

STUDIES ON TREPONEMA PALLIDUM AND SYPHILIS.

IV. THE DIFFERENCE IN BEHAVIOR IN IMMUNE SERUM BETWEEN CULTIVATED NON-VIRULENT TREPONEMA PALLIDUM AND VIRULENT TREPONEMATA FROM LESIONS.

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I. Agglutination.

In a paper published by two of the writers¹ in June, 1915, it was shown that, as other workers, notably Kolmer,² had found before, the inoculation of rabbits with culture *pallida* gives rise to the formation of powerful agglutinating properties in the sera of the animals. Definite agglutination was observed in dilutions of 1:4,000, and the serum agglutinated not only the strain with which the rabbit had been immunized, but other strains, such as those cultivated by Noguchi. On the specific relationship of these agglutinins to treponemata other than *pallida* we shall report in another paper. The discovery of specific antibodies against the *pallida* in treated animals, of course, again gave us hope that it might be possible to determine whether or not the patient afflicted with active syphilis produced antibodies against the organism, a fact which has been much discussed and written about, but which up to the present time is uncertain. It is not our intention in this communication to review the literature of antibody formation in syphilis, which is extensive though disappointing as to results. We leave this for another paper in which we shall report observations on the presence of agglutinins for culture treponemata in the sera of human beings in the various stages of syphilis.

¹ Zinsser, H., and Hopkins, J. G., *Jour. Exper. Med.*, 1915, xxi, 576.

² Kolmer, J. A., *Jour. Exper. Med.*, 1913, xviii, 18.

In this paper we wish to call attention to what seems to be to us a fundamentally important fact; namely, the existence of a definite difference between the treponemata artificially cultivated and those derived directly from the lesions of infected man and animals.

In previous studies carried on in the course of the last two years, we have frequently looked for changes in motility and for agglutination of treponemata found in exudates from syphilitic lesions to which the sera of immunized rabbits had been added. Our results on these occasions were as inconclusive as those of other writers.

Hoffmann and von Prowazek³ reported in 1906 that the serum of syphilitics in the later stages of the disease produced a diminution of motility in *Treponema pallidum*, an observation which was confirmed by Zabolotny⁴ soon afterwards. Contradictory results, however, were obtained by Landsteiner and Mucha,^{5, 6} who were unable to observe either immobilization or agglutination in syphilitic serum. Uhlenhuth and Mulzer⁷ never observed agglutination in the sera of animals treated with virulent treponemata. Neisser and Bruck,⁸ summarizing the work done up to 1911, express themselves as uncertain whether or not agglutinins in the serum of syphilitics had really been proven up to that time, when, of course, work with pure cultures had not been possible. The recent work of Kissmeyer⁹ deals with the agglutination of culture treponemata in normal and syphilitic serum and will be left for consideration in a later paper of our own dealing with the same subject.

It is plain, therefore, that in the matter of agglutinin formation in the course of syphilis, nothing is yet definitely known, a state of affairs which unfortunately describes our knowledge of the general subject of antibody formation in this disease.

Having obtained powerful agglutinins in rabbits and sheep treated with culture treponemata, we undertook to determine the action of these sera upon the microorganisms obtained directly from lesions,

³ Hoffmann, E., Cited from Neisser and Bruck, *Beitr. z. Path. u. Therap. d. Syph.*, Berlin, 1911, 205.

⁴ Zabolotny, D., and Maslakowetz, *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1907, xlv, 532.

⁵ Landsteiner, K., and Mucha, V., *Wien. klin. Wchnschr.*, 1906, xix, 1349.

⁶ Landsteiner, K., and Mucha, V., *Centralbl. f. Bakteriol., 1te Abt., Ref.*, 1907, xxxix, 540.

⁷ Uhlenhuth, P., and Mulzer, P., *Arb. a. d. k. Gsundtsamte*, 1913, xlv, 307.

⁸ Neisser, and Bruck, *Beitr. z. Path. u. Therap. d. Syph.*, Berlin, 1911, 205.

⁹ Kissmeyer, A., *Deutsch. med. Wchnschr.*, 1915, xli, 306.

since to one habitually working with treponemata, a great many differences as to morphology, agility of motion, and primarily, of course, of virulence, between the culture organisms and the lesion treponemata are apparent. We therefore carried out a number of experiments, some of which are detailed in this paper, in which the action of serum was tested, in parallel series, upon both the culture organisms and those obtained from lesions.

After a considerable amount of experimentation it seemed to us that material obtained from human lesions, however good, or from rabbit lesions consisting of small nodules and chancres, was unsatisfactory in that the treponemata were so few in number and so intimately mingled with tissue detritus and blood derivatives that washing never resulted in a sufficient yield of microorganisms for satisfactory microscopic work. Incidentally, we may mention that in all agglutination work we have found it inaccurate to rely upon macroscopic results only; our readings had to be made both macroscopically and microscopically. We therefore chose as material only the large, diffuse, gelatinous lesions obtained frequently in rabbit testicles; and we used for most of our experiments such lesions obtained with our own Strain A, which had been cultivated and with which the immune serum had been produced. This gave us a strictly homologous experiment. In addition, we tested this serum on similar lesions obtained with our F and S strains.

In preparing material the testicles were removed and thoroughly macerated with a masticator. The macerated mass was then squeezed through cheese-cloth and, in favorable cases, a thick, gummy liquid was obtained which swarmed with treponemata, actively motile. Many lesions, of course, were worked over in this way without yielding favorable material, but in the experiments finally accepted only material was used in which a great many *pallida* were present relatively free from admixtures of foreign material. To the gummy fluid salt solution was added and the larger particles which had come through the cheese-cloth were thrown down by slow centrifugation. The *pallida* have apparently a low specific gravity and hardly any of the organisms are removed when centrifugation is neither at high speed nor prolonged. The supernatant fluid from this primary centrifugation was removed and allowed to settle in the

refrigerator over night. This removed more of the detritus. Dilution with salt solution was then practiced and very prolonged centrifugation, 3 to 4 hours at high speed, brought down most of the treponemata. These were washed in salt solution, and if considerable admixtures of tissue particles, etc., were still present, repetition of the process with more or less variation according to the nature of the material was practiced. When the final preparation was obtained, it usually consisted of about 0.5 to 1.0 cc. of a salt solution suspension, relatively clean of detritus, containing from four to six treponemata to the field, sometimes less, but in some cases considerably more. With material so prepared the experiments were set up.

EXPERIMENT I.

Agglutination of Virulent Treponemata with Serum of Rabbit 1.

Readings were taken macroscopically and microscopically after 2 hours at 37°C. and after 14 to 16 hours in the refrigerator.

The suspension of the Strain A culture material was made approximately equivalent to the virulent suspension.

Tubes set up.	Agglutination.
Strain A culture + Rabbit 1 serum, 1: 10.	+++
" " " + normal serum, 1: 10.	=
Virulent Strain A + Rabbit 1 serum, 1: 10.	=
" " " + normal serum, 1: 10.	=

EXPERIMENT II.

Comparative Agglutination of Virulent Strain A and Culture Strain A.

Very good, spongy lesion used; treated as described above. A suspension of Culture A was made which corresponded in number of treponemata to the virulent suspension obtained.

Serum of Rabbit 1 was used. This serum agglutinated Culture A in dilutions of 1: 2,000.

Tubes set up.	Agglutination.
1. Virulent Strain A + Rabbit 1 serum, concentrated.	=
2. " " " + " 1 " 1: 20.	-
3. " " " + salt solution.	-
4. Culture " " + Rabbit 1 serum, concentrated.	+++
5. " " " + " 1 " 1: 20.	++
6. " " " + salt solution.	-

EXPERIMENT III.

Strain S.

Suspension of Treponemata from a Gelatinous Lesion in a Rabbit Testicle.

Readings were made macroscopically and microscopically.

The suspension of Strain A culture material was made approximately equivalent to the virulent suspension.

Tubes set up.	Results.		
	1 hr. at 37° C.	2 hrs. at 37° C.	18 hrs. in ice chest.
1. Virulent Strain S + Rabbit 1 serum, 1: 4.	0	0	0
2. " " " + normal rabbit serum, 1: 4.	0	0	0
3. Culture Strain A + Rabbit 1 serum, 1: 4.	+++	+++	+++
4. " " " + normal rabbit serum, 1: 4.	±	±	++

EXPERIMENT IV.

Suspension of Virulent Strain S Used.

Readings were taken macroscopically and microscopically after 2 hours at 37°C. and after 14 to 16 hours in the refrigerator.

Tubes set up.	Agglutination.
Strain A culture + Strain A serum, 1: 3.	+++
" " " + salt solution.	-
Virulent Strain F + Strain A serum, 1: 3.	±
" " " + salt solution.	-

EXPERIMENT V.

Suspensions unfortunately very thin. Therefore Strain A culture suspension was made equally thin, about three or four microorganisms to the field.

The different columns represent readings by three different men in order to be sure that no individual error of judgment was possible.

Tubes set up.	Results.		
1. Virulent treponemata + Rabbit 1 serum, 1: 5, 3 drops 3 drops.	-	-	-
2. Virulent treponemata + Rabbit 1 serum, 1: 20, 3 drops 3 drops.	-	-	-
3. Virulent treponemata + normal serum, 1: 5, 3 drops 3 drops.	-	-	-
4. Culture Strain A + Rabbit 1 serum, 1: 5, 3 drops 3 drops.	++	++	++
5. Culture Strain A + Rabbit 1 serum, 1: 20, 3 drops 3 drops.	++	++	++
6. Culture Strain A + normal serum, 1: 5, 3 drops 3 drops.	+	++	++

These experiments show that an immune serum produced by the inoculation of rabbits with a culture of *Treponema pallidum*, although it contained powerful agglutinating properties for the culture *pallida*, had little or no effect upon the virulent *pallida* obtained directly from lesions. In Experiments II and IV there was slight but definite indication that a very weak effect had been produced upon the virulent treponema by the immune serum. We shall refer to this again, since it seems to indicate that if a sufficiently strong immune serum could eventually be produced, even the virulent *pallida* might be influenced. We need only refer to the long delayed success in passive immunization to the pneumococcus, where the capsulated nature of the organism rendered it unamenable to anything but the most powerful immune serum.

Furthermore, this apparent inability of the virulent *pallida* to go into relation with an antibody so powerfully active against the culture organism, going hand in hand with a loss in virulence on the part of the culture treponema, suggested the possibility of a change physiologically not unlike that associated in some of the bacteria with the acquisition and loss of a capsule.¹⁰ Moreover, the difficulties of staining and general appearance variations of thickness and thinness observed in the *pallida* strains, together with the sticky, gelatinous nature of the lesions, also suggest to the observer the possibility that these organisms may either be capsulated or have a protoplasmic structure chemically not unlike that of the capsules of bacteria.

It occurred to us that we might be able to produce agglutinability in the virulent *pallida* by subjecting them to the acid and heat treatment introduced by Porges in experiments with the Friedländer bacillus group. Accordingly we carried out experiments in which virulent *pallida* material was treated with hydrochloric acid in a total concentration of 0.0625 N and heated in a water bath to from 70° to 80°C. for 15 minutes. The mixtures were made in very small agglutination tubes and measured with capillary pipettes by the Wright method, since, of course, it was difficult to obtain any quantities of the material for examination.

¹⁰ Each of the writers individually has seen in virulent *pallida* suspensions occasional sheath-like structures which resemble capsules and which we have never seen in culture suspensions. We cannot yet stain these and the uncertainty of light effects in dark-field observations causes us to hesitate in making a positive statement.

EXPERIMENT VI.

Virulent Treponemata, Strain F.

Good lesion; treated as in preceding experiments.

Washed thoroughly in order to prevent the disturbing effects of possible coagulation of protein by the acid and heat treatment. Parts of the suspension were then made up to 0.0625 N hydrochloric acid and heated for 15 minutes at 80°C., and the volumes of the other fractions made equal with salt solution.

Tubes set up.	Result.	
1. Virulent Strain F, unheated, + salt (control).	+	Many free treponemata and few clumps of 3 to 8 individuals.
2. Virulent Strain F, unheated, + serum of Rabbit 1, 1: 20.	++	Few free treponemata. Clumps larger than control.
3. Virulent Strain F, unheated, + serum of Rabbit 1, 1: 50.	+	Perhaps slightly better clumping than control, but uncertain.
4. Virulent Strain F + acid + serum of Rabbit 1, 1: 20.	+	Few treponemata caught in clumps of precipitate. Cannot make definite reading.
5. Virulent Strain F + acid + serum of Rabbit 1, 1: 50.		Few treponemata caught in clumps of precipitate. Cannot make definite reading.
6. Culture Strain A + serum of Rabbit 1, 1: 20.	++	
7. Culture Strain A + serum of Rabbit 1, 1: 50.	++++	

EXPERIMENT VII.

Suspension made from material from large, diffuse lesion, Strain A, by the technique described above. Unusually good suspension obtained.

The following tubes were set up:

1. Suspension in 0.0625 N hydrochloric acid.
 2. Suspension in salt solution.
 3. Suspension in salt solution.
- } Heated at 75°C. for 15 minutes.

After two of the tubes had been heated for 15 minutes as above, Immune Serum 1 was added in concentration of 1: 25 to all three of the tubes.

No agglutination was observed in any of them after 48 hours' observation, macroscopically and microscopically.

The acid and heat experiments detailed above (Experiments VI and VII) necessarily consumed much time, and had to be repeated a number of times before we felt sure of the results. We are confident after critical repetition, however, that heating with acid, which according to Porges is sufficient to render inagglutinable capsulated bacteria agglutinable, does not have the same effect upon *Treponema pallidum*.

II. Treponemicidal Action.

In a preceding paper two of the writers have shown that the sera of rabbits and sheep immunized with culture *pallida* possess treponemicidal action upon culture *pallida* in dilutions higher than those possessing similar properties in the case of normal sera of rabbits and sheep. The experiences detailed above which indicated differences between the cultivated microorganisms and the virulent ones obtained from lesions in respect to agglutinability, led us to investigate also the treponema-killing properties of culture immune rabbit serum for virulent *Treponema pallidum*. Accordingly, taking serum from treated rabbits we allowed this serum to act upon treated organisms of varying kinds before injection into normal rabbit testicles. These experiments were as follows (Experiment I).

EXPERIMENT I.

Material from a lesion in Rabbit 19, A XIV. The testicular mass was macerated, the coarser particles thrown down in the centrifuge, and the supernatant fluid, which contained many motile treponemata, was divided into three parts, as follows:

- I. 2.5 cc. virulent suspension + 5 cc. immune serum of Rabbit 20 (freshly taken and active).
- II. 2.5 cc. virulent suspension + 5 cc. normal rabbit serum.
- III. 2.5 cc. virulent suspension + 5 cc. salt solution.

These mixtures were allowed to stand for 1 hour at 37.5°C. There was no loss of motility or clumping in any of them. After 1½ hours it seemed that there were somewhat more non-motile forms in I than in the others. This appears to have been probably a deception. After 2 hours injections were made as follows:

0.75 Cc. Injected into Each Testicle.

- | | | | |
|------|------------|-------------------------------------|------------------|
| I. | Rabbit 21. | Died after 26 days. | Negative. |
| | " 22. | " " 26 " | Positive lesion. |
| | " 23. | Killed for transfers after 40 days. | Positive lesion. |
| | " 24. | | Negative. |
| II. | " 25. | | Positive. |
| | " 26. | | " |
| | " 27. | | Negative. |
| | " 28. | | Positive. |
| III. | " 29. | Died after 26 days. | Negative. |
| | " 30. | | Positive. |
| | " 31. | | Negative. |
| | " 32. | | " |

In this experiment it is plain that there was no protective power exerted by the action of the serum on the microorganism.

EXPERIMENT II.

Experiment carried out as before, except that no salt solution control was made. The suspension of virulent treponemata was divided into two parts, as follows:

- | | | | | |
|-----|---------------------------|---------|---------------------|---------|
| I. | Immune serum of Rabbit 33 | 5 cc. + | virulent suspension | 2.2 cc. |
| II. | Normal " | 5 " + | " " | 2.2 " |

0.8 Cc. Injected into Each Testicle.

- | | | |
|-----|------------|-----------------------|
| I. | Rabbit 34. | Negative. |
| | " 35. | " |
| | " 36. | Died before of value. |
| | " 37. | Positive. |
| II. | " 38. | Negative. |
| | " 39. | " |
| | " 40. | " |

Experiment II, also, was entirely negative.

Since we had found in our treponemicidal experiments carried out upon culture organisms that the serum, just as in the case of bactericidal reactions, could be inactivated and reactivated, it seemed to us that insufficiency of alexin or complement might have been a source of error in our previous experiments. Therefore Experiment III was done.

EXPERIMENT III.

Virulent material suspended as before. The material was then divided into three parts and the following mixtures were made:

I.	
Virulent suspension.....	1.5
Immune serum, active.....	3.0
Normal rabbit serum.....	1.0
II.	
Virulent suspension.....	1.5
Immune serum, active (dilution 1: 20).....	3.0
Normal serum.....	1.0
III.	
Virulent suspension.....	1.5
Normal serum, active.....	4.0

These mixtures were incubated for 2 hours at 37.5°C. At the end of this time rabbits were inoculated in both testicles as follows:

1.0 Cc. Injected into Each Rabbit.

I.	Rabbit 41.	Negative.
	“ 42.	“
	“ 43.	Positive.
	“ 44.	Negative.
II.	“ 45.	“
	“ 46.	Positive.
	“ 47.	Doubtful.
	“ 6.	Positive.
III.	“ 48.	Negative.
	“ 49.	Positive.
	“ 50.	“
	“ 51.	Negative.

These experiments as far as they have gone show no definite action of the immune serum upon the virulent treponemata, a result which is analogous to the agglutination experiments described in the first part of this paper.

It might be added that the technique by which these experiments had to be done still leaves considerable possibility of error. It has been impossible to obtain the virulent material free from a certain amount of tissue detritus, and dissolved protein which might in some way neutralize the action of antibodies in the immune serum. Fur-

thermore, it is possible that the serum may have definite action and yet the number of treponema placed in the mixtures in each case might have exceeded the maximum quantity that could be taken care of by the amounts of serum used, since, of course, it is well known to one who has carried out *in vitro* bactericidal experiments, that an excessive proportion of microorganisms will result in completely using up the immune serum, leaving unkilld bacteria in sufficient number to cloud by their growth all the bactericidal action. However, this latter possibility is, we think, rendered more or less unlikely by the last experiment where relatively large amounts of serum were used. We consider that these experiments justify the following conclusions, which to us indicate an important principle in questions of immunity in syphilis.

CONCLUSIONS.

Although antibodies can be produced by the immunization of animals with cultivated *Treponema pallidum*, and although these antibodies exert specific agglutinative and treponemicidal action upon the culture organisms, they possess, at least in the concentration so far obtained by us in rabbits and sheep, practically no action for virulent treponemata obtained directly from lesions. There seems to be in the infected body an inability to exert a purely serum action upon the virulent treponemata, a condition of affairs which may well lead to a lack of antigen absorption on the part of the body and a consequent failure to produce serum antibodies.

We do not think that this should in any way discourage our further investigation of the protective action of antibodies produced with culture *pallida*. On the one hand, the slight occasional agglutination and the lower proportion of takes with the concentrated serum in the last experiment at least indicate the possibility that we have been working with sera that are not sufficiently powerful and that just as with work with the pneumococcus and other highly invasive organisms, a serum of considerable antibody contents must be used before results can be expected. Again, the destruction of treponemata and the healing of lesions which undoubtedly takes place in rabbits, sometimes with surprising speed, may be a cellular destruction, and by injecting the sera either locally or intravenously and giving them time

to be absorbed by the cells before injecting virulent material, better results may be obtained. This direction of research as well as further studies on the antagonistic cellular processes against the *pallida*, the immunization of animals with killed virulent organisms, and the antibodies in rabbits and human beings during the course of infection and after recovery are being investigated, and we hope to be able to report upon them in the near future.