Immunity in syphilis
Studies in active immunity

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Summary
Support for the concept of the development of immunity during the course of syphilis is available in the literature. In experimental syphilis in rabbits, some immunity is present approximately 3 weeks after infection with *Treponema pallidum*. Resistance to re-infection increases to a maximum at approximately 3 months after infection. Termination of this state by penicillin treatment within this 3-month period may enable re-infection to be accomplished.

Attempts to reproduce this state of immunity experimentally by injection of *T. pallidum* itself, or protein derivatives, or ultrasonic disintegrates obtained from *T. pallidum* or non-pathogenic treponemes, have been unsuccessful. However, promising results in rabbits have resulted from injecting *T. pallidum* suspensions attenuated by storage at 4°C, penicillin, or gamma irradiation, and also by suspensions preserved by glutaraldehyde. In the present study, partial resistance to intratesticular challenge in rabbits with *T. pallidum* has been obtained by immunization with a variety of non-pathogenic treponemes, as exemplified by the strains Nichols, Kazan 2, 4, 5, and 8, *Treponema minutum, Treponema ambigua, Treponema refringens* and *Treponema microdentium*. Success is attributed to the processing of immunizing antigens at 4°C and storage until use at −20°C. Attempts to attenuate *T. pallidum* by immunological means, namely, passage through a limited number of immunized rabbits, were unsuccessful.

Introduction
It is well known that immunity to *Treponema pallidum* develops in the course of syphilis in rabbits and human beings. In experimental syphilis in rabbits, some immunity is present approximately 3 weeks after infection with *T. pallidum*. Resistance to re-infection increases to a maximum approximately 3 months after infection. Termination of this state by penicillin treatment within this 3-month period may enable re-infection to be accomplished (Magnuson and Rosenau, 1948; Magnuson, Rosenau, and Clark, 1949; Turner and Nelson, 1950).

Attempts to produce immunity in rabbits against experimental infection with *T. pallidum* using *T. pallidum* vaccine have been made by a number of investigators. Some have reported failure, including Magnuson, Halbert, and Rosenau (1947), Eagle and Fleischman (1948), Waring and Fleming (1951), McLeod and Magnuson (1953), McLeod (1962), and Miller, Fazzan, and Whang (1963). Others have reported success including Miller (1967, 1971), Metzger, Michalska, Podwińska, and Smogór (1969), and Jones, Zeigler, and Jones (1976).

Similarly, attempts at inducing immunity in rabbits by 'avirulent' cultivable strains of *T. pallidum*, or non-pathogenic treponemes, have given variable results. Thus, Knox, Dacres, Short, and Glicksman (1967) reported success with an avirulent cultivable treponemes, whereas Gelperin (1951), in the Reiter treponemes, and more recently Izzat, Dacres, Knox and Wende (1970), and Izzat, Smith, Jackson and Knox (1971), with the Nichols non-pathogenic treponemes, reported failure.

The present study records attempts to produce immunity in rabbits against *T. pallidum* using cultivable non-pathogenic treponemes. In experiment 1, intratesticular challenge was applied at intervals of 3 to 9 weeks after immunization, and in experiment 2, at 7 months. Experiment 3 was designed to test simultaneously immunity to challenge, the virulence of the challenging *T. pallidum*, and possible attenuation through passage.
Materials and methods

Treponemes examined in this study  The treponemal strains of this study are listed in Table I, together with their origins and their present source. T. pallidum itself is represented by the non-cultivable Nichols pathogenic strain which is maintained in the rabbit testicle. Cultivable non-pathogenic treponemes are represented by five strains of alleged cultivable strains of T. pallidum, and by four oral or ano-genital strains saprophytic to man.

Non-pathogenic treponemes, cultivation and harvest

Culture medium  All strains of non-pathogenic treponemes were cultivated in a modified Brewer (1940) thioglycollate medium using a modified culture system described by Gelperin (1949). The medium was prepared from commercially available dehydrated products—Spirolate Broth (BBL) and Brain Heart Infusion (BBL) (Hanson and Cannefax 1964). The complete reconstituted medium consisted of 14.5 g. Spirolate Broth and 18.5 g. Brain Heart Infusion to each 100 ml. of distilled water. Trypton, asparagine, and sodium thioglycollate were added to the mixture as suggested by Hanson and Cannefax (1964) in quantities of 25 mg. of each. The mixture was autoclaved at 121°C for 15 min. Sterile calf serum inactivated at 56°C for 30 min. was added to the autoclaved and cooled medium to a final concentration of 10 per cent. Semi-liquid medium was made by adding 0.1 g. ion agar No. 2 (Oxoid) to the basal medium. Solid medium was made by adding 0.75 g. ion agar No. 2 (Oxoid) to the basal medium. Serum was usually added while the medium was still warm after autoclaving.

Semi-liquid and solid media were used only to restore the activity of inactive or sluggish stock cultures. Bulk cultures in liquid medium were inoculated from these primary cultures. Media were prepared fresh and used within 48 hrs. Serum was added immediately before inoculation.

Maintenance and seed cultures  Treponemes were received from the reference laboratories either lyophilized or as cultures in solid medium, or in gelatinized serum containing a portion of meat at the bottom of the tube. Lyophilized organisms were rehydrated with distilled water. Primary cultures were made in 20 ml. semi-solid or solid medium in screw-capped bottles of 30 ml. capacity, by deep inoculation with a Pasteur pipette of approximately 0.2 ml. of culture. Incubation of semi-solid medium cultures was allowed to proceed at 35°C in 5 per cent. carbon dioxide and 95 per cent. nitrogen mixture. Growth usually became apparent in 3 to 10 days as a slight haze in the depths of the medium. Maintenance cultures, which were passaged every 2 weeks, were not incubated in the carbon dioxide and nitrogen mixture, but were incubated in screw-capped bottles of 30 ml. capacity filled with medium so as to exclude as much air as possible. The amount passaged each time was approximately 1 ml. Under these conditions the treponemes do not multiply at their maximum rates. For the preparation of seed cultures multiplying at maximum rates, larger inocula of 3-5 ml. were passaged at 2 to 3 day intervals for 6 to 8 passages, as described above. For mass cultivation the entire contents of the 30 ml. bottles were used as inocula, as described below.

Mass cultivation  5 to 10 litres of liquid medium were prepared for each strain, and distributed in aliquots of 450 ml. in screw-capped bottles of 500 ml. capacity. Each large bottle of medium was inoculated with approximately 30 ml. of the seed culture. Bottles were then filled to the top with fresh medium so as to exclude as much air as possible, tightly closed, and incubated at 35°C. Cultures were gently agitated once or twice a day.

Conditions for incubation and growth  Experience showed that treponemal growth was better when air was excluded from cultures and they were incubated tightly closed at 35°C. For maximum growth the duration of incubation varied with different strains. The longer the period for which cultures were incubated the greater the tendency for some organisms to produce cyst-like structures. For this reason, organisms were harvested before the end of the exponential phase. Optimum times for harvesting different treponemes are shown in Table II.

Harvesting and washing of treponemes  Treponemes were harvested by centrifuging cultures at 8000 × G for 10 min. at 5°C in an MSE centrifuge. The deposit, which resembled a thick paste, was washed by re-suspending in approximately 10 to 20 ml. of buffered saline depending

<table>
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<th>Table I  Human treponemes</th>
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<tr>
<td>Treponemal strains</td>
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<tr>
<td>T. pallidum (Nichols pathogenic) Nichols (non-pathogenic)</td>
</tr>
<tr>
<td>Kazan 2</td>
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<tr>
<td>Kazan 4</td>
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<tr>
<td>Kazan 5</td>
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<tr>
<td>Kazan 8</td>
</tr>
<tr>
<td>T. refringens</td>
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<tr>
<td>T. minuta</td>
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<tr>
<td>T. ambiguus</td>
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on the bulk of the deposit, using repeated aspiration and expulsion with a syringe and 20-gauge needle to homo-
genize the suspension. The suspension was then made up to 250 ml. with buffered saline and recentrifuged at
8000 × G for 30 min. at 5°C. Washing and centrifugation were repeated four times in all, using 250 ml. each time,
except in the final instance in which a pre-weighed 100 ml. polythene tube, which was re-weighed after deposition of
the cells and discarding of the supernatant fluid, was used. The weights of organisms per litre of culture medium are
presented in Table II. After the final centrifugation treponemal deposits were re-suspended in buffered saline and
the volume made up to contain 10 g. net weight per 100 ml.

Preparation and storage of preparations for immunization
For disruption, 30 ml. of the 10 per cent. suspension were
transferred to a wide-mouthed glass tube, and subjected
to ultrasonic vibration with a Bronwill Biosonil III
sonifer (10 Kc 300 W), at approximately 100 Watts per
sq. cm. using a probe of 3/4 in. (1-90 cm.) diameter,
giving a total energy of 284 W. The tube was cooled with
a jacket of crushed ice. The temperature was not allowed
to exceed 8°C.

Antigen preparations in Freund adjuvant, and whole
treponemal suspensions for injection, were stored at
—20°C before injection.

Immunization procedure for non-pathogenic treponemes
Antigens for injection were composed of treponemes which had been washed four times in saline (as previously
described) before being re-suspended in saline to form a
10 per cent. wet W/V suspension. Suspensions of
treponemes were exposed to ultrasonic disintegration for
60 sec. at 4°C, and then emulsified with an equal volume of
complete Freund adjuvant. Animals were injected with
1-5 ml. of this emulsion of which 0-5 ml. was given intra-
muscularly into each gluteal region and 0-5 ml. sub-
cutaneously in the back of the neck. This procedure was
carried out weekly for 3 wks. After 2 wks respite, injections
were recommenced using treponemal suspensions without
adjuvant. Injections were made intradermally 0-5 ml. in
each gluteal region and in the back of the neck, weekly for
4 wks. 10 days after the 4th injection rabbits were bled
2 ml. from the ear, and the antibody content estimated by
immunodiffusion. At the same time three intradermal
injections were given as before and repeated at intervals of
10 to 14 days with bleeding until titres of 1 in 32 or more
were obtained with homologous antigens at a concentra-
tion of 1 g./100 ml. For such titres the latter schedule
required to be repeated 1 to 5 times.

Propagation, maintenance, and harvesting of T. pallidum
The particular strain used in these studies was the Nichols
strain of virulent T. pallidum (Nichols and Hough, 1913).
This organism is routinely used in the Treponema pallidum
Immobilization Test (TP1) and was obtained from the
Venereal Diseases Reference Laboratory, The London
Hospital, Whitechapel, London, E1, by the courtesy of
Dr. A. E. Wilkinson.

Animals
Adult male New Zealand white rabbits weighing
approximately 3 to 4 kg. were obtained from commercial
suppliers. Before inoculation animals were allowed to
acclimatize to the animal house for 3 weeks or more. They
were kept in individual cages with food and water always
available. They were fed on pellet diet (Oxoid laboratory
animal diets). The temperature of the room was usually
below 22°C.

Before inoculation approximately 5 ml. of blood was
drawn from the middle artery of the ear of each rabbit.
The serum was separated, inactivated at 56°C for 30 min.,
and tested for the presence of treponemal antibody using
the TPI test, or the Fluorescent Treponemal Antibody
Absorbed test (FTA-ABS).

Inoculation procedure
A suspension of T. pallidum (Nichols pathogenic strain) containing approximately
10⁸ treponemes per ml. was inoculated into the body of the
rabbit testis, in a dose of 0.5 ml. for each testis. Corticoster-
oid was given to the inoculated animals to suppress
immunological response and increase thereby the yield of
treponemes (De Lamater, Saurino, and Urbach, 1952;
Turner and Hollander, 1957). This was administered
intramuscularly as cortisone acetate (Cortisul, Roussel

<table>
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<tr>
<th>Treponemal strain</th>
<th>Duration of incubation (days)</th>
<th>Treponemal harvest</th>
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<tbody>
<tr>
<td>1. T. pallidum</td>
<td>8-12 (rabbit testicle)</td>
<td>12·4 mg. dry weight/100 rabbits (trep. extract)</td>
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<tr>
<td>2. Nichols</td>
<td>5</td>
<td>1·92 g. wet weight/litre</td>
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<tr>
<td>3. Kazan 2</td>
<td>4</td>
<td>2·21 g. wet weight/litre</td>
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<tr>
<td>4. Kazan 4</td>
<td>6</td>
<td>2·4 g. wet weight/litre</td>
</tr>
<tr>
<td>5. Kazan 5</td>
<td>6</td>
<td>1·97 g. wet weight/litre</td>
</tr>
<tr>
<td>6. Kazan 8</td>
<td>6</td>
<td>1·76 g. wet weight/litre</td>
</tr>
<tr>
<td>7. T. refringens</td>
<td>9</td>
<td>1·1 g. wet weight/litre</td>
</tr>
<tr>
<td>8. T. minutum</td>
<td>9</td>
<td>1·72 g. wet weight/litre</td>
</tr>
<tr>
<td>9. T. ambigua</td>
<td>8</td>
<td>1·34 g. wet weight/litre</td>
</tr>
<tr>
<td>10. T. microdentium</td>
<td>9</td>
<td>1·65 g. wet weight/litre</td>
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Laboratories, London), 25 mg. per ml. Daily doses were given of 0.2 ml/kg. body weight, starting from the first day of inoculation until full development of syphilitic orchitis.

Harvesting of treponemes When the testes became red, indurated, and nearly twice the normal size, rabbits were killed by intravenous injection of 5 ml. pentobarbital sodium solution (Nembutal, Abbott Laboratories) 60 mg./ml. The testes were removed aseptically via a scrotal incision. In order to increase the surface area and thereby facilitate elution of the treponemes, the testes were cut into small fragments which were placed in an extraction flask to which was added 5 to 10 ml. of sterile phosphate buffer saline pH 7.2 per testis.

The air in the flask was extracted and replaced by 5 per cent. carbon dioxide and 95 per cent. nitrogen mixture, a procedure which was repeated three times. The flask was then shaken on a mechanical shaker (Griffin flask shaker) for 1 hr at room temperature. Removal of red cells and gross testicular debris was accomplished by centrifugation at 1000 × G for 10 min. Continuity of the strain was ensured by immediate inoculation of a fresh batch of rabbits.

EXPERIMENT 1

Animals For experiments on the protective effect of treponemal vaccines against *T. pallidum*, nine adult New Zealand white rabbits each weighing 3 to 4 kg. were immunized as follows:

(1) Five rabbits were immunized with non-pathogenic treponemal strains, Nichols, Kazan 4, Kazan 8, *T. minutum*, and *T. ambigua* respectively.

(2) Two rabbits with Kazan 2.

(3) Two rabbits with Kazan 5.

Subsequent challenge procedure was carried out on rabbits which had been immunized with:

(i) Nichols non-pathogenic strain (one rabbit).

(ii) Kazan 2 (two rabbits).

(iii) Kazan 4 and Kazan 5 (one rabbit each).

The last four rabbits were reserved for Experiment 3.

Challenge procedure Challenge was carried out in immunized rabbits at intervals after immunization as follows:

- Nichols (non-pathogenic) 3 wks
- Kazan 2 6 wks
- Kazan 4 and Kazan 5 9 wks

0.5 ml. of *T. pallidum* suspension (4 × 10⁷–10⁸ organisms/ml. in phosphate buffered saline) was injected intratesticularly into the testis of non-immunized and immunized rabbits on the same occasion. Both non-immunized and immunized animals were treated with cortisone daily as previously described. See also Table III, where schematic representation of passage, challenge, and immunization protocols appears.

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**TABLE III** Experiments in the protective effect of treponemal vaccines and experiments in the attenuation of *T. pallidum*

<table>
<thead>
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**DIAGRAM OF T. pallidum PASSAGE IN RABBITS**

1. **NON-IMMUNIZED RABBIT**
   - *T. pallidum*

2. **NON-IMMUNIZED RABBIT**
   - *T. pallidum* → **IMMUNIZED RABBIT**
   - Nichols non-pathogenic strain

3. **NON-IMMUNIZED RABBIT**
   - *T. pallidum* → **2 IMMUNIZED RABBITS**
   - Both with Kazan 2 strain

4. **NON-IMMUNIZED RABBIT**
   - *T. pallidum* → **2 IMMUNIZED RABBITS**
   - Kazan 4 and 5, respectively

5. **2 IMMUNIZED RABBITS**
   - Kazan 5 and 8, respectively

6. **2 IMMUNIZED RABBITS**
   - *T. minutum* and *T. ambigua*, respectively

7. **NON-IMMUNIZED RABBIT (SYPHILOMA)**
   - Treponemal count 2.5 × 10⁸
Results  None of the five immunized rabbits gave the usual testicular inflammatory response, a response which had been obtained many times previously without failure, in the maintenance of *T. pallidum*. Usually testicles of infected rabbits showed enlargement starting on the fifth day after infection and becoming marked during the second week and suitable for harvesting. By contrast, the testicles of immunized rabbits were not enlarged on the eighth day. By the 12th day the rabbits showed slight enlargement of the testicles. By the 18th day, rabbits showed moderate enlargement only, with no induration. These appearances were in marked contrast to those of non-immunized rabbits.

When treponemal counts were carried out on the excised testicles, counts in non-immunized rabbits varied from $10^8 - 2.5 \times 10^8$ organisms/ml as compared with $4 \times 10^7 - 1.5 \times 10^9$ organisms/ml in immunized rabbits.

It would appear, therefore, that all the immunizing treponemes, *viz.* the Nichols non-pathogenic treponeme, and Kazan strains 2, 4, and 5 exerted some protective effect against the challenge dose of *T. pallidum*, a dose which invariably produced a marked reaction accompanied by high treponemal counts in the testicles of rabbits used throughout this entire work.

EXPERIMENT 2

Animals  An extension of the previous experiment was carried out on two adult male New Zealand white rabbits weighing 3–4 kg. 7 mths after immunization with *T. refringens* and *T. microdentium*.

Control  One adult male New Zealand white rabbit (non-immunized) was treated with the *T. pallidum* challenge procedure intratesticularly, for comparison with the immunized animals.

Booster procedure  One week before challenge by viable *T. pallidum* suspension, a single boosting dose of the appropriate non-pathogenic treponeme was given to each of the two test animals. This dose was divided into two injections of 0.5 ml of 10 per cent. wet weight/volume suspension, which were given subcutaneously into the right gluteal region and back of the neck.

Challenge procedure  This was carried out as described in the previous experiment, one week after the boosting dose.

Results  The control rabbit showed early enlargement of the testicles which progressed to marked enlargement by the 13th day after infection with a treponemal count of $2.5 \times 10^8$ organisms/ml.

By contrast, the rabbit immunized with *T. microdentium* showed almost no enlargement of the testicles with a treponemal count of only $6 \times 10^7$ organisms/ml., while the rabbit immunized with *T. refringens* showed slow and slight enlargement of the testicles with a treponemal count of $1.5 \times 10^8$ organisms/ml.

From this experiment it would appear that both *T. refringens* and *T. microdentium* exert some protective effect against the challenge dose of *T. pallidum*.

EXPERIMENT 3

Animals  Experiments on the attenuation of *T. pallidum* used four of the adult male New Zealand rabbits reserved from Experiment 1, which had been immunized as follows:

1. One rabbit with Kazan 5.
2. One rabbit with Kazan 8.
3. One rabbit with *T. minutum*.
4. One rabbit with *T. ambigua*.

Passage procedure  For intratesticular passage, testicles of infected rabbits were pooled and homogenized, and a suspension of *T. pallidum* was prepared and injected as previously described. Rabbits were observed for 18 days before being killed and *T. pallidum* from their testicles was passaged.

Test of attenuation  Testicles of infected immunized rabbits were pooled, homogenized, and injected intratesticularly into one adult male New Zealand white rabbit which was then observed daily for evidence of infection.

Method of investigation  In these experiments, *T. pallidum* suspensions were obtained from the testicles of a non-immunized rabbit and passed to the testicles, by intratesticular injection, of two rabbits immunized with treponemal strains Kazan 4 and Kazan 5 respectively (from Experiment 1).

For the second passage, the testicles of the previous rabbits were pooled and *T. pallidum* suspension obtained therefrom for passage to testicles of two further rabbits immunized with Kazan 5 and Kazan 8 strains respectively.

For the third passage, the testicles of the previous two rabbits were pooled, and the *T. pallidum* suspensions obtained passed to the testicles of two rabbits immunized with *T. minutum* and *T. ambigua* respectively.

As a crude test of any possible attenuating effect on *T. pallidum* of the limited number of passages, the testicles of the previous two rabbits were pooled and the *T. pallidum* suspensions passed in a fourth and final passage to a non-infected, non-immunized rabbit.

Results  Within 6 days the testicles of the non-immunized rabbit showed enlargement which progressed to fully-fledged syphilomata in 12 days, with a treponemal count of approximately $2.5 \times 10^8$ organisms/ml. This was in marked contrast to the testicles of the immunized rabbits, none of which showed enlargement or treponemal counts comparable with that of the non-immunized rabbit, or indeed of the rabbits which had been used for the maintenance of the *T. pallidum* strain throughout this study.

The experiment would appear to show:

1. Confirmation of the protective effect of immunization by Kazan 5.
2. The protective effect of immunization by Kazan 8, *T. minutum*, and *T. ambigua*.
3. Attenuation of *T. pallidum* was not apparent after three passages in immunized rabbits.
Discussion

In the experiments on active immunity described above, evidence of immunity or resistance to infection by *T. pallidum* was obtained by comparing testicular appearances in non-immune rabbits with those in vaccinated rabbits. Clinical appearances in the testicles of non-immune rabbits infected with *T. pallidum* had been observed in detail in many rabbits during laboratory maintenance of the Nichols strain of *T. pallidum*. Furthermore, the naked eye appearance had also been carefully noted when the testicles were excised, together with enumeration of living treponemes present. The technique employed for maintenance of the *T. pallidum* strain, which included cortisone acetate injection, ensured marked testicular enlargement accompanied by numerous treponemes (10^8 — 2.5 × 10^9/ml). With this technique testicular enlargement reached its maximum in about 10 to 12 days, and this cycle remained constant throughout the entire period of this and other work.

Instances of testicular enlargement being less than marked, and of treponemes being present in numbers less than 10^5, were rare and occurred during the summer months when ambient temperatures were high. Where cortisone injections were not used in the maintenance of strains, the period required for testicular enlargement was longer (3 to 5 weeks).

In this way, therefore, standards for comparison were built up over a considerable period of time, so that any change in the accustomed pattern was immediately obvious.

In challenging vaccinated rabbits by intratesticular injection of *T. pallidum*, cortisone acetate was employed exactly as in the case of the non-vaccinated controls. Thus any prolongation of the period required for testicular enlargement or any other changes could be attributed to immunization and not to the absence of cortisone effect on immune response. Furthermore, it might be suggested that any such changes would most probably be due to circulating antibody formed before cortisone injection, the effect of which injection would be to abolish immune response both cellular and humoral.

In the present work partial resistance to intratesticular challenge in rabbits has been obtained by immunization using treponemal strains—Nichols (non-pathogenic), Kazan 2, Kazan 4, Kazan 5, Kazan 8, *T. minutum*, *T. ambigua*, *T. refringens*, and *T. microdentium*. This is in contrast to the results of other investigators. Apparent success is attributed to large immunizing doses and careful processing at 4°C with storage at —20°C.

In the light of the above results, it is pertinent to examine the successful results of others. The results, therefore, of three groups of investigators are of interest, viz. Tani, Inoue, and Asano (1951), Miller (1967, 1973), Metzger and others (1969), and Metzger and Smogor (1969).

Tani and others (1951) studied the effect on protective immunity against *T. pallidum* in rabbits immunized by carbolic acid saline suspensions of *T. pallidum*. The manner in which these suspensions were prepared is of interest and was as follows.

Coarsely particulate material was removed from testicular suspension by centrifugation at 1000 r.p.m. for 5 min. Stock 10 per cent. antiformin solution from the ‘ice-box’ was added to a final concentration of 0.5 to 0.7 per cent. Preparations were shaken by hand, and allowed to stand at ambient temperature for 30 min. This was followed by centrifugation at 3000 r.p.m. for 1 hr, a single washing of the ‘surface of the sediment’ by a ‘large volume of saline’, re-suspension in 0.5 per cent. carbolic acid in saline, and storage in the ‘ice-box’.

In experiments in rabbits these investigators varied; (i) Total amount of antigenic material injected for immunization, (ii) Period of immunization, (iii) Interval elapsing between termination of immunization and challenge, (iv) Size of challenge dose.

Thus the subcutaneous administration of a total of 1.4 × 10^8 *T. pallidum* to eight rabbits over a period of 7 days followed by intradermal challenge at four sites on the back of approximately 3 × 10^8, 3 × 10^7, 3 × 10^4, and 3 × 10^6 *T. pallidum* at each site, 10 days after termination of immunization produced no evidence of immunity to *T. pallidum*.

By contrast, when the total number of *T. pallidum* injected was doubled (2.8 × 10^8) and the period of immunization extended to 21 days in four rabbits, with challenge as described above 9 days after the last immunizing injection, only six of sixteen intradermal inoculation sites produced chancres, compared with ten out of twelve areas in three control rabbits.

In a further experiment, in which the previous total immunizing dose was trebled (8·4 × 10^8) and administered to nine rabbits over a period of 20 days, challenge doses at three sites of 10^8, 10^7, and 10^6 *T. pallidum*, 21 days after the last immunizing injection produced six rabbits without chancres, of which one produced infection on popliteal node transfer, and five with no demonstrable infection, which were, therefore, possibly completely immune. All the remaining test rabbits produced chancres. Seven non-immunized control rabbits produced chancres on all the challenged sites.

In a further variation of dose and immunization period, 3·6 × 10^8 *T. pallidum* were injected over a period of 6 days into seven rabbits, which were challenged 9 days after the last injection as above, with 10^6, 10^5, and 10^4 *T. pallidum*. Of these rabbits, two produced no chancres, but were infective on popliteal node transfer. By contrast, all seven control rabbits produced chancres.

In a second group of eight rabbits immunized as above but at a point 74 days after termination of
immunization, four animals were completely immune as shown by absence of chancre and negative lymph node transfer. All six control rabbits produced chancre.

These results show that effective immunization in rabbits required a large total dose of treponemes administered daily over a period of at least 3 wks. Furthermore, immunity once established lasted for at least 2 to mths. Lack of success on the part of other investigators was attributed by the authors to low vaccine dosage, in comparison with their own large total dosage of antiformin treated material. Although the authors were uncertain as to the exact part played by antiformin treatment, they believed that it was important and probably exposed underlying antigens essential for the induction of protective immunity.

A different approach was employed by Miller (1967), who used T. pallidum attenuated by gamma irradiation. In one set of experiments, rabbits immunized intravenously by T. pallidum with a total dose of 9.7 x 10^8 over a period of 12 wks were challenged at the end of this period by intradermal injection of 500 T. pallidum at each of four sites. None of the animals showed evidence of protection since dark-ground positive lesions appeared at the same time in both test and control animals.

In another set of experiments, the immunization period and the total doses of T. pallidum were increased. Thus rabbits were immunized as above for 14 wks, rested for 3 wks, and re-immunized for 7 wks. At the end of this period of 24 wks when each rabbit had received a total of 1.6 x 10^9 T. pallidum, test and control animals were challenged as before. On the basis of delayed incubation period and the development of atypical cutaneous lesions in comparison with non-immune control animals, eleven out of seventeen test animals were considered partially immune to the challenge dose. Furthermore, two rabbits showed no evidence of infection, as shown by popliteal node and testis transfer to normal non-immune animals.

In a further set of experiments (Miller, 1973), dose and immunization period were increased. Thus rabbits were injected intravenously with a total amount of 3.71 x 10^9 T. pallidum, distributed over sixty doses given once to twice weekly during a period of 37 wks with rest periods of 14 days at 12 and 24 wks. Ten days after completion of immunization, test and control rabbits were challenged intradermally with 1,000 or 100,000 T. pallidum. None of the test rabbits developed lesions during the 94-day period of observation, nor were their transferred popliteal lymph nodes infectious for other rabbits. By contrast, all control animals developed typical, dark-ground positive cutaneous lesions.

In still further experiments, rabbits challenged intradermally with 1,800 T. pallidum 1 yr after the last immunizing injection showed no cutaneous lesions, nor were their lymph nodes or testes infectious for other rabbits. All non-immunized control rabbits, however, developed cutaneous lesions.

Metzger and others (1969) used T. pallidum which had been rendered non-viable by suspension in phosphate buffer at pH 7.4 at 4°C for 10 days. Rabbits were injected intravenously with a total of 8 x 10^8 T. pallidum thus treated involving four weekly doses of 1 ml., each containing 1.8 x 10^8 treponemes, for 3 weeks, followed by a further course of four 2 ml. doses weekly, each containing 3 x 10^8, for 4 wks. Five wks after completing the immunization, the test and control rabbits were challenged by intradermal injections of 300,000 T. pallidum at each of four sites. None of the immunized rabbits developed skin lesions, in contrast to non-immune control rabbits. Of sixteen rabbits immunized, however, six were completely immune, while the remaining rabbits were considered partially immune since their lymph nodes harboured virulent T. pallidum which caused infection upon transfer to the testes of non-immunized rabbits.

A high degree of immunity was also obtained by intramuscular injection of non-viable T. pallidum, the degree of immunity depending on the number of organisms injected during the course of immunization (Metzger and Smogor, 1969). Thus ten rabbits immunized intramuscularly by T. pallidum with a total dose of 1.2 x 10^10 given over a period of 7 wks were challenged 5 wks after completing the immunization by intradermal injection at each of four sites with 300,000 T. pallidum. Of ten rabbits vaccinated, five showed no skin lesions or evidence of infection on lymph node transfer, two were asymptomatic, but showed evidence of infection on lymph node transfer, and three showed cutaneous lesions. In other experiments, in which rabbits were given doses of T. pallidum of 6 x 10^9 and 3 x 10^9, animals responded with lesser degrees of immunity.

In summary, therefore, from the results of Miller, and of Metzger and co-workers, there is little doubt that immunity against syphilis can be conferred by the injection of non-viable T. pallidum. Best results were obtained by intravenous injection of large numbers of organisms over many weeks, and to this extent their results are in agreement with those of Tani and his colleagues. There the similarity ends, for the first mentioned workers were at pains to avoid the destruction of superficial antigens considered relevant to the induction of protective immunity by gamma irradiation or by low temperature short-term storage, followed by prompt injection, whereas Tani believed that such antigens were uncovered by antiformin treatment, and were effectively preserved by 0.5 per cent. carbolic acid. Be that as it may, all three preparations were effective in large dosage in the induction of protective immunity.

It should also be noted that in most cases challenge
was by large numbers of *T. pallidum*, but whether such numbers are analogous to those likely to be met with during the course of human sexual behaviour is uncertain. Where numbers are small, intensive immunization similar to that in the experimental rabbit may not be necessary in human beings. Furthermore, it may be that a partial immunity sufficient to confine infecting *T. pallidum* to the primary lesion would give rise to a higher degree of immunity than that obtainable by vaccination alone. Elucidation of these problems, however, will require much further work.

Successful but less striking results have been obtained using non-pathogenic treponemes. Gelperin (1951) used the Reiter treponeme suspended in saline or admixed with adjuvant. Two groups of rabbits were immunized. The animals in the first group were injected intravenously and intraperitoneally with saline suspensions of Reiter treponemes three times a week for 11 wks, each rabbit receiving a total weight of organisms equivalent to 6,075 μg. nitrogen during that time. Ten days after the last injection the rabbits were challenged intradermally at four sites on the posterior part of the back with 200 *T. pallidum* at each site.

In the case of the second group, treponeme-adjuvant mixtures were injected subcutaneously on alternate weeks for the same period, each animal receiving a total weight of organisms equivalent to 1,550 μg. nitrogen. Fourteen days after the last injection, rabbits were challenged as above.

Results showed that, in immunized animals of both groups, there was a prolongation of incubation time in cutaneous lesions as compared with those in controls. Gelperin (1951), however, considered that these differences were not statistically significant. In the case of the adjuvant group, however, the cutaneous lesions were significantly smaller than those of the non-immune control group. Although other investigators commenting on the results of Gelperin (1951) state that his results did not show evidence of immunity, we think that partial immunity was established in the adjuvant group at least.

Knox and others (1967) reported partial protection in rabbits using mechanically disrupted Nichols non-pathogenic treponemes and *E. coli* lipopolysaccharide adjuvant. Animals were immunized at weekly intervals for 5 wks by subcutaneous injection in the skin of the back of 25 mg. of treponemes. The total weight of organisms injected therefore was 125 mg. Two wks after the last immunizing dose, animals were challenged intradermally on the lower back with a total of $6 \times 10^4$ *T. pallidum* distributed as $10^4$ treponemes in each of two sites, and $2 \times 10^4$ in each of two further sites. Of 23 animals immunized, nine did not develop cutaneous lesions. Significant immunity was, therefore, provided by the immunizing procedure against a considerable challenge of virulent *T. pallidum*.

More recently Izzat and others (1970) attempted to produce immunity against *T. pallidum* in rabbits using the Nichols non-pathogenic treponeme. Three types of vaccine were tested:

(i) A suspension of $2 \times 10^4$ treponemes per ml. in 2 per cent. alum potassium sulphate preserved with Merthiolate 1:10,000,

(ii) ‘Sonicated’ treponemes 100 mg./ml., and adjuvant, *E. coli* lipopolysaccharide 2-4 mg./ml.,

(iii) Lysozyme-treated treponemal suspension, and adjuvant.

The first preparation was injected subcutaneously, weekly, for 10 wks, each injection containing $10^4$ treponemes. The total number of organisms given was $10^7$.

The second preparation was injected subcutaneously, each rabbit receiving a total of 1,600 mg. of sonicated organisms over a period of 16 wks. Two groups of rabbits were used, one group receiving injections at weekly, and the other at monthly intervals.

The third preparation was injected subcutaneously, each rabbit receiving 1,600 mg. of lysozyme-treated treponemes, with adjuvant, weekly over a period of 53 weeks.

Rabbits receiving the first and second preparations were challenged with virulent *T. pallidum* 3 wks after completion of immunization in the case of the first preparation and 2 wks in the case of the second. Challenge doses consisted of $4 \times 10^4$ organisms intratesticularly and $5 \times 10^4$ organisms intradermally at two sites on the back. Animals receiving the third type of preparation were challenged with fifty organisms per site.

According to the authors, none of the rabbits showed any evidence of immunity against virulent *T. pallidum*. A possible explanation for failure to produce immunity may lie in the manner of the initial preparation of treponemal suspensions. Thus it is stated that organisms were washed thirteen times in physiological saline and stored at $-60^\circ$C until used, whereupon they were thawed and washed three times more in phosphate buffer saline (pH 7·8). It is suggested that the freezing and thawing followed by further washing may have removed material essential for the induction of protective immunity.

By contrast, treponemal preparations used in the present work were completely processed before being frozen. No further washing or processing was carried out after thawing. Moreover, the greatest care was taken to avoid denaturation of labile antigenic materials during harvesting and preparation, which were carried out at 4°C.

Recent work, therefore, by Zeigler, Jones, Jones,
and Kubica (1976) is of considerable interest in that they have shown the presence of an extra-cellular slime layer on the surface of *T. pallidum* (Nichols pathogenic strain), a layer which is absent from the non-pathogenic treponemes, *T. refringens* biotype Nichols and *T. phagedenis* biotype reiteri.

Furthermore, it would appear that, when the integrity of the slime layer and outer envelope are preserved by means of glutaraldehyde, a single intraperitoneal injection of *T. pallidum* suspension plus adjuvant may provide partial protection against intratesticular challenge with *T. pallidum*. Although no experimental evidence is produced in their paper the inference is that non-pathogenic treponemes which apparently do not possess this slime layer are unlikely to be effective as vaccines.

The results reported here, whereby non-pathogenic treponemal vaccines provide partial protection in rabbits against intratesticular challenge by *T. pallidum*, are therefore at variance with those of these authors.

In summary, there is evidence to show that the induction of protective immunity against syphilis is possible by vaccines containing non-viable *T. pallidum* or certain cultivable non-pathogenic treponemata. Both types of vaccine have until now, required prolonged courses involving high total dosage, and in view of the difficulties involved in the production of sufficient quantities of *T. pallidum* for use in vaccines, it is clear that further experiments must be undertaken as a matter of urgency to ascertain the true effectiveness of vaccines containing the comparatively easily cultivable non-pathogenic treponemes, in the induction of protective immunity.

The above work formed part of a Thesis submitted by Dr. H. T. Al-Samarrai in 1974 to the Faculty of Medicine in the University of London, in fulfilment of the requirements for the degree of Doctor of Philosophy.

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