

THE RELATIONSHIP BETWEEN IMMOBILIZING AND
SPIROCHETICIDAL ANTIBODIES AGAINST
TREPONEMA PALLIDUM

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Using a combined *in vitro* and *in vivo* technique, Ebersson (1921) demonstrated the presence of substances, presumably antibodies, in the serums of syphilitic patients and rabbits that rendered virulent *Treponema pallidum* either less infectious or noninfectious. In spite of improvements by a number of investigators (Tani, Saito, and Funada, 1935; Tani and Ogiuti, 1936; Tani and Aikawa, 1936; Turner, 1939), such *in vitro* and *in vivo* techniques have serious practical limitations. Nelson and Mayer (1949) were first to develop an *in vitro* method for the demonstration of an immobilizing antibody for virulent *Treponema pallidum*. Although most of their findings have been confirmed in this laboratory (Magnuson and Thompson, 1949), the precise relationship between "immobilizing antibody" and "spirocheticidal antibody" is as yet undetermined.

In an attempt to correlate immobilization and treponemicidal activity, Nelson and Mayer (1949) performed two experiments. The first employed three mixtures previously incubated 16 hours at 30 C. In mixture "a" containing complement and serum ultrafiltrate, 80 per cent of the treponemata were motile; in "b" containing complement plus normal serum, 78 per cent were motile; in "c" containing complement plus syphilitic serum, only 4 per cent were motile. Two normal rabbits were inoculated intracutaneously over the back with a 0.1-ml sample from each mixture. The lesions developing at sites inoculated with mixture "c" were slower to appear and were smaller in size than those at sites inoculated with "a" and "b." Since the incubation period varies with the size of the inoculum (Magnuson, Eagle, and Fleischman, 1948) and the total number of inoculated organisms was the same at all sites, it was concluded that a significant portion of the organisms in mixture "c" was noninfectious, presumably dead. In the second experiment two mixtures were used: (a) complement plus serum ultrafiltrate and (b) complement plus syphilitic serum. After 16 hours' incubation at 35 C, 90 per cent of the organisms in "a" were motile, but none were motile in mixture "b." Three rabbits were inoculated intracutaneously at 4 sites with 0.1 ml of mixture "a," and another three rabbits with mixture "b." Dark-field positive lesions developed at all 12 sites inoculated with the "a" mixture, but only one of 12 developed with the "b" mixture.

Although the results of these two experiments suggest that the immobilizing antibody is spirocheticidal, the results are neither quantitative nor conclusive. Controls of normal serum with inactive complement and of syphilitic serum

with inactive complement were lacking in both experiments. Though unlikely, the results in the second experiment could be due to differences among rabbits, since the "a" and "b" mixtures were inoculated into different animals.

It was our purpose to design a definitive experiment in which the relationship of immobilization to killing could be determined on a quantitative basis. If the immobilization and killing of *Treponema pallidum* proceeded either simultaneously or at equal rates, it would present strong evidence that the antibody responsible for immobilization was actually spirocheticidal.

MATERIALS AND METHODS

The syphilitic serum pool was obtained from a group of patients at the Eastern Medical Center, Durham, North Carolina. All these patients had high reagin titers, and through previous assays the pool of their serums was known to contain "immobilizing antibody." Donors for the normal serum pool were selected from our own laboratory personnel. Both serum pools were stored at -20°C . Complement was obtained from pools of at least 12 guinea pigs, lyophilized in 5.0-ml amounts, stored *in vacuo* at -20°C , and restored with distilled water just before use.

The emulsion containing virulent *Treponema pallidum* (Nichols strain) was prepared and the immobilization assay performed in the manner of Magnuson and Thompson (1949). One and seven-tenths ml of emulsion containing 1.2×10^7 *T. pallidum* per ml, 0.1 ml of active or heat-inactivated complement, and 0.2 ml of the inactivated test serum or its saline dilution were added to each tube. The normal and syphilitic serums were each assayed at serial fivefold dilutions from whole serum to 1:125, representing final concentrations in the test emulsions of from 1:10 to 1:1,250.

At the beginning of the *in vitro* assay, after all reagents and the emulsion had been added, samples from the 1:10 dilutions of normal and syphilitic serums with active and inactive complement were serially diluted in one part of inactivated normal rabbit serum plus three parts of 0.85 per cent NaCl. Two-tenths ml of each serial tenfold dilution ranging from 10^6 to 10^1 organism per ml were inoculated intracutaneously over the backs of three rabbits. The sites for inoculation were distributed in random fashion among the 24 sites used on each animal. The inoculated sites were examined twice weekly for a 3-month period and only dark-field positive lesions were recorded as positive.

After the assay tubes had been incubated for 18 hours at 35°C , the percentage of motile organisms was estimated from the proportion of motile organisms in the first 50 spirochetes counted. At the same time, serial tenfold dilutions for inoculation into rabbits were prepared from each of the 16 tubes in the manner employed at the beginning of the experiment. An exception was made with the tubes containing no motile organisms, in which the dilutions ranged from 10^7 to 10^2 organisms per ml rather than from 10^6 to 10 per ml.

It was feasible to inoculate each rabbit at a maximum of 24 sites. Since there were 96 treatment combinations (2 types serum \times 2 types complement \times 4 serum dilutions \times 6 emulsion dilutions) in each replicate, it was necessary to

use four animals for each replication. This necessitated the confounding of certain treatment combinations with differences between rabbits. By the use of partial confounding and rotation of the interactions confounded maximum information was retained. That is, each set of 96 possibilities was arranged on 4 rabbits so that the three degrees of freedom for confounding came from several interactions. These interactions were rotated in each of the six replications. The treatment effects thus affected were the interaction of complement with dilu-

TABLE 1

Results of the immobilization test of normal human and syphilitic human serums and the estimated percentage survival by analysis of in vivo results

SERUM	DILUTION	COMPLEMENT	PERCENTAGE MOTILE BY DARK-FIELD COUNT		ESTIMATED PERCENTAGE SURVIVAL BY ANALYSIS OF IN VIVO RESULTS
			18 hr	21 hr	
Normal human	1/10	+	94	96	
		-	98	96	
	1/50	+	92	80	
		-	96	94	
	1/250	+	94	82	
		-	92	92	
	1/1,250	+	86	68	
		-	80	80	
Syphilitic human pool	1/10	+	0	0	0
		-	100	98	
	1/50	+	6	0	12
		-	94	90	
	1/250	+	64	38	15
		-	76	72	
	1/1,250	+	84	64	100
		-	92	90	

+ = active complement. - = heat-inactivated complement.

tion, interaction of serum with dilution of serum, and interaction of serum, complement, and dilution. In all, this phase of the assay required 24 rabbits with a total of 576 inoculated sites.

RESULTS

Shown in table 1 are the percentage of motile organisms in each of the assay tubes based on direct dark-field observation. According to criteria previously set (Magnuson and Thompson, 1949), the pool of syphilitic serums was positive for immobilizing activity at the 1:10 and 1:50 dilutions. These readings, orig-

inally made at 18 hours when the rabbit inoculations were begun, were essentially unchanged at 21 hours when the inoculation had been completed.

The infectiousness of the mixture containing syphilitic serum and active complement was estimated in the following manner: For each tube in the *in vitro* assay, the number of spirochetes required to produce dark-field positive lesions at 50 per cent of the inoculated sites was estimated. These 50 per cent end points were estimated by the method of Behrens (1929) from the results of the serially diluted emulsions. The values so obtained for the mixture of syphilitic serum and active complement were then compared with the combined results of the other three mixtures. By the use of the latter group as a base line representing 100 per cent infectiousness, the values in the last column of table 1 were obtained.

The *in vivo* results check the *in vitro* results rather well. The *in vivo* measure subject to greatest variation is the number of organisms required to produce infection in 50 per cent of inoculated sites. This estimate was least precise in the emulsions from the immune serum and heated complement in which there were only 6 rabbits at each inoculation level for each serum dilution.

Only a portion of the information available in an experiment such as this can be recovered from an analysis of 50 per cent end points. Such analysis ignores the information contributed by the incubation periods of the lesions. As Magnuson, Eagle, and Fleischman (1948) have shown, the length of the incubation period may be considered a quantitative response to the number of inoculated organisms. Expression of all incubation periods as reciprocals, as suggested by Turner and his co-workers (1948), has indicated that reasonable weight may be attached to all observations including those sites that remained negative, i.e., the incubation period was infinite. Further investigations in this laboratory as to the nature of the variance after such transformations have shown that the variance is stabilized and additional information may be obtained.

The analysis of variance with the reciprocal transformation was straightforward for a factorial design and followed the procedure outlined by Yates (1937). There were 19 sites on one animal that were not used because of death during the observation phase of the experiment. These values were estimated in accordance with standard formulae for missing values and resulted in the loss of 19 degrees of freedom from the total and error mean square. The analysis of variance on an individual rabbit-site basis is shown in table 2.

The addition of active complement and variation of the number of inoculating organisms had a significant effect on both the percentage of successful inoculations and the incubation periods. These were the only primary factors to show significant variation. Immune serum and normal serum differed significantly only when complement was present, as shown by the significant interaction between complement and serum. Other interactions which were significant at the 1 per cent level included the interaction between complement and the dilution of serum, the interaction between serum and dilution of serum, and the interactions between complement, serum, and serum dilution, and between complement, serum dilution, and the number of organisms inoculated. Interactions that were

significant only at the 5 per cent level were those between complement and and the number of organisms, and among all four factors, complement, serum, dilution of serum, and number of organisms.

TABLE 2
Analysis of variance for 18-hour incubation and inoculation

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total	556	0.406622	
Replications	5	0.007136	
Rabbits within replication	18	0.036814	
Treatment combinations	92*	0.184490	
C	1	0.009892	0.009892†
S	1	0.000043	0.000043
D	3	0.001876	0.000625
N	5	0.093125	0.018625†
CS	1	0.002571	0.002571†
CD	2*	0.003789	0.001895†
CN	5	0.004568	0.000913‡
SD	2*	0.012641	0.006321†
SN	5	0.000826	0.000165
DN	15	0.005525	0.000368
CSD	2*	0.011821	0.005911†
CSN	5	0.000859	0.000172
SDN	15	0.008481	0.000565
CDN	15	0.016719	0.001115†
CSDN	15	0.011754	0.000784‡
Error	441	0.178182	0.000404

C = effect of active vs. heat-inactivated complement.

S = effect of normal human vs. syphilitic human serums.

D = effect of fivefold dilutions (1/10 to 1/1,250) of normal human or syphilitic human serums in the presence of active and inactive complement at each dilution.

N = effect of varying the number of virulent *Treponema pallidum* in the inocula.

* The use of 4 rabbits to test the full 96 treatment combinations resulted in three degrees of freedom being sacrificed from the possible 95. The source of these was one each from CD, SD, and CSD.

† Significant at 1 per cent level.

‡ Significant at 5 per cent level.

A similar analysis of variance for the controls inoculated at 0 time is shown in table 3. As is there shown, a significant effect on the percentage of takes and rate of development of lesions is produced only by variation in the number of inoculated organisms. The average incubation period for all groups at 0 time

was 37.4 days. The number of organisms required to produce dark-field positive lesions at 50 per cent of inoculated sites was essentially the same for all groups: for normal serum plus active complement $10^{8.77}$ *T. pallidum*, for normal serum plus inactive complement $10^{8.50}$, for immune serum with active complement $10^{8.50}$, and with immune serum plus inactive complement $10^{8.44}$.

The harmonic mean incubation periods of lesions developing after inoculation of the various serum and complement mixtures are shown in table 4. As is

TABLE 3
Analysis of variance for 0 hour incubation and inoculation

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE
Total.....	71	0.065752	
Treatment combinations			
S.....	1	0.000036	0.000036
C.....	1	0.000136	0.000136
N.....	5	0.033815	0.006763*
SC.....	1	0.000024	0.000024
SN.....	5	0.001068	0.000214
CN.....	5	0.003101	0.000620
SCN.....	5	0.002039	0.000408
Error.....	48	0.025532	0.000532

See table 2 for explanation of C, S, and N.

* Significant at 1 per cent level.

TABLE 4
Observed harmonic mean incubation periods in days using the 1:10 and all dilutions of normal and syphilitic human serums with active and heat-inactivated complement

COMPLEMENT	NORMAL SERUM		IMMUNE SERUM	
	1:10 dilution	All dilutions	1:10 dilution	All dilutions
Active.....	38.5	52.1	270.7	74.2
Heat-inactivated.....	46.4	43.0	39.6	37.1

there apparent, the striking increase in incubation periods occurs with the active complement and immune serum at the 1:10 dilution, but an increase is also noted when all serum dilutions from 1:10 to 1:1,250 are included.

DISCUSSION

Comparison of the 0 time inoculations with the 18-hour inoculations clearly indicates that a period of *in vitro* incubation of virulent spirochetes with immune serum in the presence of active complement is required to render the spirochetes noninfectious. The reasons for this needed period require further in-

vestigation. Analogy with other antigen-antibody reactions would suggest that the initial combination of antibody with *T. pallidum* occurs almost instantaneously. It is also reasonable to assume that the lethal effect of this combination becomes apparent only after prolonged exposure to complement. If one assumes adequate concentrations of complement were present *in vivo* following the 0 hour inoculations, the data suggest that either the initial binding of antibody to antigen was not immediate or that the antigen-antibody combination dissociated *in vivo* after intracutaneous inoculation.

One may speculate as to the order in which infectiousness and motility are lost. Comparison of the motility counts in the present experiment with the calculated percentage of infectious organisms yields relatively good checks for the 1:10, 1:50, and 1:1,250 dilutions of syphilitic serum plus active complement. At the 1:250 dilution the estimated motility was much higher than the estimated infectiousness obtained *in vivo*. It is possible that this represents a transition point at which *T. pallidum* retains motility but has become noninfectious because of some change in the organism, either making it unable to reproduce *in vivo* or so altering the parasite that it is more susceptible to immune mechanisms of the normal host. This suggests that motility may represent the final effective activity surrendered by virulent *T. pallidum*. This is perhaps analogous to the observation of Eagle and Musselman (1944) that in the presence of penicillin the cultured nonpathogenic Reiter strain of *T. pallidum* lost its ability to multiply before it became nonmotile.

The present experiment demonstrates that spirochetes immobilized in the immobilization test are not infectious and are, therefore, presumably dead. The parallel though not necessarily simultaneous action of immobilization and killing of *T. pallidum* suggests that the antibodies responsible for these phenomena are identical.

SUMMARY

There is a corresponding loss of infectiousness of virulent *Treponema pallidum* (Nichols strain) as it is rendered nonmotile by the immobilizing antibody. This relationship is not directly proportional and suggests that infectiousness may be lost slightly before motility ceases. This noninfectiousness may be due to some alteration in the *Treponema pallidum* rendering it incapable of reproducing *in vivo*, or it may be that the *Treponema pallidum* is so altered as to render it more susceptible to the natural immune mechanisms of the host.

A period of *in vitro* incubation is necessary to demonstrate immobilization and reduction of infectiousness. This suggests that the initial antigen-antibody reaction was either not immediate or that the antigen-antibody complex dissociated *in vivo*.

For both the *in vitro* demonstration of immobilization and the *in vivo* demonstration of loss of infectiousness, syphilitic serum in adequate concentration and active complement are required.

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