THE ANTIBODY RESPONSE IN RABBITS TO KILLED SUSPENSIONS OF PATHOGENIC T. PALLIDUM

By HARRY EAGLE, M.D., and RALPH FLEISCHMAN

(From the Laboratory of Experimental Therapeutics of the United States Public Health Service, and The Johns Hopkins School of Hygiene and Public Health, Baltimore)

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Although reinfection with syphilis is not uncommon, it is usually observed in patients who have been treated early in the disease, and is observed only rarely in patients treated after the secondary lesions have spontaneously resolved (1). In syphilitic rabbits also, a solid immunity develops after a period of approximately 3 months (2). If such animals are cured by appropriate treatment and then reinoculated, even several million organisms usually fail to produce a second infection.

The mechanism of this immunity is not clear. It bears no obvious relationship to the presence of serum agglutinins or lysins; and opsonins have not been demonstrated. Syphilis does regularly cause the production of an antibody ("reagin") reactive with a ubiquitous lipoidal component of normal mammalian tissue. It is not yet clear, however, whether this reagin is actually a specific antibody to *T. pallidum* which happens to cross-react with a serologically related component of mammalian tissue (3), or whether, as suggested by Sachs, Klopstock, and Weil (4), syphilis causes the breakdown of host tissue and the release of tissue haptenes which are activated by spirochetal protein to form a complete antigen. In either case, the presence of so called Wassermann or flocculation reagin is apparently not the basis of the observed resistance to reinfection. In both rabbits and man, the titer of this antibody is highest in the early stages of the disease, long before there is a significant degree of immunity, and the titer may have dropped to negligible levels at a time when the immunity is maximal.

The only experimental evidence yet adduced for the presence in the serum of syphilitic animals or men of antibodies directed specifically against the causative organism is the demonstrations by Tani and his coworkers (5, 6) and by Turner (7) that when such serum is incubated with suspensions of *T. pallidum* and the mixtures injected into rabbits, the production of syphilitic lesions is then either delayed or prevented. In the latter instance, it is not yet known whether the admixture with serum actually prevents infection, or merely suppresses the appearance of the primary lesion, and makes the infection asymptomatic.

The present experiments were undertaken to determine the antibody response in rabbits to killed suspensions of pathogenic *T. pallidum*, administered in sa-

line suspension, or incorporated in water-in-oil emulsions as described by Freund (8). As will be here shown, the injection of even large numbers of the organisms, up to a maximum of 38 billion injected over a period of 13 weeks, did not produce demonstrable resistance to infection, since as few as ten organisms then sufficed to cause infection. The serum of such "immunized" animals had no demonstrable direct effect on the organisms *in vitro*.

The injection into rabbits of killed *T. pallidum* did, however, regularly cause the production in high titer of antibodies similar to those which develop in the course of syphilitic infection, the serum giving positive complement fixation (Wassermann) and flocculation tests with lipoidal extracts of normal tissue. The serum dilution titers reached a maximum level within 2 to 3 weeks. Thereafter, and despite continuing injection, the titers decreased progressively.

The present experiments therefore throw no light on the mechanism of immunity in syphilis. They do, however, constitute strong evidence that, as originally postulated by Wassermann, so called reagin may be simply an antibody to *T. pallidum*. Its anomalous reactivity with alcoholic extracts of normal mammalian tissue is probably to be attributed to the presence in such extracts of a lipid substance serologically related to one or more of the antigenic components of the organism (*cf.* (3)). Methods of preparing treponematal suspensions from rabbit chancres have not yet yielded suspensions sufficiently concentrated and sufficiently free from tissue components to warrant either their chemical fractionation or their use in absorption experiments.

EXPERIMENTAL

Antibody Response to the Injection of Saline Extracts of Testicular Chancres

Experiment 1. Intradermal Injection.—

Rabbit testicular chancres were removed at the height of the inflammatory reaction, minced with a scissors, and ground in a mortar with sand. The supernatant fluids, containing 10 million organisms per cc., were heated at 56°C. for 1 hour to kill the organisms, and were then kept frozen at −25°C. until used. Three rabbits were injected intradermally three times weekly, in amounts of 0.2 to 0.5 cc. One animal died after eight injections. The remaining two rabbits received 3.7 cc. (37 million organisms) over a 4-week period. Nine days after the last injection the serum Wassermann titers were 1:24 and 1:16. One of the two rabbits, inoculated intradermally with an unmeasured inoculum 7 days after the last injection, developed a darkfield positive lesion after a normal incubation period.

Experiment 2. Intradermal and Intraperitoneal Injection.—

A second group of four rabbits was injected both intradermally and intraperitoneally, the individual dose by the former route increasing from 0.2 to 0.5 cc., and by the latter route from 0.5 to 7 cc. In the course of twelve injections over a period of 4 weeks, the rabbits received a total of 3.7 cc. intradermally and 50 cc. intraperitoneally, and a grand total of 537,000,000 organisms. Nine days after the last injection, the serum Wassermann titers were 1:48, 1:32, 1:8, and 1:24. Three of the four rabbits inoculated at that time intradermally with an unmeasured inoculum developed typical darkfield positive lesions after a normal incubation period.

Experiment 3. Intravenous Injection.—

In a third experiment, begun in January, 1940, the organisms were injected intravenously and in much larger numbers than had previously proved feasible. Similarly prepared extracts of rabbit chancres were kept frozen at −25°C. until large amounts had been pooled. Two suspensions were prepared: one (325 cc. from a total of 132 testes) contained 75 million organisms and 5 mg. of N per cc.; the second suspension (235 cc. from 110 testes) contained 50 million organisms and 5.4 mg. of N per cc. Ten rabbits were injected intravenously three times weekly. Five died in the course of the injections. The remaining five received 35 cc. in

TABLE I

Antibody Response in Rabbits to Suspensions of T. pallidum in Testis Emulsion (Suspension Injected Intravenously Three Times Weekly)

Rabbit No.	No. of injections	Duration of immunization period	Total No. of organisms $\times 10^9$	Wassermann* titers on day No.			Flocculation* titers on day No.			Resistance to infection
				1	20	37	1	20	37	
		days								
42-87	13	37	4.4	0	6	32	0	16	48	Approximately 2 × 10^6 organisms were inoculated intradermally 2 days after the last immunizing injection. All the rabbits developed a darkfield positive lesion in 13 days, simultaneously with a group of non-immunized control animals.
42-88	13	37	4.4	0	18	48‡	0	48	96‡	
43-98	13	37	4.4	4	12	12	0	24	24	
44-40	13	37	4.4	0	<4	6	0	12	24	
44-49	13	37	4.4	2	<6	6	0	2	4	
43-22	13	37	4.4	0	4	—	0	3	—	
53-91§	12	33	4.8	0	—	½	0	—	12	Not done
53-92§	12	33	4.8	½	—	0	0	—	16	
53-97§	12	33	4.8	0	—	6	0	—	12	

* Wassermann and flocculation technics as described in (12), pages 1 and 5. Titers are expressed as the highest dilution of serum giving a positive result.

‡ This serum had no demonstrable agglutinating effect when added to an equal volume of a spirochetal suspension containing 10^8 organisms per cc., did not affect their motility, and the suspension remained infectious for rabbits (*cf.* Table II).

§ Injections by intravenous drip.

eight injections, and after a 10 day rest period were given a second course of 36 cc. in five injections. A total of 4.4 billion organisms was thus injected over a period of 35 days. The serum Wassermann and flocculation titers of this series of rabbits during the immunization period are summarized in Table I. As there shown, every animal injected developed a significantly increased reactivity with the alcoholic extracts of beef heart used as "antigen" in these tests.

This increased reactivity with mammalian tissue extractives presumably represents an antibody response to the organisms, although the necessary presence of tissue derivatives in the material used for injection complicates the inter-

pretation of the results (*cf.* page 381). It is true that similar extracts of normal testes, containing even larger amounts of solid and of N, did not cause the appearance of these antibodies in any one of fourteen rabbits tested. Further, such antibodies were not obtained when suspensions of cultured treponemata (Reiter strain) were added to the normal testis extracts in numbers corresponding to the number of pathogenic *T. pallidum* present in the chancre extracts. Antibodies reacting specifically with the saprophytic organism were, however, produced in high titer (9). These findings confirm the results obtained by many previous workers who have studied the antibody response to cultured spirochetes (*cf.* (4), page 292).

There was the further possibility that the treponematal suspensions used for immunization might have been infectious, despite the fact that they had been heated at 56°C. for 1 hour and stored at −25°C. for at least 2 weeks prior to use. This possibility was excluded by the demonstration that the popliteal lymph nodes of the immunized animals were not infectious for normal animals at the end of the immunization period.

Despite the appearance of a reagin-like substance in the serum in dilution titers comparable to those observed in the natural infection, when four of the animals of Table I were inoculated intradermally 2 days after the last immunizing injection, all developed typical darkfield positive lesions after a normal incubation period. Although the size of the inoculum was not accurately determined, it follows from the fact that many motile organisms were present in each microscopic field that on the order of 1 million to 10 million were injected in this challenge inoculation (10). This experiment must, therefore, be qualified by the fact that the inoculum used was many thousand times the minimal infectious dose (10), a complicating factor which was rectified in a following experiment.

Corresponding to the fact that the animals were not resistant to infection, when serum from rabbit 42-88, obtained at the end of the immunization period, was mixed with living organisms, there was no effect on either their motility or infectiousness (*cf.* Table II).

Experiment 4. Intravenous Drip.—

Three rabbits were injected, by slow intravenous drip, with a suspension containing 85 million organisms per cc. In thirteen such injections, given three times weekly over a period of 35 days, the animals received a total of 57 cc. of chancre extract, representing 4.8 billion organisms. The serologic response in those animals (bottom section of Table I), although not as pronounced as that observed in the preceding series, was nonetheless definite.

Antibody Response in Rabbits Immunized with a Concentrated Suspension of Sedimented T. pallidum

In later experiments concentrated suspensions of *T. pallidum* containing relatively small amounts of tissue extractives were prepared by differential centrifugation of the chancre emulsion.

Experiment 5.—

Testes were removed at the height of the inflammatory reaction 10 to 14 days after their inoculation, finely minced with scissors, and ground in a mortar and pestle with 0.85 per cent NaCl (approximately 5 cc. per testis). The mixture was lightly centrifuged to remove the tissue particles, and the sediment reextracted with a second portion of salt solution. The combined supernatant fluids were centrifuged in a conical head (International Centrifuge Co. No. 923) for 1 hour, at the end of which time from 75 to 90 per cent of the organisms had been sedimented as a whitish pellicle. The number of organisms in the sediment was estimated by counts on the suspension before and after centrifugation. The supernatant fluid was drained, the sediment was resuspended in salt solution to give a final concentration of 1 billion organisms per cc., and the suspension frozen at −25°C. Over a period of 2 months, nine such suspensions totalling 140 cc. were prepared from 192 testicular chancres. Some of these suspensions were killed before freezing by the addition of merthiolate to a 1:1000 concentration, and warming to 37°C. for 1 hour. The others were kept frozen until they were no longer infectious. No difference was noted between the two types of suspension, and they are not distinguished in the text.

TABLE II

The Failure of Serum from an "Immunized" Rabbit (42-88 of Table I) to Affect Either the Motility or Infectiousness of a Suspension of T. pallidum

"Immune" serum, with or without fresh guinea pig serum as complement, was added to 0.4 cc. of a suspension of *T. pallidum* containing 10^8 actively motile organisms per cc.

Tube No.	Normal serum	"Immune" serum, rabbit 42-88*	1:10 fresh guinea pig serum	Proportion of motile organisms after 4 hrs. in anaerobe jar at 37°C.	Infectiousness of mixture on intradermal inoculation of 0.2 cc. into normal rabbit
	cc.	*cc.*	*cc.*		
1	0.4	0	0	92	All the rabbits developed a darkfield positive lesion in 10 days
2	0	0.4	0	92	
3	0	0.4	0.4	90	
4	0	0	0.4	82	
5	0	0	0	74	

* Serum from rabbit 42-88 of Table I, drawn 37 days after beginning immunization with killed *T. pallidum*, when the serum Wassermann and flocculation dilution titers had reached 1:48 and 1:96, respectively.

In the first experiment with this material, thirteen rabbits were injected three times weekly to a total of sixteen injections in a period of 46 days. After the seventh injection, there was a rest period of 12 days before injections were resumed. The dosage per injection averaged 0.5 cc., or 500 million organisms, and the total number of treponemata injected during the immunization period was 7 billion.

As is indicated in Table III, every animal injected developed Wassermann and flocculation antibodies within a period of 16 days, the flocculation titers at this time varying from 1:16 to 1:64. No significant increase in this titer was observed on prolonged immunization. Instead, in most of the animals there was an indication that the serologic titer had reached a maximum in the first 2

weeks and thereafter decreased. (This was more clearly shown in the following experiment.) Three days after the last injection, a popliteal node was removed from the surviving animals, emulsified in 25 per cent serum, and injected into a normal rabbit to demonstrate that the suspensions used for immunization had actually been killed, and that the immunized animal had not been infected. None of the nodes proved infectious. The following day, and 4 days after the last immunizing injection, the animals were challenged either intradermally or intratesticularly with living inocula which varied from 100,000 down to 10 or-

TABLE III

The Antibody Response (Wassermann and Flocculation Tests) in Thirteen Rabbits Injected Intravenously with Sedimented Suspensions of T. pallidum

Sixteen injections totalling 7 billion organisms, over a period of 46 days: rest period of 12 days midway during immunization period.

Rabbit No.	Time after beginning of immunization, *days*						
	1	16	1	16	36	42	49
	Wassermann titer*		Flocculation titer*				
66-62	<2	6	0	16	12	8	12
66-87	<2	8	0	8	12	—	8
66-93	<2	12	0	16	12	Died	—
67-03	<2	12	0	16	Died	—	—
67-05	<2	24	½	64	16	24	32
67-22	<2	12	1½	16	24	24	8
67-25	<2	32	0	48	32	Died	—
67-28	<2	16	0	32	32	16	16
67-61	<2	—	0	6	16	8	8
67-70	<2	8	½	16	—	8	8
67-79	<2	6	½	8	12	6	8
67-80	<2	6	0	16	8	8	8
67-81	<2	24	0	32	24	8	—

* Technics as described in (12), pages 1 and 5.

ganisms. As is seen in Table IV, every animal inoculated intradermally, even those receiving as few as 10 organisms, developed a darkfield positive primary lesion after incubation periods which did not significantly differ from those in a control series of normal rabbits simultaneously inoculated. In the same rabbits injected intratesticularly, inocula of 100,000 and 1,000 organisms were regularly infectious, but two of three animals inoculated with 10 organisms did not develop demonstrable involvement of the testis within the period of observation (92 days). There was a suggestion also that the incubation period may have been prolonged in some of the animals which developed a darkfield positive lesion after intratesticular inoculation.

Experiment 6. Effects of Prolonged Immunization.—

In the infected rabbit, resistance to reinfection becomes manifest within a few weeks, but increases in degree progressively and slowly. From 3 to 6 months must pass before the animal becomes immune to reinfection with large inocula (2, 11). Accordingly, in a second experiment with sedimented organisms, an average of 1 billion killed organisms was injected three

TABLE IV

Incubation Period on Inoculation of Rabbits with living T. pallidum 4 Days after the Last Immunizing Injection

Rabbits of Table III

No. of organisms inoculated	Rabbit No. (*cf.* Table III)	Incubation period after	
		intradermal inoculation	intratesticular inoculation
		days	*days*
100,000	67-75	17	67
	67-70	13	42
	Normal controls simultaneously inoculated	9, 13, 13	21, 28, 35
1,000	66-62	21	28
	67-28	28	77
	67-61	21	35
	Controls	21, 21, 21	28, 28, 28
10	66-87	28	—*
	67-79	28	—*
	67-80	28	42
	Controls	28, 35	42, 42, 92

* No obvious testicular involvement for 3 months after inoculation, and testis darkfield negative at that time.

times weekly for a period of 4 months, and the total number of organisms injected was 38.5 billion.

As seen in Table V, and as is shown graphically in Fig. 1, the serologic antibody response previously noted was again observed. Maximum titers were again obtained within 2 weeks. Thereafter, however, the titers fell continuously, despite continuing injections at the same high level. Thus, the flocculation titer at the end of 2, 4, 6, 9, and 18 weeks averaged 28, 14, 6.5, 4.5, and 4.5, respectively. When 100 or 10 organisms were injected into two animals intradermally at the end of the immunization period, a syphilitic lesion developed within a normal incubation period (*cf.* Table V). As in the previous experi-

TABLE V

The Antibody Response in Rabbits Immunized for 4 Months with Suspensions of T. pallidum Sedimented from a Testicular Emulsion

Injections repeated three times weekly to a total of 40 injections and 38 billion organisms

Rabbit No.	Day No. 1	Day No. 14	Day No. 28	Day No. 42	Day No. 63	Day No. 126	Resistance to infection with *T. pallidum*		
	Flocculation titer*						Site of inoculation	No. of organisms injected	Result‡§
70-08	1½	32	16	4	3	8	Testis	100	+37
70-88	1½	16	12	2	4	2			+72
71-06	0	32	12	1½	2	1		10	+37
71-39	1	32	8	4	2	2			∞ §
70-30	½	16	8	1½	1	2			
71-38	0	32	16	4	2	8	Skin	100	+22
70-99	1	24	24	32	16	8			+22
71-07	0	16	16	2	1	1½		10	+33
70-67	0	16	8	4	4	—			+37
70-94	½	48	—	—	—	—			
70-97	1½	64	—	—	—	—	Conclusion: No demonstrable resistance to infection in 4 rabbits inoculated intradermally. Although 1 of 4 rabbits inoculated intratesticularly failed to develop a lesion, the significance of this fact is questionable (*cf.* text).		
71-09	1	32	—	—	—	—			
71-13	1	32	—	—	—	—			
71-14	0	16	—	—	—	—			
71-42	0	16	—	—	—	—			
Average flocculation titer..	0.5	28	14	6.5	4.5	4.5			

* Result of Eagle flocculation test, expressed as the highest dilution of serum giving a positive result.

‡ + = darkfield positive lesion developing in the inoculated area after the incubation period (days) indicated in the table. These results are to be compared with the following results in 4 normal rabbits simultaneously inoculated:

No. of organisms injected	Incubation period in control rabbits inoculated into	
	Skin	Testis
	days	*days*
100	37, 37, 33, 33	33, 29, 33, 37
10	27, 54, 37, ∞§	37, 37, 37

§ ∞ = No lesion at site of inoculation within period of observation. However, lymph nodes from rabbit 71-39 removed 110 days after inoculation proved infectious for normal rabbits, indicative of an asymptomatic infection.

ment, however, there was a suggestion of resistance in animals inoculated intratesticularly. Although two rabbits inoculated with 100 organisms both

developed a chancre, the incubation period was prolonged in one of the two. Of two other rabbits inoculated with 10 organisms, one failed to develop a lesion. A popliteal lymph node transferred to a normal animal 110 days after the original inoculation proved infectious, indicating that the animal had undergone an asymptomatic infection. Despite these suggestions of an altered response, it is nevertheless clear that no significant degree of immunity had developed in these animals under the impact of relatively large numbers of dead organisms.

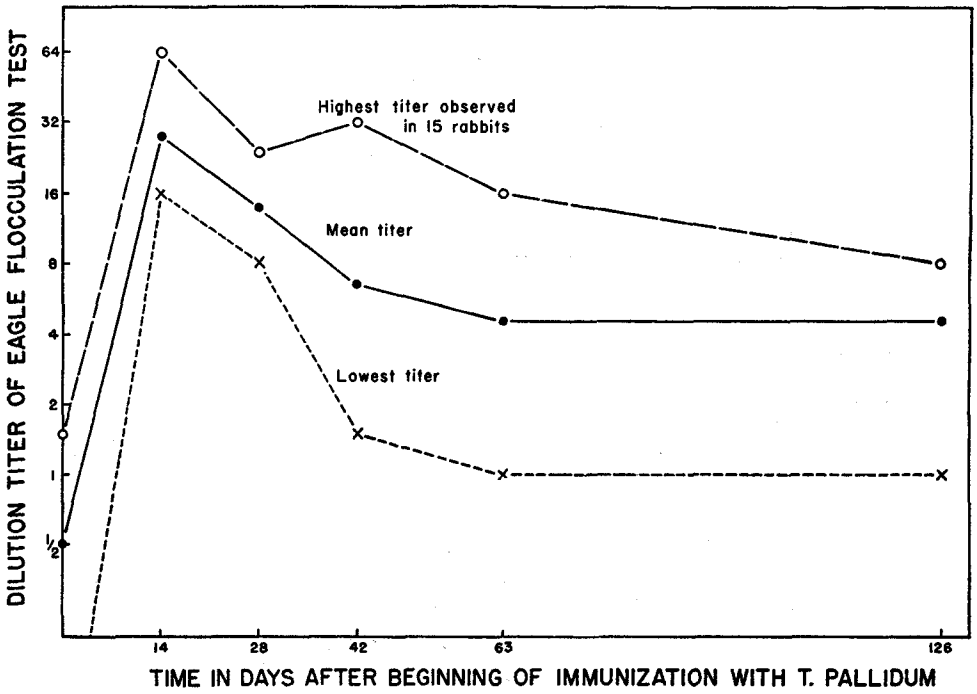

FIG. 1. The antibody response (Wassermann and flocculation reagin) in rabbits immunized by the intravenous injection of killed *T. pallidum* (after data of Table V).

As is indicated in Table VI, the serum from one of these rabbits, obtained midway in the immunization period, 63 days after the first injection, had only a questionable direct effect on pathogenic *T. pallidum*. The serum, either whole or diluted 1:10, was incubated with an equal volume of a chancre emulsion containing 10^8, 10^6, 10^4, or 10^2 organisms per cc., and 0.2 cc. of each mixture, representing inocula of 10^7, 10^5, 10^3, or 10 treponemata, was then injected into normal rabbits intradermally or intratesticularly. The three larger inocula caused the appearance of typical darkfield positive chancres within a normal incubation period. However, the suspension containing the smallest number of organisms proved non-infectious on intratesticular inoculation into two rabbits, this

despite the fact that one to two treponemata have been shown to be regularly infectious for rabbits on intratesticular inoculation (10). When the same suspension was similarly incubated with normal serum and inoculated, typical chancres developed in 28 to 35 days. The significance of this observation is nevertheless open to question. When 1:10 serum was used instead of whole serum, the "immune" and normal sera had the same effect; in each instance one of two rabbits inoculated developed a chancre.

TABLE VI

The Effect in Vitro of Serum from an "Immunized" Animal on the Virulence of T. pallidum

Serum from rabbit 70-08, obtained midway in immunization period, 63 days after the first injection (*cf.* Table V), was added to an equal volume of freshly prepared chancre extract diluted with 25 per cent rabbit serum to contain 10^8, 10^6, 10^4, and 10^2 treponemata per cc. After 1 hour at 37°C., 0.2 cc. of each mixture was injected both intradermally and intratesticularly into a normal rabbit. A fresh normal rabbit serum was simultaneously tested; and both the "immune" and normal serum were tested as both whole serum and in a 1:10 dilution.

No. of organisms inoculated after incubation with serum for 1 hr. at 37°C.		10^7		10^5		10^3		10	
Concentration of serum incubated with treponemata	Type of serum used	Incubation period on inoculation of mixture into							
		Skin	Testis	Skin	Testis	Skin	Testis	Skin	Testis
		days	*days*	*days*	*days*	*days*	*days*	*days*	*days*
Whole serum	Antiserum	14	Died	14	49	21	28	∞*	∞
	Normal serum control	14	21	14	21	21	35	28	35
1:10	Antiserum	14	43	21	21	21	35	∞	49
	Normal serum control	14	21	21	21	28	35	∞	49

* No lesion developed during period of observation (85 days).

Experiments 7 and 8. The Immunization of Rabbit with Killed T. pallidum Suspended in a Water-in-Oil Emulsion.—

Freund and his coworkers (8) and others (13, 14) have shown that the antigenic activity of bacteria is often considerably enhanced if they are suspended in the aqueous phase of a water-in-oil emulsion. This was produced by mixing an aqueous suspension of the organisms first in lanolin (or falba) and then emulsifying the mixture in *e.g.* mineral oil. They further showed that the incorporation of killed tubercle bacilli in the oil phase of such emulsions enhanced the sensitizing activity of the suspension.

A series of treponematal suspensions was therefore prepared in which extracts of rabbits' syphilitic testes, or the organisms sedimented from such extracts, were emulsified in anhydrous lanolin and then in mineral oil to form suspensions containing from 47 to 370 million organisms per cc. Rabbits were injected subcutaneously with varying amounts of these emulsions as indicated in the footnote to Table VII. At varying periods thereafter, specimens of blood were obtained by cardiac puncture, and their Wassermann and flocculation titers determined. In only one rabbit of the five (rabbit 60–66 injected with suspension E) was there a significant

TABLE VII

The Incubation Period of Syphilis in Five Rabbits Immunized with Killed T. pallidum, Suspended in a Water-in-Oil Emulsion

Suspension used for immunization*	1		2			3		
	Incubation period after inoculation with 100 organisms, 28 days after last injection		Incubation period on intradermal inoculation, 53 days after last injection, with			Results in intradermal inoculation with 2×10^6 organisms, 66 days after last injection		
						Live organisms		Dead organisms
	Skin	Testis	10^6 organisms	10^4 organisms	10^2 organisms	Incubation period	Size of lesion at that time	
						days	*min.*	
A	20	35	8	8	13	2	5×5	3×3 mm. in 24 hrs. 5×5 mm. in 48 hrs. 0 in 72 hrs.
B	17	45	8	14	13	2	Minute	0
C	14	39	23	20	14	7	3×6	0
D	20	48	8	14	14	2	5×5	0
E	17	27	14	20	14	7	3×5	0
Normal controls	>57‡	55				7	2×2	0
	27	48				7	Minute	0
	45	45				7	Minute	0
	38	39				10	5×5	0
	>55‡	38				7	Minute	0
Syphilitic controls			13	13	27	3	Minute	0
			10	13	23	7	3×7	0
			14	16	27	2§		

* One part of a treponematal suspension (either a direct extract of syphilitic testes, or the sedimented organisms from such an extract, resuspended in salt solution) was mixed with 1 to 1.5 parts of anhydrous lanolin, and the paste then stirred into 1 to 1.5 volumes of mineral oil. The composition of suspensions A to E listed was as follows:—

Suspension used for immunization	Final concentration of *T. pallidum*	Final concentration of tubercle bacilli	Total No. of treponemata injected $\times 10^6$
	No. per cc. $\times 10^6$	*mg./cc.*	
A	47	0	132
B	47	0.6	132 + tubercle bacilli
C	200	1.0	560 + tubercle bacilli
D	370	0	1040
E	370	1.75	1040 + tubercle bacilli

From 0.8 to 1 cc. of each suspension was injected subcutaneously three times at 6 day intervals.

‡ Animal died.

§ No lesion had developed in this time at the site of inoculation, but the expressed interstitial fluid contained actively motile organisms.

increase in antibody reactive with alcoholic tissue extracts, the Eagle flocculation titers on days 1, 6, 13, 23, 27, and 37 being 1½, 4, 4, 8, 12, and 8, respectively.

Forty-one days after the first immunizing injection, and 28 days after the last, the rabbits were inoculated intradermally and intratesticularly with 100 living organisms. Every immunized animal developed darkfield positive lesions. The incubation periods at the intratesticular sites did not differ significantly from those in control normal animals. There was, however, an indication that at the intradermal sites, the incubation period had perhaps been decreased. In the five immunized animals, the incubation period varied from 14 to 20 days, averaging 17; while in five control rabbits simultaneously inoculated, the incubation periods were 27, 38, and 45, and more than 55 days in the remaining two.

In order to retest this apparent increase in skin reactivity, 24 days after the first inoculation the same animals were reinoculated intradermally at three different sites with 10^6, 10^4, and 10^2 organisms. The incubation periods after these varying inocula are indicated in section 2 of Table VII. The incubation periods at the sites receiving large inocula did not differ significantly from those in control syphilitic rabbits. In the sites receiving a small inoculum, however, there was again a definite indication of a reduced incubation period, the observed period varying only between 13 and 14 days in the "immunized" animals as against 23 to 27 days in the control animals.

Further to test this apparent increase in sensitivity, 66 days after the last injection of the killed organisms, and 38 days after the first inoculation with living treponemata, fresh skin areas were injected with 2 million killed and live organisms. A third site in each rabbit was injected with a similarly prepared control extract of normal rabbit testes. Normal and syphilitic control animals were simultaneously injected. The results are summarized in section 3 of Table VII. The normal testis extract produced no reaction in any of the aninals. In one of the five immunized animals, but none of the controls, the killed organisms caused a significant erythematous reaction within 24 hours, which reached a peak in 48 hours, and had disappeared after 72 hours. The live organisms produced a darkfield positive lesion in 2 to 7 days, averaging 4, in the immunized animals, and in 2 to 10 days, averaging 6, in the control series.

In summary, the immunization of rabbits with killed *T. pallidum* suspended in water-in-oil emulsions after Freund, with or without the simultaneous injection of an adjuvant antigen (killed tubercle bacilli), did not cause the development of demonstrable resistance to infection with *T. pallidum*. There was, however, an indication that such immunization may perhaps have sensitized some of the rabbits to *T. pallidum*. The incubation period on intradermal inoculation with live organisms was decreased, and one of the five experimental rabbits developed an erythematous wheal 24 hours after the injection of a heat-killed suspension of the organisms.

DISCUSSION

Every one of thirty-seven rabbits injected intravenously with a total of 4.4 to 38 billion *T. pallidum* in aqueous suspension developed positive Wassermann or flocculation tests (complement fixation and precipitation with alcoholic extracts of beef heart) in significantly increased titer. The dilution titers of these tests in the normal serum controls varied from 0 to 1:4, averaging less than 1:2; the titers in the immunized series rose to as high as 1:96. This antibody response reached its maximum levels within 2 to 3 weeks, and thereafter either remained constant during the period of immunization or, in two experiments involving the continuing injection of large numbers of organisms, fell steadily in the course of the following 6 to 7 weeks.

The organisms used in these experiments were derived from rabbit testicular chancres, and the suspensions of necessity contained tissue extractives. The present experiments therefore do not constitute a rigorous demonstration that Wassermann and flocculation reagin is a specific antibody to *T. pallidum.* The possibility remains that the tissue extractives contain a haptene activated by the treponematal protein to form a complete antigen. However, there are two aspects of the present experiments which make that explanation unlikely. The first is the demonstration that control animals injected with extracts of normal testes, either with or without the addition of non-pathogenic Reiter spirochetes, failed to develop these antibodies. One would therefore have to assume either that pathogenic *T. pallidum* differs qualitatively from the cultivated organisms in its ability to activate the tissue haptene to a complete antigen, or that the syphilitic testes contained a haptenic substituent not present in normal tissue. The second point is the present demonstration that sedimented organisms containing relatively small amounts of tissue extractives were just as antigenic as the crude chancre emulsion from which they had been concentrated. The simplest explanation of the present data is that Wassermann or flocculation reactivity induced by the injection of these organisms, and presumably also the similar "reagin" elaborated during syphilitic infection, represent an antibody response to pathogenic *T. pallidum.* This was the thesis originally postulated by Wassermann when he developed the complement fixation test for syphilis which bears his name, and which was apparently negated by the subsequent demonstration that alcoholic extracts of normal mammalian tissue could be used as antigen. One need only assume that the treponemata and the mammalian tissues contain an immunologically related antigen. The final demonstration of that fact must await either the cultivation of the pathogenic organism, or the preparation of suspensions sufficiently concentrated or sufficiently free from tissue extractives to warrant their chemical fractionation or their use in cross-absorption experiments. Attempts in this direction are now in progress.

Paradoxically, the "immunized" rabbits in the present series did not regu-

larly develop a significant resistance to infection. Intradermal inoculation, in some experiments with as few as 10 treponemata, regularly resulted in a typical darkfield positive primary lesion at the site of inoculation, whether the animals had been immunized with totals of 30 million organisms intradermally, 537 million organisms subcutaneously, 4.4 to 38 billion organisms intravenously, or 130 to 1040 million organisms in a water-in-oil emulsion, administered over periods which varied from 13 days to 4 months. Recently, Magnuson, Halbert, and Rosenau (15) have also reported failure to produce a significant measure of resistance to infection by the injection of pathogenic *T. pallidum* suspended in oil-in-water emulsions of the type here used.

Three of five immunized rabbits which were challenged by the intratesticular inoculation of ten organisms failed to develop a primary lesion, while every one of five simultaneously inoculated controls was infected. In the one such animal tested, there had been an asymptomatic infection, the organisms having disseminated without producing a primary lesion at the site of inoculation. The significance of this observation, in the light of the results after intradermal inoculation, is open to question. The at best small measure of resistance to infection in these artificially immunized rabbits contrasts sharply with the fact that in the course of an actual infection the animals develop a solid immunity, to the degree that after they have been cured, inocula of many million organisms fail to produce even an asymptomatic second infection. Equally paradoxical, and perhaps related to the foregoing, is the fact that the immunized animals, while developing antibodies to a non-specific antigen (alcoholic extract of beef heart), failed to develop antibodies directly active against the treponematal suspension itself. The organisms were not specifically agglutinated, the sera did not give specific complement fixation with the treponematal suspensions, and as few as ten living organisms incubated for 1 hour with a high titered (Wassermann and flocculation) serum from an immunized animal, retained their infectiousness on inoculation into a normal animal. It is possible that the rabbits had been overimmunized, and were in a "negative phase" at the time they were tested for resistance to infection, or at the time their sera were tested for direct antitreponematal reactivity. There is the further possibility that the surface of the organisms contains a relatively non-antigenic material, and that the most effective antigen is intracellular. This might explain the development of serum antibodies which cross-react in high titer with non-specific antigens, despite the absence of reactivity (specific agglutination, complement fixation, lysis, or protection) with intact *T. pallidum*. However, this would not explain the pronounced immunity which develops in the course of actual syphilitic infection, but not in rabbits immunized with killed organisms. The final, if unlikely, possibility is that in none of the animals was there an antibody response to the treponemata as such, and that the Wassermann reagin was an antibody response to the small amounts of tissue extractives present in the

treponematal suspension. Under ordinary circumstances, those rabbit extractives are non-antigenic for rabbits, even if injected simultaneously with cultured treponemata. The pathogenic organisms may nevertheless possess a unique ability to activate the homologous tissue haptene to a complete antigen.

Of particular interest is the fact that in one series of animals, injected with organisms suspended in a water-in-oil emulsion, there was a suggestion that some of the rabbits may have been sensitized to the treponemata by the preceding immunization. The incubation period on intradermal inoculation with small numbers of organisms was decreased, and one of five rabbits reacted to the intradermal injection of killed organisms. The possible relationship of this observation to the late manifestations of the disease, in which relatively small numbers of organisms produce a disproportionately large tissue reaction, is apparent.

SUMMARY

The intravenous injection into rabbits of suspensions of dead *T. pallidum* derived from rabbit testicular chancres regularly caused the appearance of Wassermann and flocculation antibodies in significantly increased titer. Control suspensions of cultured treponemes (Reiter strain) added to extracts of normal testes were ineffective. This suggests that the Wassermann and flocculation reagin elaborated during syphilitic infection may be an antibody to *T. pallidum* which happens to cross-react with alcoholic extracts of mammalian tissue.

The antisera did not cause the agglutination of suspensions of pathogenic *T. pallidum*, living or dead, did not give specific complement fixation with those suspensions, and did not usually cause the living treponemata to lose their infectiousness.

Animals immunized with such aqueous suspensions for as long as 4 months, or with organisms suspended in a water-in-oil emulsion, were not demonstrably resistant to infection. As few as ten living organisms inoculated intradermally into animals "immunized" with as many as 38 billion dead treponemata regularly produced typical darkfield positive infections; and two of five animals inoculated intratesticularly with ten organisms were also infected.

The contradiction involved in the production of antibodies cross-reacting with a non-specific antigen, and the non-appearance of specific antibodies against the organism used as antigen, is discussed in the text.

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