. The dose, 0.2 mg., has been selected because it is approximately half of that which causes suppuration within 2 days when injected into the skin of normal rabbits. Injection of 0.2 mg. of heat killed tubercle bacilli produces slight redness and edema in an area about 3 mm. across, raised very little above the skin surface and usually recognizable by touch. In a few instances the area of edema is 5 mm. across.

The progress of sensitization as shown by allergic inflammation in animals treated with heat killed tubercle bacilli has characters that deserve consideration. In some animals with successive injections there is a continuous increase in the diameter and height of the inflammatory edema that follows intracutaneous injection, but in others no significant change can be observed after the first 4 or 5 injections. In most animals local edematous swelling maintains its maximum extent during a period of from 5 to 10 weeks after the beginning of injections, when it usually measures between 30 and 50 mm. in diameter. Later there is less edema and the lesion at the site of injection is less extensive, but more elevated, reaching occasionally 10 mm. in height. It is now firmer than in the period of maximum edema, and suppuration, indicated by a yellow spot at the summit of the elevation, often occurs within 48 hours after injection. Suppuration is promptly followed by ulceration and usually after 1 or 2 weeks by complete healing.

The progress of sensitization induced by BCG and measured by the reaction to BCG does not differ essentially from that induced by heat killed tubercle bacilli and tested with heat killed bacilli, but it proceeds in general somewhat more rapidly and edema is more extensive.

With the quantity of old tuberculin that has been used to test sensitization in rabbits, namely, with 0.2 cc. of a 1 in 5 dilution of old tuberculin (40 mg.), allergic inflammation as measured by the diameter of the area of inflammatory edema 48 hours after injection

has been almost identical with that produced by 0.2 mg. BCG. When reactions to this quantity of tuberculin are compared with those produced by 0.2 mg. of heat killed tubercle bacilli, the reactions are in general more extensive with tuberculin.

Variations in the Progress of Sensitization in Different Animals

Rabbits vary widely in the rapidity with which they undergo sensitization following repeated injection with dead tubercle bacilli or with BCG, as indicated by the local inflammatory reaction to the injected substance when introduced into the cutis or to old tuberculin.

TABLE I

Variation in Progress of Sensitization among Rabbits Repeatedly Injected into the Cutis with 0.2 Mg. of Heat Killed Tubercle Bacilli

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left			
	Tested with heat killed tubercle bacilli		Tested with tuberculin	
	After 4 to 5 wks.	After 6 to 8 wks.	After 4 to 5 wks.	After 6 to 8 wks.
mm.				
0–9	9	1	4	2
10–19	5	3	2	3
20–29	5	7	3	2
30–29	3	8		3
40–49	1	4	4	2
50–59			1	2
Total.....	23	23	14	14

Table I shows the maximum diameter of redness and edema produced by heat killed tubercle bacilli or by tuberculin after 4 to 5 and after 6 to 8 injections of the former.

Edema less than 10 mm. in diameter may be regarded as evidence of insignificant sensitization and after 4 to 5 weeks 9 of 23 animals have failed to reach this level (Table I). Nevertheless, after 8 weeks only one animal remains below this figure and the general trend in the interval has been conspicuously toward more extensive reactions. Sensitization tested by tuberculin in a smaller number of animals shows similar variation in intensity.

Sensitization of animals repeatedly injected with BCG proceeded more rapidly than that produced by heat killed tubercle bacilli. The trend between 4 and 20 weeks was toward somewhat more severe reactions but differences were inconspicuous.

The foregoing observations show that individual rabbits vary widely in the readiness with which they are sensitized by heat killed tubercle bacilli or by BCG, but few escape conspicuous sensitization after repeated injection of the antigen.

The Fate of Lesions of Allergic Inflammation Produced by Heat Killed Tubercle Bacilli and by BCG

It has been considered desirable to study in rabbits the changes in local lesions produced by heat killed tubercle bacilli because in inoculated persons a single injection of BCG produces skin ulceration and often suppuration of adjacent lymph nodes in a large proportion of instances. The fate of skin lesions produced by the repeated injection of heat killed tubercle bacilli is modified by developing sensitization (Fig. 1).

The lesions, which in general have been produced at intervals of 1 week, have been remeasured each week. The lesion of the first injection is small throughout its course and has disappeared after 5 or 6 weeks before sensitization is well established (see graph at top of Fig. 1). The local lesions of the next 2 or 3 injections persist through a longer period and are still present at a time when sensitization is increasing rapidly. At this time they undergo noteworthy increase in size (see Fig. 1), which is doubtless analogous to the focal increase of tuberculous lesions produced by injection of tuberculin into tuberculous animals. It recalls the enlargement of the local lesions observed by Andrewes, Derick and Swift (2), 8 days after they injected hemolytic streptococci into the skin and coincident with the appearance of sensitization to filtrate from cultures of the microorganism. The progress of sensitization and the corresponding fate of lesions produced by BCG is shown diagrammatically in Fig. 2.

The rapidity with which suppuration and subsequent ulceration occurs at the site of injection into sensitized animals determines the duration of lesions because ulceration in most instances is followed within from 1 to 3 weeks by healing and complete disappearance of the lesion. Lewandowsky (3) showed that ulceration at the site of

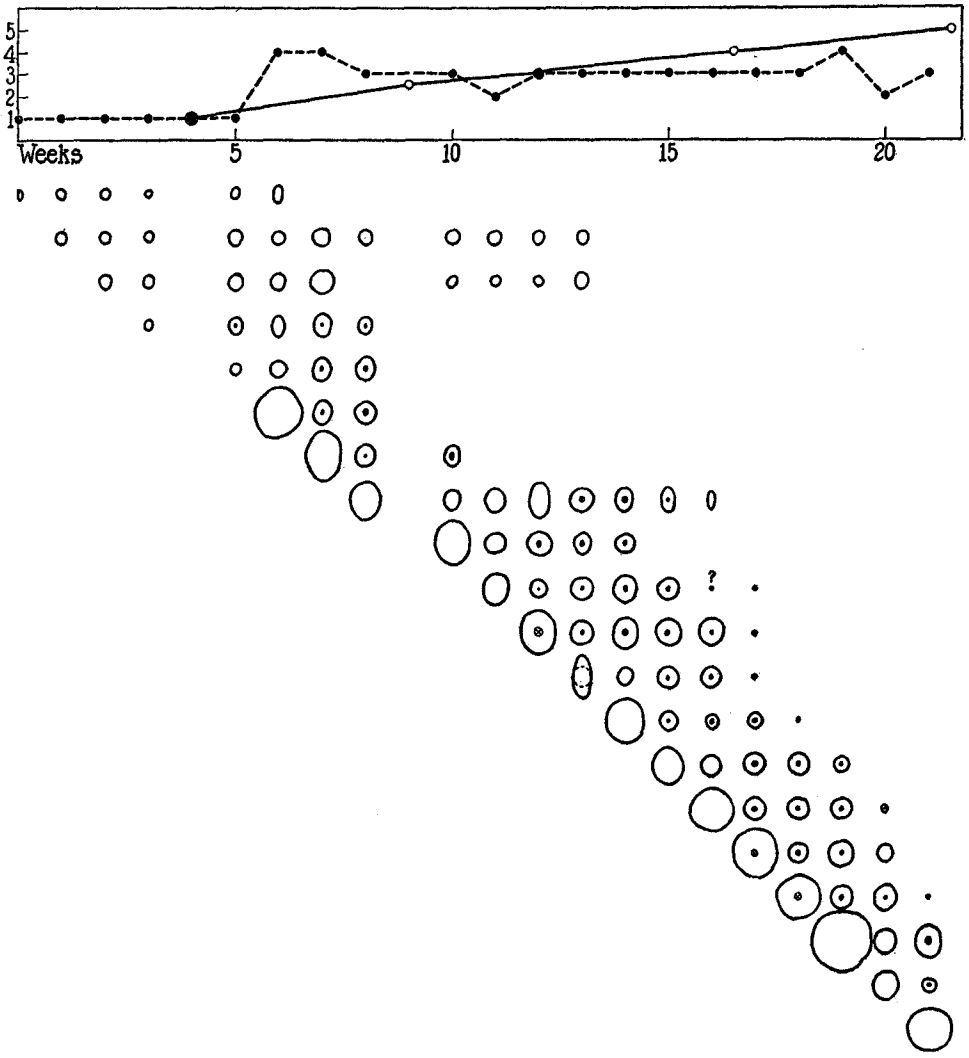

FIG. 1. At the top of the figure is a graph showing the progress of sensitization in a rabbit that received repeated intracutaneous injections of heat killed tubercle bacilli at intervals of 1 week. The solid line represents the relative diameters of skin lesions produced by old tuberculin and the broken line those of lesions produced by heat killed tubercle bacilli. The relative size of the lesions produced by weekly intracutaneous injections of tubercle bacilli is shown below the graph and the time at which observations were made is shown by the horizontal scale of weeks, simultaneous observations being in vertical lines below it. In this and in subsequent graphs the figures at the left of the ordinate indicate sensitization measured by the diameter of allergic inflammation, 1 being 8 to 9 mm.; 2, 10 to 19 mm.; 3, 20 to 30 mm.; 4, 30 to 40 mm., etc.; or the titer of complement fixation, 1 being 1:5; 2, 1:10; 3, 1:20; 4, 1:40; 5, 1:80, etc.

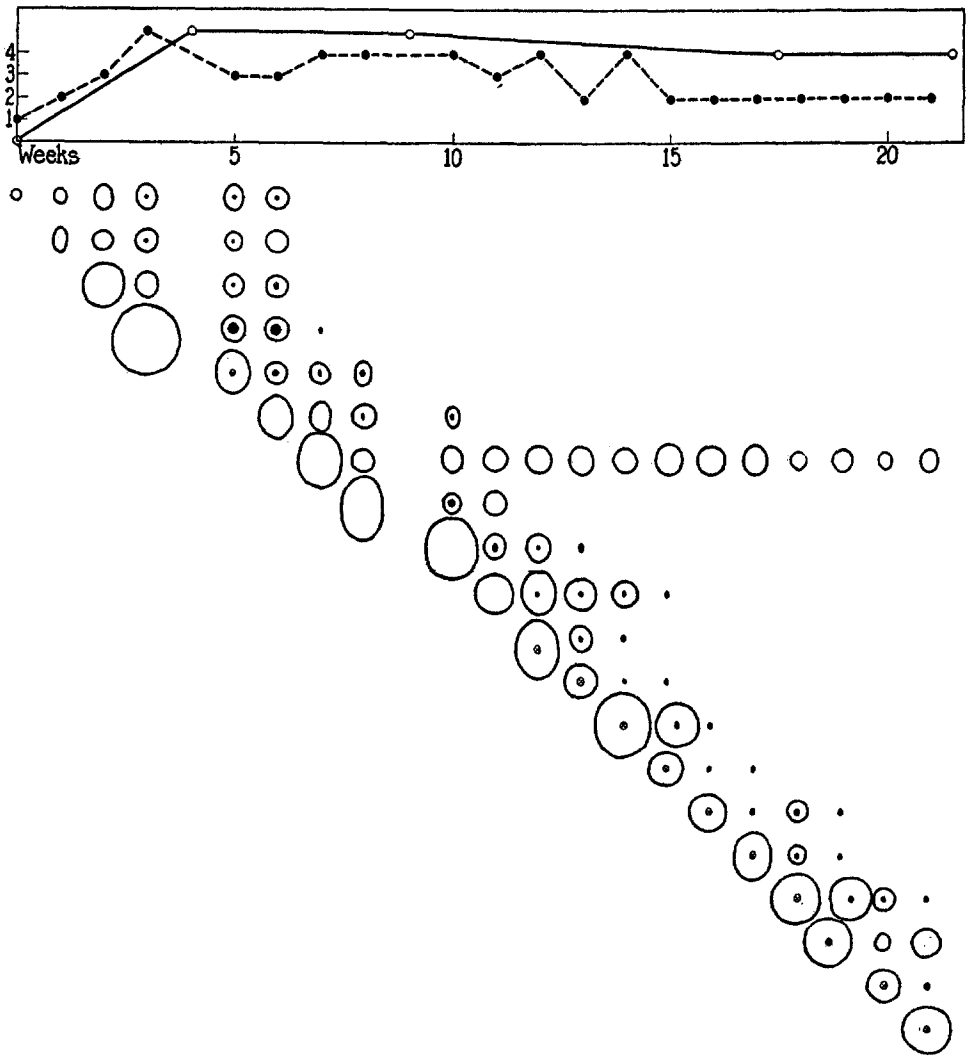

FIG. 2. The progress of sensitization and changes in intracutaneous lesions produced by repeated injections of BCG at intervals of 1 week (for explanation see Fig. 1). The broken line shows the progress of sensitization as measured by the size of skin lesions produced by BCG.

inoculation of living tubercle bacilli into sensitized animals was associated with the discharge of the microorganism to the exterior in the material that sloughed away. Table II shows the average duration of local lesions produced in 9 rabbits at intervals of 1 week.

When sensitization caused by heat killed tubercle bacilli reaches its maximum lesions persist only about 5 weeks. Intracutaneous lesions following injection of BCG produce suppuration and subsequently heal more promptly than those induced by heat killed

TABLE II

Average Duration of Intracutaneous Lesions in Nine Rabbits Produced at Weekly Intervals by Intracutaneous Injection of 0.2 Mg. of Heat Killed Tubercle Bacilli and of BCG

Time after beginning of injections when lesions were produced	Average duration of intracutaneous lesions produced by heat killed tubercle bacilli	Average duration of intracutaneous lesions produced by BCG
wks.	*wks.*	*wks.*
1	8.2	6.8
2	11.7+	6.5
3	12.0+	6.0
4	8.1+	6.7
5	6.4+	7.1
6	4.7	7.5+
7	9.0+	8.3+
8	9.4+	5.7+
9	6.4	6.5
10	4.4	5.7+
11	5.5	3.8
12	5.0	5.1

tubercle bacilli. The persistence of lesions at the site of injection of heat killed tubercle bacilli is a functional index of the persistence of the antigen at the site of its injection. It is improbable that its immunizing activity outlasts its ability to produce a local lesion.

Repeated subcutaneous injection of heat killed tubercle bacilli at intervals of 1 week produces no palpable nodules until sensitization appears. After sensitization small subcutaneous nodules are formed but ulceration rarely occurs.

Sensitization Produced by Intracutaneous and by Subcutaneous Injection of Heat Killed Tubercle Bacilli

Experiments have been undertaken to determine if the progress

of sensitization measured by old tuberculin differs when heat killed tubercle bacilli are introduced into the cutis on the one hand, or into the subcutaneous tissue on the other.

The progress of sensitization following intracutaneous and subcutaneous injection of 0.2 mg. of heat killed tubercle bacilli at intervals of 1 week is shown by composite graphs in Fig. 3, obtained from 9 rabbits injected intracutaneously and 10 injected subcutaneously. Sensitization proceeds more rapidly with the former but after 8 weeks sensitization following subcutaneous injection has reached the same level as that after intracutaneous injection.

In the experiments that follow (Table III), 6 injections of 0.2 mg. of heat killed tubercle bacilli have been given at intervals of 1 week; in the first group of experiments this quantity has been given each week in a single injection; in the second group it has been divided into 4 simultaneous injections, of which each is 0.05 mg.; and in the third group it has been similarly divided into 8 simultaneous injections, each of 0.025 mg.

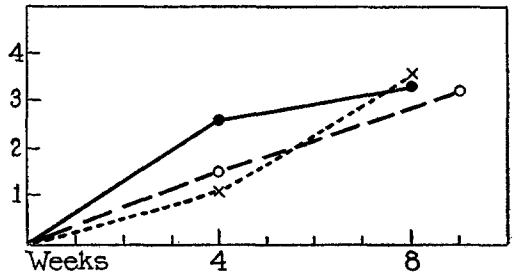

FIG. 3. Composite graph showing the progress of sensitization measured by tuberculin in 9 animals following the intracutaneous (solid line), in 10 animals following subcutaneous (coarsely broken line), or in 13 animals following intravenous (finely broken line) injection of heat killed tubercle bacilli.

The three groups of experiments show uniformly that sensitization proceeds more rapidly following intracutaneous than following subcutaneous injection, so that after 4 weekly injections a smaller number of animals resist sensitization and in general reactions are more intense. After 8 weekly injections reactions are stronger and all animals have become well sensitized.

Effect of Different Quantities of Heat Killed Tubercle Bacilli upon the Progress of Sensitization

In order to determine the effect of a minimal stimulus upon the production of sensitization a single injection of 0.2 mg. of heat killed

tubercle bacilli has been injected into the subcutaneous tissue and the progress of sensitization compared with that produced by 4 injections of the same quantity of heat killed tubercle bacilli adminis-

TABLE III

Progress of Sensitization to Tuberculin with Cutaneous and Subcutaneous Injection of Heat Killed Tubercle Bacilli and with Undivided and Divided Doses

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left			
	With intracutaneous injection		With subcutaneous injection	
	During 4 wks.	During 8 wks.	During 4 wks.	During 8 wks.
mm.				
With injections of 0.2 mg. each week				
0–9	1		2	
10–19				
20–29	1		2	
30–39		1		1
40–49	3	2	1	1
50–59		2		2
60–69				1
With 4 simultaneous injections of 0.05 mg. each week				
0–9			2	
10–19	1		1	
20–29				
30–39	1	1	2	
40–49	2	2		4
50–59		1		1
With 8 simultaneous injections of 0.025 mg. each week				
0–9			2	
10–19			1	
20–29	2			
30–39	2	1		1
40–49		2	1	1
50–59	1	1	1	3
60–69		1		

tered at intervals of 1 week (Table IV; the progress of antibody formation in the same experiment is shown in Table VIII).

The experiment shows that 0.2 mg. of heat killed tubercle bacilli in 1 injection is ineffective in the production of sensitization in rabbits and even after 4 injections at intervals of 1 week few animals have become sensitized. Sensitization with BCG in 1 or in 4 injections

has been much more successful. Nevertheless, after an interval of rest, that is, after 6 weeks (Table IV), these differences have in considerable part disappeared.

Injections of 0.2 mg. of heat killed tubercle bacilli continued during 6 weeks have been much more effective in the production of sensitization that 1 or 4 similar injections (Table IV). In one experiment increasing the initial dose from 0.2 mg. of heat killed tubercle bacilli (10 animals) to 7 times this amount (39 animals) has had little effect upon the progress of sensitization judged by the number that resist sensitization (with lesions less than 10 mm. in diameter) after

TABLE IV

Progress of Sensitization to Tuberculin Produced by a Single Injection of Heat Killed Tubercle Bacilli or of BCG Compared with That Following Four Similar Injections at Intervals of 1 Week

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left											
	Heat killed tubercle bacilli						BCG					
	With 1 injection			With 4 injections			With 1 injection			With 4 injections		
	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.
mm.												
0–9	9	6	7	9	6	7	4	2	5	3		5
10–19		2	2		1		2	1	1	2		2
20–29		1			1		1		1	1	1	1
30–39					1	1	2	5	1	2	5	1
40–49								1		2	2	1
50–59											2	

4 weeks, but has increased the proportion of those with stronger reactions (30 to 49 mm. in diameter). With an increase of the injected dose from 0.2 to 0.4 mg. (24 animals) there has been uniform diminution in the proportion of rabbits that resist sensitization and an increase of the proportion with strong reactions.

Sensitization Following Intravenous Injection of Heat Killed Tubercle Bacilli

Intravenous injection of dead tubercle bacilli has proven to be a relatively ineffective and very dangerous method of producing sen-

sitization. The progress of sensitization following intravenous, intracutaneous and subcutaneous injection of 0.2 mg. of heat killed tubercle bacilli at intervals of 1 week is shown by the composite graphs in Fig. 1, of which that showing sensitization induced by intravenous injection has been obtained from 13 animals. It is evident that sensitization following intravenous and subcutaneous injection of heat killed tubercle bacilli proceeds more slowly than that after intracutaneous injection, so that after 4 weeks sensitization with intracutaneous injection is much stronger. After 8 weeks, sensitization following both subcutaneous and intravenous injection has increased and reached the level of that induced by intracutaneous injections. There has been no essential difference in the progress of intravenous and subcutaneous sensitization.

TABLE V

Progress of Sensitization after Intravenous Weekly Injections of 0.2 Mg. of Heat Killed Tubercle Bacilli

Diameter of inflammatory reaction	After 4 wks.	After 8 wks.
mm.		
0–9	8	5
10–19	1	
20–29	2	2
30–39	1	2
40–49	1	
50–59		3

The range of reactions is better shown by Table V, in which it is evident that a considerable number of animals resist sensitization though a few become well sensitized.

Intravenous injection of heat killed tubercle bacilli into animals that are highly sensitized is attended by considerable risk. 2 of the rabbits that have developed intense sensitization, included in Table V, have died within 1 day, and 2 after 1 week, following the intravenous injection of heated tubercle bacilli.

Antibody Formation

Antibody formation has been studied in the animals that have been used to follow the progress of sensitization and the plan of the experiments has been the same.

The complement fixation tests have been performed with sera heated for 30 minutes at 55°C. and a suspension of the Ravenel strain has been used as antigen. The latter is prepared by suspending a culture with the aid of grinding in normal saline so that 1 cc. of the suspension contains 10 mg. of tubercle bacilli. The suspension is heated for 30 minutes at 60°C. and 0.35 per cent tricresol is added as a preservative. About one-fourth of the self-inhibition dose is used for the complement fixation. The three ingredients, namely, the serum to be tested, the antigen suspension and the guinea pig serum containing complement, are mixed and incubated in a water bath at 37°C. for 1 hour. Then rabbit serum containing 2 units of hemolysin and a 5 per cent suspension of sheep blood cells are added to the mixture and incubated in the water bath until the control serum indicates the completion of the reaction. To check the accuracy of the complement fixation tests, a standard serum which is obtained from a rabbit immunized by a series of

TABLE VI

Variation in Progress of Antibody Production among Rabbits after Six Injections of Heat Killed Tubercle Bacilli

Titer	Number of rabbits with antibody titers indicated in column at left					
	With intracutaneous injection			With subcutaneous injection		
	After 4 wks.	After 6 wks.	After 10 wks.	After 4 wks.	After 6 wks.	After 10 wks.
0	2	1		4	1	
5	2		2	1		2
10		1	1		2	1
20		2	2		2	2
40	1					
80		1				

injections of heat killed tubercle bacilli is included in each test. Measured quantities (0.5 cc.) of the serum are dried *in vacuo* in the frozen state by the method of Elser, Thomas and Steffen (4), and a sample of the serum, recovered by adding 0.5 cc. of distilled water to the dry material, is tested for complement fixation. Tests made simultaneously with three samples of dried serum show that the method of drying yields uniform samples.

Individual Variation of Antibody Production in Rabbits after Repeated Injections of Heat Killed Tubercle Bacilli

One group of 5 animals has received into the skin weekly injections of 0.2 mg. heat killed tubercle bacilli for 6 weeks and another group has received the same injections into the subcutaneous tissue. Antibody titers are determined by complement fixation test 5 days after

the fourth injection, 5 days after the sixth injection, and approximately 10 weeks after the beginning of the experiment. The results are shown in Table VI.

The titers of the sera that have fixed complement vary widely in different rabbits. Individual variation of the same order is present in the group with both intracutaneous and subcutaneous injections.

Progress of Antibody Formation after Intracutaneous, Subcutaneous and Intravenous Injection of Heat Killed Tubercle Bacilli

Parallel experiments with varied methods of administration of heat killed tubercle bacilli have been made, on the one hand, with

TABLE VII

Progress of Antibody Formation after Subcutaneous and after Intravenous Injection of Heat Killed Tubercle Bacilli

Titer	Number of rabbits with antibody titers indicated in column at left					
	With weekly injections of 0.2 mg.					
	With subcutaneous injection			With intravenous injection		
	After 2 wks.	After 4 wks.	After 6 wks.	After 2 wks.	After 4 wks.	After 6 wks.
0						
5	1					
10	2	2				
20	1	3	2			
40	1		2	1		
80			1	1		
160				1	1	
320					1	2
640					1	1

intracutaneous, and on the other, with subcutaneous, injection. Antibody formation has proceeded somewhat more rapidly with intracutaneous than with subcutaneous injections but the difference is not conspicuous.

Antibody titers of the blood rise faster and reach higher levels after weekly intravenous than after weekly subcutaneous inoculations of 0.2 mg. of heat killed tubercle bacilli (Table VII). In another group of seven rabbits injected intravenously at intervals of 1 week with amounts of heat killed tubercle bacilli ranging from 0.003 to

0.05 mg. the antibody titers have been lower than in animals that have received weekly intravenous injections of 0.2 mg. of vaccine, but higher than in those treated with weekly subcutaneous injections of 0.2 mg. heat killed tubercle bacilli.

Antibody Formation with Multiple Simultaneous Injections

Multiple simultaneous intracutaneous or subcutaneous injections of heat killed tubercle bacilli produce antibodies in greater titer than the same quantity in a single injection. Of 10 animals that received a single injection of 0.2 mg. each week (see Table III), only one had

TABLE VIII

Progress of Antibody Formation after One Subcutaneous Injection or after Four Weekly Injections of Heat Killed Tubercle Bacilli and of BCG

Titer	Number of rabbits with antibody titers indicated in column at left							
	Heat killed tubercle bacilli				BCG			
	At 2 wks.	At 4 wks.	At 6 wks.	At 8 wks.	At 2 wks.	At 4 wks.	At 6 wks.	At 8 wks.
After 1 subcutaneous injection of 0.2 mg.								
0	9	8	9	9	9	6	3	4
5		1				3	3	1
10							2	2
20								1
After 4 weekly subcutaneous injections of 0.2 mg.								
0	9	9	7	7	9	1	2	2
5			1	1	1	4	4	5
10						2	3	3
20						3	1	

antibodies in a titer of 40 or more after 6 weeks; of 9 animals that received 4 injections of 0.05 mg. each week, 5 had titers of 40 or more, and of 10 animals that received 8 injections of 0.025 mg. each week, 6 had titers of 40 or more.

Progress of Antibody Formation after Injection of Heat Killed Tubercle Bacilli or of BCG

When rabbits have received one subcutaneous injection of 0.2 mg. of heat killed tubercle bacilli or the same quantity 4 times at intervals of 1 week, antibody formation has been scarcely demonstrable

(Table VIII; the progress of sensitization in the same experiment is shown in Table IV), but when BCG has been administered by the same procedure antibody formation has been observed in most of the rabbits.

In experiments with 4 simultaneous injections of 0.1 mg. repeated at weekly intervals for 4 weeks (Table IX), the antibody titers in the group treated with BCG are slightly higher after 4 weeks than in those injected with heat killed tubercle bacilli, but the relationship is reversed at the end of 6 and 8 weeks.

The experiment shows that small quantities of BCG have induced much more abundant antibody formation than heat killed tubercle

TABLE IX

Progress of Antibody Formation after Four Simultaneous Injections (0.1 Mg.) of Heat Killed Tubercle Bacilli or of BCG at Intervals of a Week for 4 Weeks

Titer	Number of rabbits with antibody titers indicated in column at left							
	Heat killed tubercle bacilli				BCG			
	After 2 wks.	After 4 wks.	After 6 wks.	After 8 wks.	After 2 wks.	After 4 wks.	After 6 wks.	After 8 wks.
0	8	3	1	1	17	2	4	7
5	1	2	2	3	1	5	2	3
10		2	3	2		3	6	5
20		1	1	3		5	4	3
40		1	2			2	2	
80						1		

bacilli, but when the amount of vaccine has been increased the two kinds of vaccine have been almost equally effective in producing antibodies measured by complement fixation.

The Relation of Sensitization and Titer of Antibody (Complement Fixation) Preceding Infection to Resistance against Infection

Animals repeatedly inoculated with heat killed tubercle bacilli or with BCG become, on the one hand, sensitized as indicated by the cutaneous tuberculin reaction and form antibodies actively as measured by complement fixation, and on the other hand acquire increased resistance to infection as revealed by complete recovery from

infection, prolonged survival after infection, or diminished extent of tuberculous lesions. Animals that exhibit increased resistance are sensitive to products of the tubercle bacillus and have humoral antibodies at the time when they are infected, but no exact parallel has been found between either the intensity of sensitization or the titer of antibody and resistance. It is doubtful if any parallel should be expected even though sensitization, antibody formation and resistance are dependent upon a common factor, for each may be dependent upon other variable factors which cannot be kept constant.

Sensitization or allergic inflammation measured by the maximum intensity of the tuberculin reaction before infection may be compared with the extent of pulmonary lesions, the duration of life after infection, or complete recovery from infection. Gradations in resistance to infection may be roughly defined: (*a*) The least resistant of the "vaccinated" animals are those that die within 300 days and at autopsy, like most of the infected controls, are found to have one-half or more of the lungs consolidated by tuberculous lesions. (*b*) Animals that die within 300 days and have less than half of the lungs involved have evidently shown greater resistance, not exactly measurable by the length of survival after infection because intercurrent disease may have hastened their end. (*c*) Another group of animals, in general more resistant than the foregoing, are those that survive more than 300 days. (*d*) The most resistant animals are those that have had no tuberculosis discoverable after death. Control animals after infection with 0.00001 mg. of virulent bovine tubercle bacilli have died in all instances and with few exceptions within 200 days after infection; from four- to nine-tenths of the cut section of the lung substance is usually occupied by tuberculous tissue. Table X shows the fate of animals with varying intensity of sensitization preceding inoculation with virulent tubercle bacilli.

A few animals of both the susceptible and the resistant groups (Table X), on the one hand, have exhibited scant sensitization before infection (reactions with diameter from 0 to 9 mm.). On the other hand, a small number of animals that have become intensely sensitized (reactions with diameter from 50 to 69 mm.) have exhibited minimum resistance to infection.

The attempt has been made to determine if there is any relation

between the titer of complement fixation of immunized animals before infection and their subsequent resistance to the disease (Table XI).

There is evidently no relation between the titer of complement fixation before infection and resistance of the animal so constant that it may be used to predict the fate of the animal.

TABLE X

Relation of Sensitization before Infection, as Indicated by the Maximum Tuberculin Reaction, to Resistance

Diameter of inflammatory reaction	Number of rabbits with maximum reactions of diameter indicated in column at left			
	Surviving infection less than 300 days and with half of lung involved by tubercu-lous lesions	Surviving infection less than 300 days and with less than half of the lung involved	Surviving from 300 to 600 days	Living after 700 days
mm.				
0–9	2		3	2
10–19	3		3	2
20–29	2	4	13	3
30–39	5	20	20	10
40–49	6	7	8	9
50–59	3	1		
60–69	2		1	1
Total number of animals.....	23	32	48	27

Sensitization and Complement Fixation during the Course of Tuberculous Infection and Their Relation to Resistance

Sensitization or complement fixation before infection is doubtless a poor index of its influence upon the latter because during the course of long continued disease there is abundant opportunity for alteration of preexisting relations. To measure the influence of sensitization, as determined by intracutaneous tuberculin tests, and of antibody formation, determined by complement fixation with blood serum, upon the progress of infection, it is desirable to compare: (*a*) sensitization and complement fixation during the course of infection with no preceding immunization (infected controls); (*b*) changes in

sensitization and complement fixation in immunized animals that have not been infected; (*c*) sensitization and complement fixation in immunized animals that have been infected.

Changes in sensitization and antibody formation during the progress of tuberculous infection in rabbits have been studied by Freund, Laidlaw and Mansfield (5). In animals that have received 0.00001 mg. of bovine tubercle bacilli intravenously, sensitization becomes evident from 2 to 6 weeks after infection, increases rapidly and then fluctuates somewhat in intensity. During a period that has usually

TABLE XI

Relation of Complement Fixation before Infection to Resistance

Titer of complement fixation	Number of rabbits with maximum complement fixation as indicated in column at left			
	Surviving infection less than 300 days and with half of lungs involved by tuberculous lesions	Surviving infection less than 300 days and with less than half of the lungs involved	Surviving from 300 to 600 days	Living after 700 days
0				
5				
10	1			
20		2	1	2
40	3	7		
80	5	5	3	4
160		3		
Total number of animals.....	9	17	4	6

varied from 2 to 8 weeks before death, the animals have failed to react to tuberculin. Complement-fixing antibodies have appeared in the blood within 2 weeks after infection, increased during 6 to 10 weeks, and subsequently maintained a fairly constant level, remaining elevated during the terminal period in which sensitization has disappeared. Fig. 4 is a composite graph showing the course of sensitization and complement fixation in animals infected intravenously with 0.00001 mg. of bovine tubercle bacilli. It is noteworthy that a few animals infected with this dose form no antibodies demonstrable by complement fixation.

The changes in sensitization and complement fixation following immunization produced by repeated subcutaneous injection of heat killed tubercle bacilli during a period of 8 to 12 weeks is shown in Fig. 5, which is a composite graph recording observations on 17 animals. 4 weeks after the beginning of immunization both sensitiza-

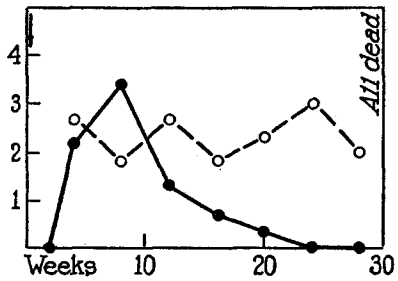

FIG. 4. Composite graph showing the progress of sensitization measured by tuberculin (solid line) and of antibody formation determined by complement fixation (broken line) in 9 animals that were intravenously infected with 0.00001 mg. of bovine tubercle bacilli.

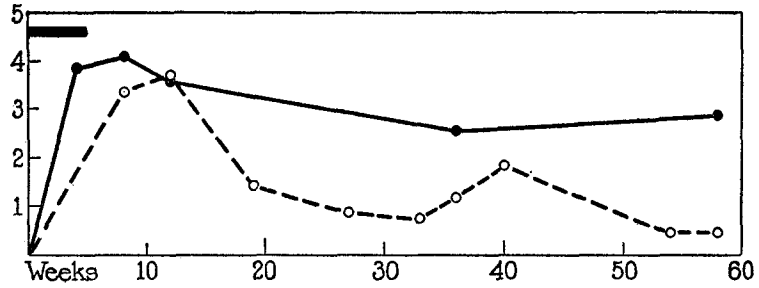

FIG. 5. Composite graph showing the progress of sensitization (solid line) and of antibody formation determined by complement fixation in 17 animals that received intracutaneously 8 to 12 injections of heat killed tubercle bacilli at intervals of 1 week.

tion and complement fixation have reached a high level. With no immunizing injections after the 12th week, tuberculin sensitization remains elevated with only slight diminution for at least 1 year but complement fixation, which has reached a maximum about 12 weeks after the beginning of the injection of heat killed tubercle bacilli, gradually falls and in most animals (12 of the 17 included in the

graph) disappears for a time completely. An almost constant feature of these curves is the subsequent rise of complement fixation. It has occurred between the 30th and 40th weeks and has been sharply defined in 14 of the 17 animals included in Fig. 5, and is present, though ill defined, in the remaining 3.

In animals infected after immunization with heat killed tubercle bacilli, the resistance of the animal is indicated by the duration of life greater or less than 300 days, and by the extent of tuberculosis found after death, measured roughly by the proportion of lung occupied by tuberculous tissue, in accordance with the groups defined above (*e.g.* in Tables X and XI). In an animal that has died within 300 days after infection with half or more of the lung replaced by tuberculous lesions there has evidently been little resistance to the disease (Fig. 6, graph 1), and it is noteworthy that sensitization and antibody formation demonstrable by complement fixation have followed the same course as that of the infected control (Fig. 4), sensitization disappearing several weeks before death although complement fixation remains elevated. In an animal that has shown more resistance to infection so that within 300 days after infection only one-tenth of the lung is occupied by tuberculous tissue (Fig. 6, graph 2), the course of sensitization has been at first approximately the same as that of the infected control and of the immunized animal that has exhibited no resistance. Following infection sensitization measured by the tuberculin test falls rapidly and after from 20 to 30 weeks has for a time completely disappeared, but complement fixation, as in the infected control, remains at a high level. In the least resistant of the immunized animals as in the control, sensitization fails to reappear, but in the more resistant animals with longer period of survival and less extensive tuberculosis sensitization reappears but disappears again before death occurs.

In animals that have shown maximum resistance, indicated by complete recovery from infection, the early course of sensitization and antibody formation (Fig. 6, graph 3) has differed little from that of the control infected animal or of the immunized animal that has died with tuberculosis; sensitization has disappeared after 30 weeks but complement fixation has remained elevated. Sensitization again increases and then falls to zero; this rise to a considerable

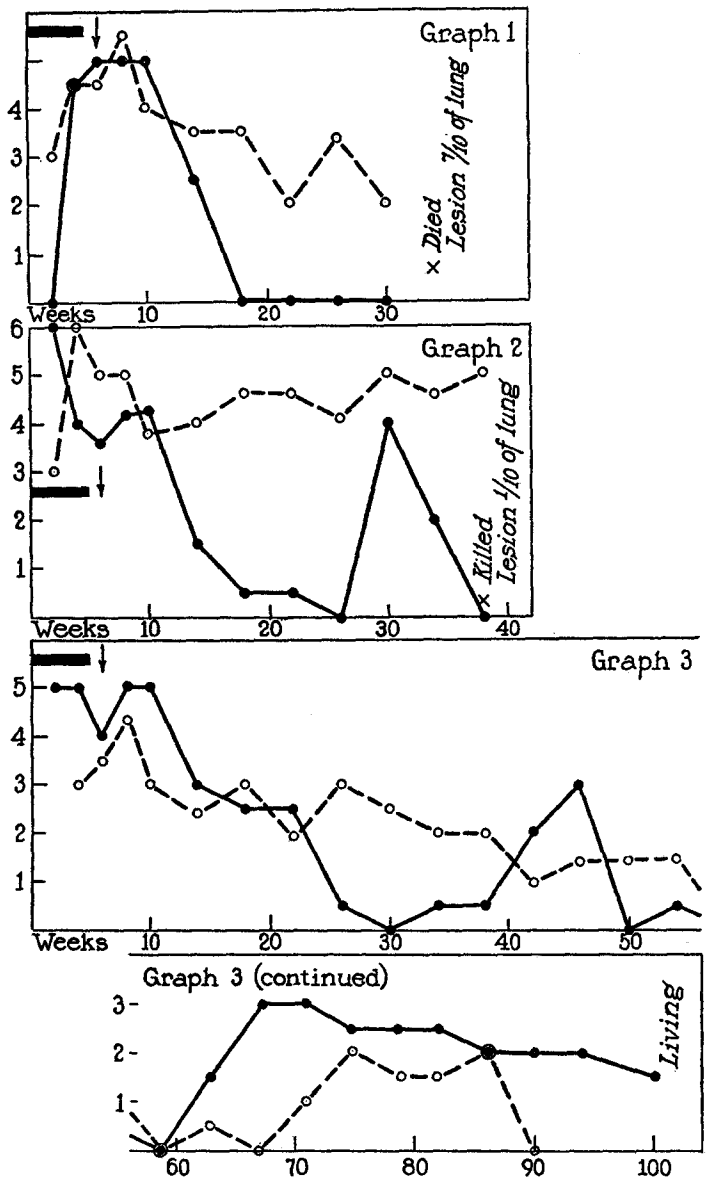

FIG. 6. Graph 1 shows the progress of sensitization (solid line) and of antibody formation (broken line) in a rabbit that received six intracutaneous injections of heat killed tubercle bacilli and showed scant resistance to intravenous infection with bovine tubercle bacilli, dying after 30 weeks with seven-tenths of the lung substance occupied by tuberculous lesions. Graph 2 shows the progress of sensitization and antibody formation in an animal that showed moderate resistance to infection, dying after 40 weeks with only a tenth of the lung occupied by tuberculous lesions. Graph 3 shows the same in an animal with such complete resistance that no tuberculosis was found when the animal was killed 111 weeks after infection.

height, followed by complete disappearance, may occur repeatedly (Fig. 6, graph 3). Complement fixation fluctuates less conspicuously and its course does not coincide with that of sensitization.

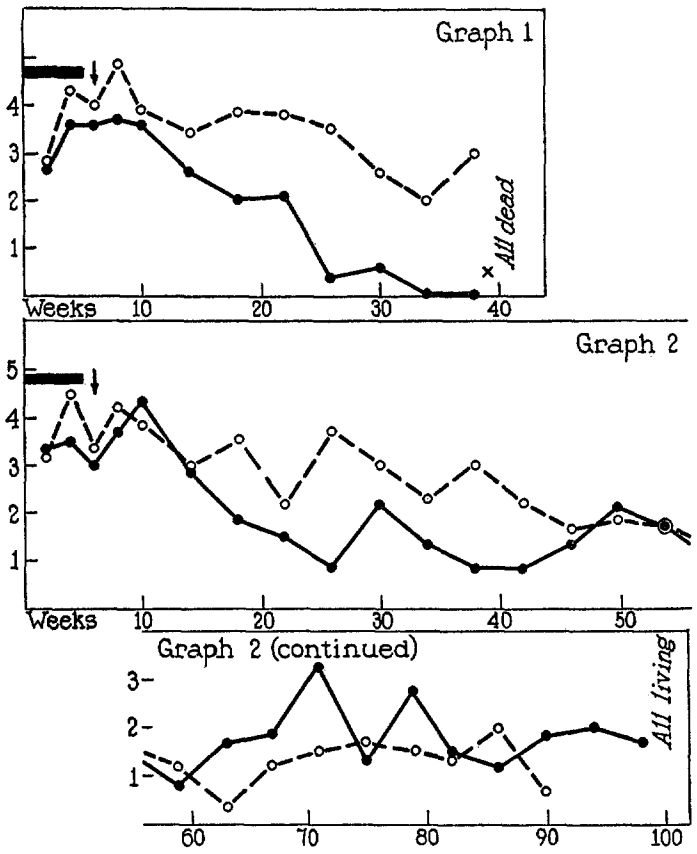

FIG. 7. Graph 1 is a composite showing the progress of sensitization and antibody formation in 9 "vaccinated" animals that showed scant resistance to tuberculous infection (see Fig. 6, graph 1). Graph 2 is a composite showing the progress of sensitization and antibody formation in 6 "vaccinated" animals that resisted tuberculous infection (see Fig. 6, graph 3).

It tends to diminish gradually in animals that recover from infection. Its course resembles that following immunization with no infection but the fall is more gradual and more prolonged.

The trend of sensitization and of complement fixation is well shown in Fig. 7, in which graph 1 is a composite of 9 immunized animals that died within 300 days after infection with more than half of the lungs occupied by tuberculous lesions. It resembles closely those from which it is formed (see Fig. 6, graph 3). The composite graph (Fig. 7, graph 2) of animals that have been most resistant to infection, surviving more than 700 days, does not show the usual course of sensitization as well as the selected graph (Fig. 6, graph 3), because in 5 of the 6 animals from which it is prepared sensitization has repeatedly fallen to the base line and then reappeared, whereas it has remained continuously elevated in only one animal. The titer of complement fixation is more constantly elevated but has gradually and almost continuously decreased.

DISCUSSION

It has long been recognized that resistance to tuberculosis may occur in the absence of sensitization revealed by allergic inflammation; the dog and rat which unlike the guinea pig and the rabbit acquire no skin sensitization to tuberculin when they are infected with tubercle bacilli are highly resistant to the disease. Examples of specific acquired immunity in the absence of sensitization or "allergy" might be multiplied readily. It is more important to know in what degree and within what limitations sensitization or allergic inflammation (the word is here used in the sense defined by von Pirquet (6), who introduced it) when it occurs, increases or decreases resistance against tuberculous infection.

When rabbits are immunized by BCG under varied conditions, some, Clawson (7) states, react to tuberculin and others acquire no demonstrable sensitization; nevertheless, both groups, he says, show some resistance against infection, indicated by the extent of tuberculous lesions in animals killed 3 months after inoculation. It is noteworthy that these experiments in which sensitization has been measured before infection give no decisive information concerning its relation to resistance because during the long course of infection there is abundant opportunity for fluctuation in its intensity. Our experiments in which rabbits were given from 1 to 6 or more injections of heat killed tubercle bacilli (Tables I and IV) show that the ap-

pearance of sensitization may be much delayed and fail to appear after several injections. Successive injections seem to have a cumulative effect so that when they are continued sensitization finally ensues and similar increase of sensitization may follow infection of a partially sensitized animal.

The graphs of sensitization and antibody formation during the course of tuberculous infection show unexplained fluctuations. Changes of sensitization and of antibodies are not parallel. The intensity of sensitization diminishes and disappears with advancing disease and with fatal infection sensitization is usually absent during a considerable period before death, though antibodies indicated by complement fixation persist. Also with tuberculosis in immunized animals that pursues a favorable course, sensitization usually disappears but may reappear in waves throughout the course of recovery. When the animal loses its capacity to react with restored sensitization, death occurs. The disappearance of sensitization in infected animals preceding death has not been satisfactorily explained; the fluctuations in its intensity with its periodic disappearance, observed in immunized and infected animals, are doubtless of similar character. It is probable that desensitization by excess of antigen formed during the progress of infection has a part in this phenomenon.

These relations of antigen to antibodies, and of sensitization to resistance, suggest that we are measuring antibodies or other factors that are formed in excess of the quantity needed to combat infection. Those that are actually concerned in agglutination or phagocytosis or other antibacterial activity are perhaps absorbed by the tubercle bacillus and are no longer demonstrable in the serum or measurable by skin tests. With the progress of recovery stimulation of antibody formation diminishes and the measurable excess in the serum falls.

SUMMARY AND CONCLUSIONS

Rabbits (and human beings) differ widely in the rapidity with which they undergo sensitization with heat killed tubercle bacilli, but after repeated injections all animals become sensitized.

Intracutaneous injection of a small quantity of heat killed tubercle bacilli into a previously normal animal produces a nodule which persists from 8 to 12 weeks; the same injection into well sensitized

animals produces a lesion which ulcerates within from 1 to 3 weeks and is completely healed after about 5 weeks. Complete healing is functional evidence of the disappearance of the antigen.

Intracutaneous injection of heat killed tubercle bacilli induces more rapid sensitization than subcutaneous or intravenous injection, but after repeated injections the difference disappears.

Increasing quantities of heat killed tubercle bacilli or the same quantity divided into several simultaneous injections accelerates sensitization.

The rapidity of antibody formation measured by complement fixation varies in different rabbits under the same conditions but complement fixation is always demonstrable after repeated injections of heat killed tubercle bacilli. Antibody formation is more rapid and reaches higher titers with intravenous than with intracutaneous or with subcutaneous injections. It is accelerated by division of the injected antigen into multiple simultaneous injections.

Small quantities of BCG induce rapid sensitization and more abundant antibody formation measured by complement fixation than heat killed tubercle bacilli but with repeated injections the difference disappears.

Animals that are sensitized and immunized (allergic) before infection are in most instances more resistant to infection than previously normal animals, but there is no correlation between the intensity of sensitization or the titer of antibodies, on the one hand, and resistance to infection on the other.

A previously normal animal subjected to infection differs essentially from a sensitized and immunized animal during the first few weeks of infection when sensitization and immunity are developing as the result of infection, but subsequently the progress of sensitization and antibody formation measured by the means at our disposal follows for a time the same course in both. Sensitization diminishes and in most instances disappears, whereas the titer of complement fixation remains elevated.

When infection pursues a fatal course sensitization permanently disappears, but in animals that proceed toward recovery sensitization measured by injection of tuberculin into the skin repeatedly diminishes, usually to complete disappearance, and then increases in suc-

cessive waves which tend to diminish in height with recovery from infection.

The titer of complement fixation gradually diminishes with recovery from infection.

It is probable that the skin test for sensitization and complement fixation applied to the blood serum measure antibodies or other factors determining sensitization and immunity that are in excess of those actively concerned in the maintenance of resistance.

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