

INFECTIVITY TESTS IN SYPHILIS*†

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Renewed interest in the occurrence of spiral micro-organisms morphologically resembling *Treponema pallidum* in various tissues and body fluids of human beings and animals, especially in those which have received supposedly curative doses of penicillin, has forced a critical reappraisal of methods for identifying the causative agent of syphilis as we know it today. Among the most definitive tests for the presence of syphilitic infection in man and experimental animals is transfer of the infection to laboratory animals. Despite a large experience with these methods over the past 60 years, the notion is being promulgated that the successful transfer of *T. pallidum* from man to animals is fraught with difficulty, and is therefore an unreliable method for determination of infectivity in the original host. Before accepting such a conclusion and drawing from it other far-reaching conclusions, it will be well to look at the experience in this field.

In this paper we shall review some of the pertinent experiences of ourselves and others, particularly from the standpoint of utilization of optimum methods. From this we believe will emerge the sense of the infectivity test as an entity, just as the TPI, the FTA, and the VDRL tests are entities, a test which if properly used can be a research tool of great value in determining the significance of the more recent findings.

Consideration of Technical Factors

Transfer of syphilitic infection from the tissues and body fluids of man to animals has been made for two principal purposes, either to isolate a strain of *T. pallidum* for the study of experimentally-induced disease, or to test the infectiousness of the tissue in question. Such transfer of infection has become known as the infectivity test, and our experience, together with that reported by many

other investigators, provides a fairly clear picture of the uses, optimum techniques, and limitations of this procedure.

The overwhelming majority of infectivity tests have employed the rabbit as test animal, a choice based on much comparative experience, including that derived from experimental infections in primates. While most tests are susceptible of improvement, it is generally best to adhere to a technique which has yielded satisfactory results in many hands, which is reasonably reproducible under a given set of conditions, and which is not unnecessarily complicated. The rabbit infectivity test for syphilis meets these criteria, and should be the standard to which other methods are compared. This is not to say that a better method of obtaining the same information may not be found, and certainly explorations of this point should be continued. It can be said categorically that there is no evidence at present available which indicates that primates are more satisfactory test animals than rabbits, even apart from such questions as cost, availability, and convenience.

To anticipate some of the conclusions to be reached from a review of the data presented in this paper the following statements may be made emphatically:

(1) *That the rabbit infectivity test, as defined here, yields one kind of essential information.*

(2) *That no other readily performed test with which there has been adequate experience yields this information in such clear cut form.*

Workers in this field are now generally aware of the possibility of confusing results because of the presence of natural *T. cuniculi* infection, but with reasonable precautions this potential source of error can be minimized. In this connexion good background material is presented by Smith and Pesetsky (1967).

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Conditions for the Infectivity Test:

- (1) Mature male rabbits free of *T. cuniculi* infection as determined by clinical inspection and cardiolipin serological tests;
- (2) No antibiotics in food shortly before or following inoculation;
- (3) Maintenance of the rabbit at a cool environmental temperature (circa 16°C.);
- (4) Inoculation into the body of one testis;
- (5) Observation for 120 days.

A Positive Test is Defined as:

(1) Development of a definite or suggestive induration in the inoculated testis with demonstration of motile *T. pallidum* by darkfield examination of testicular fluid,

or

(2) Transfer of the recipient rabbit's tissue, preferably lymph nodes, to second passage rabbits with induction of lesions and demonstration of motile treponemes in the latter.

A positive test in these circumstances shows within a very low margin of error that the donor individual or animal harboured pathogenic *T. pallidum* in the transferred tissue. Moreover, in the present state of knowledge, *consistent failure to obtain a positive infectivity test is strong presumptive evidence that pathogenic T. pallidum is not present in the transferred tissue.*

This latter statement requires elaboration. It is well known that human material from clinically recognizable syphilitic lesions produces a positive rabbit infectivity test in a high proportion of cases. In each instance the presumption is that the strain of *T. pallidum* has been in a human host for millenia yet production of a lesion in the rabbit is usually prompt (under 60 days), the lesion is usually clinically unmistakable to an experienced observer, and treponemes are readily demonstrated in the testicular lesion by darkfield. In other words, no long adaptation to the rabbit is required. Moreover, incubation periods of over 90 days are rare, and secondary transfers, if the initial test is carried out under optimum conditions, yield very few additional positive tests, as witness the extensive series of tests made by Yobs, Clark, Mothershed, Bullard, and Artley (1968). It should also be noted that positive tests have a high degree of predictability in the same general set of circumstances.

Perhaps it would be well at this point to say what in our opinion is *not* acceptable as a positive infectivity test:

(1) The mere fact that an animal develops a positive serological test with either lipoidal or

treponemal antigens during a long observation period cannot now be accepted. It is known that a high proportion of rabbits show weakly reactive tests from time to time for reasons which are not clear and such tests have not been made on large numbers of uninoculated rabbits held in the laboratory for long periods of time.

(2) Demonstration of treponemes by FTA or silver stain, simply because the nature of spirochaetes found on staining is one of the questions we are trying to prove.

(3) Acceptance of bizarre skin lesions, ruffled fur, alopecia, snuffles, infection about the eyes or nose etc., as evidence of syphilis in the test animal. Experience has shown that these lesions are rarely due to *T. pallidum*; they are most often due to some intercurrent infection, usually, *Pasteurella pseudotuberculosis*, formerly called *Bacterium lepi-septicum*. It should be emphasized that syphilitic infection itself does not significantly impair the health of the rabbit.

One other brief word of caution: to the inexperienced microscopist filaments from red blood cell membranes may resemble spirochaetes, especially when floating free of the blood cell; darkfield results should be interpreted with caution in the presence of much blood in the preparation.

Brief comments may be made about each of the criteria for the test referred to above.

Mature rabbits. This refers mainly to the development of the testis, for we have the impression, but no data, suggesting that the immature testis is less favourable than the mature testis as a growth environment for *T. pallidum*.

T. cuniculi infection. If feasible, breeding stocks should be checked for the presence of *T. cuniculi* infections. On the basis of our own experience as well as that of the Russians (cited by Smith and Pesetsky, 1967) it is reasonably easy to keep the incidence low by clinical surveillance of the colony. In any event, animals should be examined for lesions on the genitalia, anus, or ears upon admission to the laboratory and all animals with suspicious lesions should be rejected. We carry out Brewer card test routinely on all rabbits before use and eliminate those reacting with a 1:4 or higher dilution of serum. Many seemingly otherwise normal animals have weakly reactive sera to cardiolipin tests.

Antibiotics in food. It has become increasingly difficult in America to find rabbits fed with prepared food that does not contain antibiotics which have some suppressive action on treponemal infection. Special arrangements should be made with dealers to avoid this contingency.

Cool environmental temperature. It has been well documented that an environmental temperature of approximately 16°C. favours development of *T. pallidum* in the rabbit host (Turner and Hollander, 1957). Before the common use of air conditioning it was generally known that results of infectivity tests in the warm months of the year would be inferior to those obtained in the cooler months.

Intratesticular inoculation. Most investigators over the years have inoculated emulsified material into the body of the testis, and until other methods are clearly demonstrated to be superior this method should be followed. Our experience indicates that inoculation intracutaneously on the shaved back is a less satisfactory route, in part because subsequent growth of hair significantly increases the local temperature. It is probably desirable to inject one testis only, reserving the other for comparison, although it is conceivable that inoculation into both testes might increase the proportion of positive results. The dependent rabbit testis is about 3°C. cooler than the internal body temperature of the rabbit and it is presumed that this is the principal basis of the good results obtained by this route. Ordinarily the testis can absorb up to 0.5 ml. of emulsified tissue material without significant structural change. The chance of irreversible damage to testicular tissue increases as larger amounts are injected. The initial testicular reaction to infection may be very slight and all suspicious changes should be tested by aspiration and darkfield examination of the testicular fluid for the presence of motile treponemes. Non-motile treponemal forms in early infections are extremely rare and should be regarded with a high degree of scepticism.

McLeod and Arnold (1951) described a technique in which lymph nodes from infected rabbits were inserted into the scrotal sac of normal rabbits through a small lower abdominal incision. With donor rabbits that had received subcurative doses of penicillin this procedure gave a higher proportion of positive results than when aliquots of the same tissue were emulsified and inoculated intratesticularly. This technique certainly deserves further comparative study, but it may not be applicable to human tissue explants. Problems related to transplant rejection and other unanticipated factors might serve to make this method less acceptable. Introduction of the explant into the scrotal sac by direct incision does in our experience frequently lead to secondary infection and vitiation of the results. The results of Collart, Dunoyer, and Dunoyer (1968) who, using scrotal implants of skin lesions from patients with secondary syphilis, obtained seven positive results in ten patients, suggest that this method has no advantages over that referred to above.

Collection and preparation of material for inoculation. This should be carried out as follows: If the material is to be obtained from a surface lesion, the area should first be washed thoroughly with a clean gauze sponge and physiological saline. Crusts, when present, should be softened by soaking and then removed. After

cleaning, the lesion is grasped firmly with a haemostat, and gentle pressure is made to effect haemostasis and hasten extrusion of tissue juices. Tissue fluids are collected with a capillary pipette or small syringe, using physiological saline with 10 per cent. rabbit serum added if a diluent is needed.

Lymph node aspirates are customarily inoculated directly. Blood may be inoculated as whole blood, with or without heparinization, or as plasma after brief centrifugation. Cerebrospinal fluid and other tissue fluids may be injected directly, or after concentration by centrifugation. Biopsy and autopsy specimens should be emulsified in saline, the heavy particles sedimented, and the supernatant fluid used for injection. *T. pallidum* can be readily sedimented by centrifugation, and it is probably advisable to concentrate organisms in this fashion in situations where the number of treponemes may be small but a large volume of fluid is available, e.g. blood and cerebrospinal fluid. We have accomplished this by centrifuging infectious material at approximately 2,000 G for 30 minutes, although a higher gravitational force may be desirable (Chandler and Cannefax, 1969).

Lymph node transfer. The most readily accessible and identifiable nodes are the popliteal. While in rabbits with untreated syphilis these nodes are often enlarged, we have encountered nodes in uninfected rabbits that are quite large also, possibly as a result of non-treponemal infections about the hind feet. The nodes lie slightly lateral to the midline of the popliteal space and are often imbedded in a fat pad in which they may be very difficult to identify. It was noted by one of us (B.N.) that removal and immediate chilling of the fat pad causes the fatty tissue to harden and turn white but leaves the lymph node as an easily identifiable reddish body. The node should be identified microscopically by examination of an emulsion or impression smear for lymphocytes. We usually emulsify either one or both nodes in 1 ml. physiological saline and inoculate half the emulsion into one testis of two rabbits. Although smears of the material can be stained by various methods for revealing treponemes, these are rarely found by other than intensive search as reported by Yobs, Olansky, Rockwell, and Clark (1965) and Yobs and others (1968) among others. We have on rare occasions observed motile *T. pallidum* in popliteal lymph nodes during the course of active syphilitic infection of the testis in rabbits.

Duration of observation. The proposed duration of observation of 120 days for inoculated rabbits has been arrived at more or less arbitrarily on the basis of experience. To state categorically that a syphilitic infection never becomes clinically evident after an incubation period of 120 days is manifestly unsupportable, but available evidence indicates that this event rarely occurs. Clearly slight lesions may be missed, and it is suggested that clinically negative animals which develop positive results to serological tests should be subjected to secondary passage of their popliteal nodes. Experience has shown that the yield of positive tests

from secondary transfers is very small when the original infectivity test was done under optimum conditions. Support for these statements will emerge from data to be given later in this paper, but particular attention is called to the work of Yobs and others (1968), whose studies included observation of inoculated animals over many months.

While observation periods of longer than 120 days might yield an occasional additional positive test, longer observation periods for either initial or subsequent transfers are scarcely justified as a routine procedure when the yield is balanced against the best use of limited facilities and manpower.

Man to Rabbit Transfers in This Laboratory

Our experience in the transfer of infective material from patients with one of the treponematoses covers a period of many years. The sole purpose of most of the transfers was to isolate new strains of treponemes, and not to test for infectivity *per se*. Since potential sources of new strains were usually plentiful, and unfortunately still are, often only one rabbit was inoculated from each patient; a higher proportion of positive results would probably have been obtained had at least one additional animal been inoculated. For the same reason less

than exhaustive studies were made of each inoculated rabbit.

Early Syphilis

Transfers of material were made to rabbits from 51 patients with early syphilis, fourteen of whom were in the primary stage, and 37 in the secondary. The results of transfers from the patients with primary syphilis are shown in Table I.

It will be noted that, of twelve transfers made from the primary lesion, a strain of *T. pallidum* was recovered and successfully passed a second time in each. Two instances in which aspirates from the regional lymph node were injected into rabbits likewise resulted in isolation of the strain; with one of these patients lymph node material as well as material from the lesion yielded positive results. In one instance the inoculation of whole blood failed to produce evidence of infection in the rabbit. Incubation periods from inoculation to development of a palpable lesion varied from 33 to 69 days in all animals but one which became positive after 89 days.

Table II (opposite) shows the results of the transfer of material from skin or mucous membrane lesions

TABLE I
SYPHILIS INFECTIVITY TESTS IN MAN
RESULTS OF TRANSFERS TO RABBITS IN FOURTEEN CASES OF PRIMARY SYPHILIS

Date	Patient No.	Source of Inoculum	Result in Rabbit*	Incubation period in First Passage (days)†	Result of Second Passage
19 August, 1932	1	Lesion	Pos.	60	Pos.
10 February, 1933	2	Lesion Lesion Lesion Lesion	Pos. Pos. Neg. Neg. (died)	60 150 32	Pos. Pos. ND§ ND
24 November, 1933	3	Node	Pos.	33	Pos.
1 December, 1933	4	Lesion Lesion Node	Pos. Pos. Pos.	51 89 51	Pos. ND ND
16 February, 1934	5	Lesion	Pos.	36	Pos.
14 September, 1937	6	Lesion	Pos.	34	Pos.
25 October, 1937	7	Lesion	Pos.	56	Pos.
2 November, 1937	8	Lesion	Pos.	48	Pos.
30 November, 1937	9	Whole blood	Neg.	69	ND
18 January, 1938	10	Lesion	Pos.	64	Pos.
24 January, 1938	11	Lesion	Pos.	39	Pos.
30 November, 1939	12	Lesion	Pos.	41	Pos.
30 December, 1950	13	Lesion Lesion	Pos. Pos.	34 25‡	Pos. Pos.
9 February, 1951	14	Lesion	Pos.	11‡	Pos.

*Intrasticular inoculation unless otherwise noted

†In rabbits that remained negative, number indicates days of observation

‡Intracutaneous inoculation of rabbit

§ND = Not done

TABLE II

SYPHILIS INFECTIVITY TESTS IN MAN
RESULTS OF TRANSFERS TO RABBITS OF MATERIAL
FROM SKIN OR MUCOUS MEMBRANE LESIONS IN 21
CASES OF SECONDARY SYPHILIS

Date	Case No.	Result in Rabbit*	Incubation period (days)†	Result of Second Passage
7 July, 1934	1	Pos.	30	Pos.
1 Mar., 1937	2	Pos.	30	Pos.
9 Sept., 1937	3	Died	18	ND‡
1 Oct., 1937	4	Neg. (abscess)	90	ND
25 Oct., 1937	5	Pos.	53	Pos.
8 Nov., 1937	6	Neg.	95	ND
16 Nov., 1937	7	Pos.	34	Pos.
22 Nov., 1937	8	Died	2	ND
3 Dec., 1937	9	Neg.	90	ND
10 Jan., 1938	10	Pos.	42	Pos.
12 Sept., 1939	11	Pos.	30	Pos.
18 Sept., 1939	12	Pos.	39	Pos.
13 Oct., 1939	13	Pos.	45	Pos.
18 Oct., 1939	14	Pos.	59	Pos.
1 Nov., 1939	15	Pos.	50	Pos.
12 Nov., 1947	16	Pos.	21	Pos.
6 May, 1950	17	Pos.	25	Pos.
		Pos.	53	ND
6 May, 1950	18	Pos.	66	Pos.
5 Sept., 1950	19	Pos.	50	Pos.
5 Sept., 1950	20	Pos.	56	Pos.
12 Nov., 1958	21	Pos.	41	Pos.

*Intratesticular inoculation in all.

†In rabbits that remained negative, number indicates days of observation

‡ND = Not done

of 21 patients with secondary syphilis. In all instances the lesions showed *T. pallidum* by darkfield microscopical examination, but treponemes were not always demonstrated in the material actually

transferred to rabbits. Of 21 patients studied, material from sixteen produced syphilis in rabbits, with incubation periods varying from 21 to 66 days. Two of the remaining animals died prematurely, and one developed a testicular abscess, caused by secondary infection shortly after inoculation. If these unsatisfactory tests are excluded, then a strain was recovered in sixteen of eighteen transfers.

Table III shows the results of transfer of material from various tissues of patients with secondary syphilis. In all of six instances infection was established in rabbits with aspirates from an enlarged lymph node. Results of the inoculation of whole blood or blood serum were less constant. Of nine instances in which whole blood was the inoculum, the results were positive in four, and negative in five. In two instances in which a strain was recovered, one of two rabbits inoculated failed to show evidence of infection. Liver biopsy material from one patient with syphilitic hepatitis produced infection in one of two rabbits into which it was inoculated, and kidney biopsy material from a patient with syphilitic nephrosis failed to produce infection, even though material from a lymph node of the same patient was infectious. Incubation periods in infected rabbits varied from 16 to 69 days.

It should be noted that strains isolated from patients were passed serially in rabbits without difficulty.

TABLE III

SYPHILIS INFECTIVITY TESTS IN MAN
RESULTS OF TRANSFERS TO RABBITS OF LYMPH NODE, BLOOD OR TISSUE BIOPSY MATERIAL IN
SIXTEEN CASES OF SECONDARY SYPHILIS

Date	Case No.	Source of Material	Result in Rabbit*	Incubation Period (days)†	Result of Second Passage
18 January, 1934	1	Lymph node	Pos.	35	Pos
2 February, 1934	2	Lymph node	Pos.	48	Pos.
25 November, 1947	3	Lymph node	Pos.	16	Pos.
17 November, 1947	4	Lymph node	Pos.	54	Pos.
5 December, 1947	5	Lymph node	Pos.	25	Pos.
23 September, 1966	6	Lymph node	Pos.	40	Pos.
		Lymph node	Pos.	47	ND‡
		Blood serum	Neg.	102	ND
		Blood serum	Neg.	110	ND
		Kidney biopsy	Neg.	102	ND
30 November, 1937	7	Whole blood	Neg.	90	ND
30 November, 1937	8	Whole blood	Neg.	129	ND
14 December, 1937	9	Whole blood	Pos.	69	Pos.
14 December, 1937	10	Whole blood	Neg.	150	ND
16 December, 1937	11	Whole blood	Neg.	150	ND
21 March, 1941	12	Whole blood	Pos.	43	ND
		Whole blood	Neg.	115	ND
25 March, 1941	13	Whole blood	Pos.	58	ND
		Whole blood	Neg.	110	ND
29 March, 1941	14	Whole blood	Neg.	92	ND
		Whole blood	Neg.	92	ND
2 November, 1966	15	Whole blood	Pos.	56	Pos.
		Whole blood	Pos.	(?)§	Pos.
21 January, 1948	16	Liver biopsy	Pos.	55	Pos.
			Neg.	90	ND

*Intratesticular inoculation in all

†In rabbits that remained negative, number indicates days of inoculation.

‡ND = Not done

§Lymph node transfer positive

Late Syphilis

In late syphilis, in contrast to early syphilis, treponemes are less abundant in the infected host and more difficult to demonstrate. Indeed, it is often difficult to be sure that *T. pallida* are present. Modern staining techniques, especially that in which fluorescein tagged antibody is used, have rendered demonstration of *T. pallidum* less difficult, but artefacts are common and can be misleading.

Our own data are meagre; they include only two patients in whom tertiary syphilis was suspected, but from each virulent *T. pallidum* was recovered by rabbit inoculation. One of these (W.M.-2-XI-48) had jaundice, and liver-biopsy material was inoculated into three rabbits. One rabbit was positive at 63 days, and the other two were still negative when last examined after 90 days. The other patient (C.C.-20-I-49) had a stomach lesion originally thought to be a carcinoma, but which at operation was considered to be a gumma; biopsy material from a retroperitoneal lymph node was positive in each of two rabbits at 39 and 43 days respectively. Liver biopsy material from the same patient gave a negative infectivity test. These two cases have been previously reported by Calkins, London, Mellinkoff, van Meter, and Turner (1950).

Man to Rabbit Transfers by Others Earlier Studies

Early Syphilis

The early history of the isolation of strains of *T. pallidum* in laboratory animals was well reviewed by Willcox and Guthe (1966). Even before the discovery of *T. pallidum*, the disease had almost certainly been successfully transferred to both the higher apes and to rabbits, but shortly after the organism was discovered syphilis was definitely established in both monkeys and rabbits. While successful transfers from patients with early syphilis to laboratory animals have been made by many individuals since that time, few large series have been reported and few data are available on the proportions of positive results obtained in the probably varying circumstances then prevailing. Likewise, reports have appeared only sporadically on the results of transfer of material from patients with late or latent syphilis; with such cases it is probably fair to conclude that in most instances only successful attempts were reported.

No attempt will be made to review the numerous reports in the literature describing the isolation of *T. pallidum* in rabbits from skin and mucous

membrane lesions of patients with early syphilis; a good summary of the early experiences has been given by Mulzer (1927). There has been recovery from various secretions including blood, tears, milk, urine, joint and cerebrospinal fluid, and lymph nodes, but most of these reports do not permit assessment of the ease of isolation in rabbits, assuming the presence of treponemes in the transferred tissue. Details of some of the earlier results are to be found in the papers by Uhlenhuth and Mulzer (1913) for blood, Chesney and Kemp (1924) for cerebrospinal fluid, and Lake and Bryant (1930) for lymph nodes.

Of 23 cases of primary syphilis in which blood was transferred to rabbits by Uhlenhuth and Mulzer (1927), the results were positive in sixteen, negative in three in which one or more rabbit survived, and unsatisfactory in four. In the cases of 38 patients with secondary syphilis, blood from 27 invoked a lesion in the rabbits' testes, blood from nine failed to produce a lesion in at least one or more surviving animal, and in two the results were unsatisfactory. It should be noted that, in the two series, of 59 rabbits which showed lesions, the incubation period was between 30 and 60 days in 23, between 61 and 90 days in 32, and between 91 and 109 days in four. The duration of observation was at least 120 days in most surviving animals.

Chesney and Kemp (1924) summarized all the reported instances in which *T. pallidum* had been isolated from the cerebrospinal fluid of patients in various stages of syphilis and they reported the results in their own series of 34 patients. From the literature there were 46 instances in which *T. pallidum* was demonstrated in the spinal fluid: 24 by rabbit inoculation, one by monkey inoculation, six by darkfield, fifteen by staining techniques, and one unspecified. Apparently only one method was used by a particular group of investigators so no comparison of method could be made. Of those cases from which *T. pallidum* was isolated by animal inoculation, fourteen were in patients with early syphilis and ten in patients with either paresis or tabes. The cases studied by Chesney and Kemp were patients with early syphilis, but with no changes detected in the cerebrospinal fluid by the customary tests. Of the 34 patients studied, *T. pallidum* was isolated in five, which together with similar cases studied up to that time, gave a positive result in approximately 15 per cent. It should be noted that in two of the five cases in Chesney's series, *T. pallidum* was recovered on the second animal passage, not the first.

The most impressive of the earlier series of transfers of lymph nodes was that reported by

Lake and Bryant (1930) in which lymph node biopsy was performed on 34 patients with untreated early syphilis. Emulsions of the excised nodes were injected intratesticularly in rabbits with positive results in all 34 cases. Incubation periods in the rabbits varied from 16 to 89 days, all but six being less than 60 days. In four of these cases lymph node material was also transferred to rabbits after antisyphilitic treatment, with negative results in all.

It is evident from this experience not only that the infectivity test is positive in most cases of early syphilis but also that in the vast majority of instances the incubation period in the recipient rabbit is under 60 days and only rarely longer than 90 days. The observations of Beerman (1936) suggest, too, that there is no pronounced shortening of the incubation period in subsequent passages. Working with a strain of *T. pallidum* isolated from a patient with treatment-resistant syphilis, he found that the average incubation period in rabbits following intratesticular inoculation was consistently about 42 days from the first to the eleventh animal passage, which was about the same as for the Nichols strain. It was noted in the days before air-conditioning that the incubation period tended to be longer in summer.

Ecker (1933), studying nine freshly-isolated strains as compared with four well-established strains, found that the "mean incubation period of the newer group varied from 30 to 44 days, while that of the older group varied from 24.75 to 33.85 days".

Late and Latent Syphilis

Apart from the cerebrospinal fluid studies noted above, there is little information on attempted isolations from patients with longstanding syphilis; most have been made from patients with clinically recognizable late lesions, although a few have been made from cases of inapparent infection. Among the latter have been the isolations from semen reported by Ebersson and Engman and the lymph node isolates of Cottini and Hamburger (both cited by Calkins and others, 1950). Lake and Bryant (1930) reported negative results from lymph node transfer in the cases of eight patients with longstanding latent syphilis who had received no antisyphilitic treatment and in five who had received "inadequate" treatment. Positive results were obtained from four other patients, two of whom had tertiary lesions at the time of transfer. The duration of syphilis was estimated to be 24, 9

6 and 4 years respectively, and incubation periods in the rabbits to which node material was transferred were 31, 40, 62, and 49 days respectively.

It appears, therefore, that, regardless of the stage of syphilis from which *T. pallidum* is obtained, the incubation period in the rabbit is usually within the same general range.

Recent Studies on Persistence of Treponemal Forms

In the past few years a new dimension has been introduced into the determination of the presence of syphilitic infection in a human or animal host. Beginning with the work of Collart, Borel, and Durel (1962, 1964) and Collart and others (1968), followed by that of Yobs and others (1965, 1968) and, from a somewhat different angle, by Smith and Israel (1967, 1968) and Smith and Pesetsky (1967), treponeme-like forms have been found in various tissues, principally by staining techniques. Search has been directed particularly to lymph nodes, aqueous humour, and cerebrospinal fluid from patients, many of whom had received penicillin in amounts generally regarded as curative for syphilis. These studies on human beings have been supplemented in some instances by studies in rabbits and primates.

It is especially noteworthy that up to the time of writing this paper few instances have been reported in which pathogenic treponemes have been recovered from such tissues, either because attempts at recovery have not been made or having been made they have proved to be negative. Since these treponeme-like organisms are being referred to as *T. pallidum* by some investigators we are being asked to ignore two touchstones of the past (recovery of virulent *T. pallidum* in animals and response of the lesion to specific therapy) and to accept a definition of *T. pallidum* based solely on stains, including the fluorescent antibody stain. Subordination of the lessons of Koch's postulates has led, to borrow a sociological phrase, to a "crisis of identification". When is a treponemal form *T. pallidum*, the causative agent of syphilis? While the answer to that question will not be found in this paper, the results of infectivity tests, as defined in the preceding sections, will be reviewed in relation to the overall problems.

The initial studies of Collart and others (1962) were made in rabbits in studies covering a 4-year-period. Fifty rabbits which had been infected with the Nichols strain and subsequently developed typical syphilitic lesions were treated with large

doses of penicillin 24 months after inoculation. One popliteal node was transferred shortly before treatment by intrascrotal transplant to one normal rabbit, and the other node was transferred 5 to 15 months after treatment. The pretreatment infectivity test was positive in 29 of 39 survivors of the original inoculation and holding period; incubation periods in the node-recipient animals varied from 30 to 145 days. Post-treatment node transfer gave no positive infectivity test results in 20 surviving rabbits, whereas positive infectivity tests were obtained in two of seven untreated control rabbits infected some 2½ to 3 years previously. Later, twelve treated rabbits and one control were given cortisone. The control and ten treated rabbits remained free of lesions, but two treated animals developed annular lesions on the ears; in one animal the lesion was darkfield positive for treponemes. It should be noted in passing that the ear is a favourite location for lesions in natural *T. cuniculi* infection.

In contrast to the infectivity tests cited above, silver stains on the nodes showed the following:

Pretreatment node examination. Thirteen of fifteen positive;

Post-treatment examination. Of twenty animals eleven were positive for "typical" treponemal forms and nine for "atypical" forms. The nodes of seven untreated controls examined about the same time showed "typical" forms in three and "atypical" in four.

Extensive serological tests were made in both the original animals and in the node-recipient rabbits; these will not be reviewed in detail here.

Collart and others (1964) then studied the nodes of twelve patients, all of whom had a clear history of syphilis treated with large amounts of penicillin, although in some instances treatment had not been recent. By means of stains, typical treponemal forms were found in seven and "atypical" in five. Infectivity tests by tissue implants into the scrotum of rabbits were made in six cases, with positive results in three; in the other three the recipient animals were described as still under observation.

Mention has already been made of the study by Collart and others (1968) in which seven positive infectivity tests were obtained from ten patients with secondary syphilis.

The most extensive studies in terms of numbers of subjects, animals used, and sheer manpower commitment, have been those of Yobs and others (1965, 1968). It is difficult to determine from the text of the published papers (especially the second of the two) precisely what were the results, as determined by infectivity tests, demonstration of trepo-

nemes by fluorescent antibody techniques (FA), demonstration of treponemes by silver stains and development of positive serological tests by recipient rabbits.

The published charts, however, permit regrouping of their data. Beginning with a group of 45 patients, all of whom had received substantial anti-syphilitic treatment, the following subgroups may be recognized (one patient with untreated early syphilis has been eliminated from consideration):

Group A. Two patients whose inguinal nodes gave positive infectivity tests in rabbits, with incubation periods of 29, 35, 36, and 36 days respectively. Treponemal forms were demonstrated in the nodes of one patient but not in the other.

Group B. Three patients in whose nodes treponemal forms were demonstrated by stains, but in which the infectivity test was negative.

Group C. Forty patients in whose nodes treponemal forms were not demonstrated by stain, and in whom the infectivity test was negative.

Group D. Five patients originally in Groups A or B who were subjected to re-treatment with penicillin after the initial node transfer.

No treponemes were demonstrated in the nodes of any after the treatment and the infectivity tests were negative.

Secondary transfers of either popliteal nodes or blood following cortisone treatment were made from the first passage recipient rabbits after 12 months and these second passage rabbits in turn were observed for 12 months or longer. The nodes of many of both the first passage and second passage rabbits were searched for treponemes after staining.

It should be noted that the infectivity test was positive in only two of the 45 patients, and in these the incubation period in each recipient rabbit was less than 40 days. Despite observation of a total of 175 rabbits of the first and second passages, all of which were observed for 6 months and most for 12 months or longer, no additional positive infectivity test was encountered from these 45 patients.

Turning now to the recipient rabbits from Groups B and C, of those inoculated with human node material in which treponemes had been demonstrated (Group B), two rabbits from one patient (No. 20) were both negative, but a popliteal node from one of two second passage rabbits was positive for stained treponemal forms. From patient No. 29, one recipient rabbit was positive and the other negative, but the second passage rabbit from the latter was positive. In both instances, therefore, the treponemal form skipped a passage, assuming that the forms in fact were being transferred. From

the third patient in this group (No. 22) no search of rabbit nodes was made.

The first passage animals from 21 patients of Group C we examined for nodal treponemes, and five rabbits and four patients were positive. Of two second passage animals from those that were examined, the nodes of one were positive for treponemal forms and those of the other were negative.

24 rabbits inoculated with inguinal nodes from seventeen patients of Group C were negative for nodal treponemes, but second passage from sixteen of them yielded a positive result in three rabbits and a negative result in thirteen, all stemming from different patients originally.

Among Group D patients, all of whom had either a positive infectivity test or treponemal forms demonstrated in their nodes before the last round of antisypilitic treatment, no treponemes were demonstrated by stain in their inguinal nodes. However, the first passage rabbits whose nodes were examined showed the following:

- Patient No. 10, one rabbit positive;
- No. 12, one negative, second passage two negative;
- No. 20, one positive, one negative and from the latter one positive and one negative;
- No. 22, two negative, no second passage;
- No. 29, one positive, one negative, and second passage to two rabbits each, yielded negative results in all four.

It is difficult to detect any pattern in these results. Percentage figures for any combination of supposedly transmission pathways that we can assemble are without statistical significance. One is led to speculate, therefore, whether the results are indeed distributed randomly without relation to the original source of the inoculum.

Unfortunately, for obvious reasons, no comparable observations have been made on large groups of animals originally inoculated from non-syphilitic patients, or perhaps better still not inoculated at all, and observed over long periods of time in the laboratory. It seems reasonable to question, therefore, whether use of the term "transmissible" agent is justified under the circumstances.

Supplementing the foregoing studies on nodes from human beings with treated syphilis, was a study of seventeen rabbits with long-standing untreated syphilis. These were given cortisone and their blood then transferred to two to six rabbits each. Five positive infectivity tests were obtained from the seventeen rabbits, but the result of the search of recipient rabbits nodes for treponemal forms bore no obvious relationship to the results of the infectivity tests. Of four rabbits that developed orchitis and whose nodes were examined,

treponemal forms were found in one, but not in the other three. Treponemal forms were found in the nodes of only one of eighteen recipient rabbits which showed a negative infectivity test, although all had been inoculated with blood following cortisone treatment of rabbits with long-standing untreated syphilis. Again, no predictable pattern for the occurrence of nodal treponemes by staining can be detected.

It should be emphasized that the conclusions of Yobs and others (1968) are conservative and the foregoing should in no way be construed as a criticism of their studies, indeed quite the contrary.

Data of some relevance may be obtained from an experiment carried out in rabbits in this laboratory. 48 rabbits were divided into four groups of twelve rabbits each. The animals of three groups, B, C, and D, were infected with *T. pallidum* by intratesticular inoculation. The animals of Group A were not infected but maintained as a "normal" control group. The infected rabbits developed typical syphilitic lesions and went through the customary evolution of the experimental disease, retrogressing eventually into a latent stage in which no evidence of clinical activity could be detected.

Six months after infection, the animals of Group B were given penicillin in doses believed to be curative and at 18 months after infection the rabbits of Group C were similarly treated; those in Group D remained untreated. Surviving animals of all groups were killed at approximately 2½ years and various tissues studied for the presence of *T. pallidum*. Only the results of the infectivity test made with popliteal nodes and blood plasma will be given here, since studies on other aspects of the problem have not yet been completed.

A number of rabbits in each of the groups, including the uninfected controls, died during the long holding period, usually of intercurrent infection and often unexpectedly at night or during the weekend, in which case the tissues were not suitable for study. It should be noted that the proportion of rabbits which died during the 2½ years observation period was essentially the same for the four groups. Of those in which infectivity tests could be done, the results of organ transfer were largely those which could have been expected from previous studies. Lymph nodes from untreated animals invariably gave positive infectivity tests, whereas those from the treated groups, as well as the uninfected rabbits, were negative. The blood plasma of only one of three untreated rabbits gave a

positive infectivity test. The results are summarized in Table IV.

In this series, secondary transfers were made after 120 days from all surviving animals inoculated with the nodes and plasma of the original animal. The secondary transfers were made by pooling the popliteal lymph nodes of the animals inoculated from a common source and inoculating this material intratesticularly into two normal rabbits. No strain of treponeme was recovered during the subsequent observation period of 120 days.

The foregoing results merely confirm many years experience of a similar nature in this laboratory, as reported originally in a number of papers by Chesney and Kemp (1925).

Demonstration of Spiral Organisms in Aqueous Humour and Cerebrospinal Fluid of Patients

Willcox (1964) reviewed the findings of Collart and others (1962, 1964) and suggested that patients with clinical lesions which have always been of a "problem" nature (*i.e.* those with interstitial keratitis, nerve deafness, optic atrophy, and lightning pains) might offer promising material for study. Reports were not long in coming.

In some eight papers, Smith and his associates in Miami reported finding spiral organisms in the aqueous humour of patients with various forms of ocular disease which could be due to syphilis (*cf.* Smith and Israel, 1967, 1968). In many of these patients the history and serological findings strongly suggested that the patient had been infected with *T. pallidum*. At the same time, study of the aqueous humour often revealed treponeme-like organisms by darkfield examination and by stains. In many instances the treponemes gave a positive fluorescent antibody test with syphilitic serum. Somewhat the same findings were obtained with cerebrospinal fluid from patients suspected of having syphilis.

Goldman and Girard (1967) in Boston found treponemes in the aqueous humour of two patients

with interstitial keratitis. Christman, Hamilton, Heaton, and Hoffmeyer (1968) in Philadelphia reported the presence of spiral organisms in the aqueous humour from twelve of 36 patients suspected of having syphilitic ocular disease. Golden, Watzke, Lindell, and McKee (1968) in Iowa City reported the findings in the aqueous humour from 47 unselected patients subjected to cataract surgery. Spiral organisms were found in one only and this patient had no evidence of syphilis; it is especially significant that these spirochaetes failed to stain immunofluorescently with syphilitic serum. It is known that the aqueous humour and cerebrospinal fluid from many other patients have been studied but at the time of writing no estimate can be made of the total number. Until such figures are available, the incidence of spirochaetal forms in aqueous humour or cerebrospinal fluid and their relation to clinical and serological evidence of syphilis cannot be determined.

The point to be made here is that infectivity tests with aqueous humour have been reported in only three patients (Smith and Israel, 1967, 1968; Smith, Israel, McCrary and Harner, 1968), and one of these we would not accept as a positive test. In only one patient was a positive infectivity test obtained with cerebrospinal fluid (the one which in our view showed the equivocal test on aqueous humour). Further details of these cases are given in the Addendum (p. 195). Each patient had lesions suggestive of syphilis. One had received substantial amounts of antisyphilitic treatment in the past. It is worth noting that no mention was made of finding spiral organisms in the aqueous humour of two of the three cases, although treponemes were found in the iris of one of these two. Thus far, it appears that the sole criterion for characterizing as *T. pallida* treponeme-like organisms found in the aqueous humour and cerebrospinal fluid has been staining with fluorescent-tagged antibody. This we believe is not justifiable in the present state of our knowledge.

TABLE IV
RESULTS OF INFECTIVITY TESTS WITH LYMPH NODES AND BLOOD PLASMA FROM TREATED AND UNTREATED SYPHILITIC RABBITS

Group	Category	No. Surviving in Group	Results of Transfer of Tissue					
			First Passage				Second Passage	
			Nodes		Blood Plasma		No. Pos.	No. Neg.
			No. Pos.	No. Neg.	No. Pos.	No. Neg.		
A	Not infected	3	0	3	0	3	0	3
B	Treated 6 mths	6	0	6	0	4	0	6
C	Treated 18 mths	3	0	3	0	3	0	3
D	Not treated	5	5	0	1	2	ND	ND

ND = Not done

Discussion

This paper is concerned with a consideration of the value of the rabbit infectivity test as an index of syphilitic infection in any given host. Few studies have been directed primarily to this point, and the data cited here have been gathered from a variety of sources and provide, at best, less than a complete answer to the main question.

Nevertheless, when the data presented are examined critically some clearer notion of the role of the infectivity test does emerge. In the first place, it seems abundantly clear that a strain of *T. pallidum* which is producing disease in human beings, and which presumably has been perpetuated in the human species for millenia, is readily pathogenic for rabbits when inoculated under conditions believed to be optimum. Not only is the incubation period of the disease in this new species usually under 90 days, but failure to isolate *T. pallidum* from overt syphilitic lesions is relatively uncommon, even by the less-than-exhaustive effort made in most of the reported experiments.

It seems justified therefore to conclude with the general proposition that *failure consistently to isolate T. pallidum from human tissue* (when the test in rabbits is performed under specified conditions) *places the burden of proof on those who contend that pathogenic treponemes are nevertheless present.*

The clinical concept of latent syphilis grew out of the discovery of the Wassermann test, and the biological concept was firmly established when tissue infectivity during this period was demonstrated. Of course, experiments in nature were all the while demonstrating the same phenomenon, when syphilis in man would move from an asymptomatic to a symptomatic phase.

It soon became accepted that sero-reactivity in general indicated that the individual had been infected with a treponemal organism, but it has never been clearly shown that positive test results with either the cardiolipin antigen or the treponeme as antigen, necessarily indicate persistence of infection. Indeed, the weight of evidence in experimental syphilis clearly indicates that persistently positive results, especially in tests in which the virulent treponeme is used as antigen, occurring after antisyphilitic treatment, do not necessarily mean that the infection persists in a biological sense.

More recently another dimension has been introduced in the form of better techniques for staining treponemes in tissues, coupled with remarkable industry and persistence on the part of a few investigators. These workers have suc-

ceeded in demonstrating spiral organisms in various human and animal tissues where their presence was not suspected on the basis of previous experience. These demonstrations are of the greatest importance and imaginative speculation about them is both justified and desirable. But the new findings are scarcely yet of such a transcendent nature as to render the older data useless; rather, there should be a strong effort to relate the new and the old, if possible.

To be specific, what criteria should be at hand clearly to establish these spiral organisms as *T. pallida*? What data can be obtained concerning their ability to produce disease and their ability to serve as an antigenic stimulus?

The important question is:

Are *T. pallida* residing in long-infected hosts less virulent for laboratory animals than those in hosts with infections of shorter duration?

Convincing data on this question are most difficult to obtain. It has long been known that many infected untreated rabbits yield *T. pallida* of undiminished virulence for the rest of their lives, even though the infection remains below the clinical threshold. It is not known with certainty whether the same situation obtains in man. Yet *T. pallida* virulent for rabbits have been recovered from enough cases of late human syphilis to prove that the treponemes often remain virulent for laboratory animals for years.

Clearly, failure to establish infection in rabbits does not eliminate the possibility that the patient harbours virulent treponemes, for we have seen that even in early syphilis transfer is not always immediately successful. Nevertheless, continued failure to recover virulent organisms in large groups of patients does raise doubts whether such organisms are in fact present. In other words, the evidence to support the contention that *T. pallidum* through long residence in a single human host gradually loses its virulence for the rabbit is meagre indeed, and mostly of an indirect nature.

There is evidence from the realm of comparative microbiology to suggest that in certain instances something does happen to the metabolism of a micro-organism during a long stage of latency in a human host that makes it respond less readily to a new environment. Some of these studies have been cited by Yobs and others (1968). For example, McDermott (1958) found in some instances that *Mycobacterium tuberculosis* recovered from patients in whom the disease was latent required prolonged periods on artificial media before multiplication again began. It is known, too, that organisms of the

Mycoplasma genus and so-called "L" forms may remain in human hosts for many years (Eaton, 1965), but these, like *M. tuberculosis*, are essentially intracellular parasites, whereas the evidence indicates that *T. pallidum* is commonly located extracellularly. It is not known whether the treponeme also has an "L" form which may hide for years in disguise within the host's tissue. But it is incumbent upon us to try to go beyond mere speculation.

The surprising does indeed happen in science, but each new finding should be well established in fact before being used to invoke new surprises and brush away current concepts. Our task is to assess, with all the tools at our disposal, both old and new, the significance of these newer findings in terms of human disease. In this assessment the infectivity test as developed and applied to the study of syphilis should be used not casually but expertly and as an essential tool in our approach to these problems.

In the present state of our knowledge it seems wise to place rather restrictive criteria around the definition of a positive infectivity test; for when so used the test can provide information of value. This is not to imply that experimentation should not continue toward making this or similar tests more definitive. Moreover, other methods aimed at the detection of transmissible treponemal forms should be explored to the fullest in the hope that, whether through serological, histological or immunological techniques, the presence or absence of such forms can be ascertained with a degree of assurance.

The infectivity test as traditionally interpreted yields highly useful information. A positive test indicates that the donor harbours treponemes that are capable of inducing disease. Almost certainly the test is negative in some cases in which virulent treponemes are still present in the host, and doubtless repetitive testing would yield a higher percentage of positive results.

Nevertheless, the predictability of results under various situations is high, and this has been further refined by the extensive studies of the past few years. It should be observed also that positive infectivity tests are ordinarily manifested within 3 months of inoculation; beyond that period virtually no additional positive tests have been observed, although second passage of material at 3 to 4 months may be positive.

While the long holding periods used in some of the experiments referred to in this paper were justified, there is no indication that such long periods of observation are needed or yield a sig-

nificantly higher proportion of positive results than an observation period of 4 months. In other words, there is no evidence that *T. pallidum* regains pathogenicity in a new host after a 4-month period, to pick an arbitrary limit.

In our opinion a second passage, 3 months after the original inoculation, would be preferable to observation of the first passage animal for longer periods. In making such secondary transfers tissues from animals inoculated from the same original source might be pooled.

One of the central facts to emerge from both old and recent observations is that *T. pallidum*, after thousands of years in human hosts and after many years in a single host, is still highly pathogenic for the rabbit.

In the face of this evidence great caution must be exercised in assuming that treponemal forms which seem not to have the capacity to produce disease in rabbits, are capable of inducing disease in the host in which they are discovered. In the final analysis the diagnosis of syphilis in man must rest primarily on clinical and epidemiological evidence; serological and bacteriological findings are of the greatest practical value, but evaluation of these procedures, overtly or by implication, rests on their relationship to specific disease processes in man. It is often forgotten that the clinical characteristics of syphilis as we know them today were, on the whole, well known in the two decades preceding the discovery of *T. pallidum*, as witness the successive editions of Osler's "Textbook of Medicine" (Harvey and McKusick, 1967). *T. pallidum* is still by definition an organism that produces a characteristic disease picture in man or animals. While treponeme-like organisms have been cultivated *in vitro*, their disease-producing proclivities have never been convincingly demonstrated. We suggest that it will be wise to apply the same rigorous criteria to such forms as might be demonstrated by darkfield microscopy or stains.

In our opinion specific fluorescent antibody staining of a treponeme-like organism does not prove that the organism is a true *T. pallidum*, although the method has great promise and even in the present state of knowledge constitutes an important research tool.

Such an attitude need in no way inhibit the vigorous search for further information on the nature of these treponemal forms and their role in disease. Indeed, as so well pointed out by Willcox (1964), these new observations should serve as a tremendous spur to further studies, keeping in mind that the long-range objective is to assess the results of the studies in terms of human health and

disease. In such investigations infectivity tests will constitute an indispensable research tool. Regardless of the transformations virulent *T. pallidum* may undergo, at some point in the biological cycle of either the organism or its host there must be evidence of its reversion to a pathogen if the findings are to be meaningful to the clinician or the epidemiologist.

Summary and Conclusions

When syphilitic lesions of man are transferred to rabbits under favourable conditions, syphilitic disease is invoked in these laboratory animals in a significant proportion of instances. While such infectivity tests have obvious limitations, they nevertheless yield information not readily obtainable by any other method.

Certain criteria for the performance of infectivity tests as a research tool are proposed.

Consistent failure to obtain a positive infectivity test is strong presumptive evidence that pathogenic *T. pallidum* is not present in the transferred tissue.

The implications of the recent demonstration of treponeme-like organisms in tissues of man and animals and the possible role of the infectivity test in these important new studies are discussed.

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ADDENDUM

In support of the statements on page 192 with reference to positive infectivity tests obtained by Dr. J. Lawton Smith and his colleagues, the following data from their case reports are cited. We shall assign an alphabetical designation to each case.

Case A. White male aged 63. This case was referred to in sufficient detail to permit identification in two papers by Smith and his colleagues; as Case 3 in *Trans. Amer. Acad. Ophthalm. Otolaryng.* (1968) **72**: 63, and in *Amer. J. Ophthalm.* (1968) **65**: 242. He had optic atrophy and chorioretinitis. Serology negative or equivocal. Gonorrhoea 42 years earlier. On April 17, 1967, fluid obtained by paracentesis from left eye according to first paper and right eye according to second was inoculated into right testis of rabbit 8. On June 1, 1967, right testis of this rabbit was said to be a "bit enlarged" and 44 days after inoculation darkfield examination of the testis was "unequivocally positive". No mention was made of finding treponemes in the aqueous humour.

Case B. Negro male. This case was also referred to in *Trans. Amer. Acad. Ophthalm. Otolaryng.* (1968) **72**: 63 and in *Amer. J. Ophthalm.* (1968) **65**: 242. In both papers the patient's age was given as 68; serology was negative or equivocal; and the patient had a diastolic murmur over the aortic area. Aqueous humour was inoculated into left testis of rabbit 5 on April 6, 1967. On May 4, 1967, this left testis was enlarged and fluid from the testis showed treponemes by darkfield examination and by stains. No mention was made of finding treponemes in the aqueous humour.

A small portion of the iris was inserted into a subcutaneous abdominal pouch of squirrel monkey 6. Treponemes were found in the iris by fluorescent antibody stains. The monkey was deemed to show a positive