SINGLE CELL INOCULATIONS WITH TREPONEMA PALLIDUM*

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The development of syphilis in the rabbit following intratesticular inoculation of test material affords positive evidence of the presence of the virus in the inoculum. Failure to thus infect has been interpreted as indicating the absence of Treponema pallidum in the inoculum. Inasmuch as this interpretation is widely used in the study of experimental syphilis, it seemed desirable to test the validity of the assumption upon which it is based. The assumption is that if the virus is present in any quantity whatsoever it will lead to infection in the inoculated animal and that when infection does not result from such an inoculation it is because of the complete absence of Treponema pallidum in the inoculum.

When saline suspensions are made of the tissues of acute lesions in rabbits infected with syphilis the Treponema pallidum is usually, although not always, demonstrable by dark-field examination. Such preparations will lead to infection when injected into rabbits (the tissue transfer method of inducing infection). If a tissue suspension in which there are great numbers of visible organisms is subjected to a series of increasing dilutions with salt solution, a point is reached where no organisms are visible on careful and painstaking search, and yet the material will prove infectious when injected into rabbits. This observation is in accord with those of Truffi (1), Brown and Pearce (2), and Levaditi, Schoen, and Sanchis-Bayarri (3). Observations of this character have been cited as favoring the existence of a granular or ultramicroscopic form of the virus (3–5).

It is admittedly possible that visible Treponema pallidum are present

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in inocula inducing infection though not seen in the very small fraction subjected to dark-field study. Although this possibility does exist there seems to be no way to definitely prove or disprove it. Assuming that visible forms are always present, does this fact warrant the abandonment of the hypothesis of an ultramicroscopic form of the virus? If infection is produced only by the visible, morphologically typical forms of Treponema pallidum, how many organisms must be present to induce the disease in a susceptible rabbit? It has evidently been assumed by many workers in the immunity and chemotherapy of experimental syphilis that the quantity of virus is not an important factor in the initiation of disease. The tissue transfer method for determining the presence or absence of infection in an animal is based upon the assumption that if Treponema pallidum is present in the tissue it will induce disease when a saline suspension of this tissue is injected into another susceptible animal—and this without regard for the number of organisms present. Conclusions based upon this assumption are abundant in the literature. It has even been suggested that the method might be useful in determining either persistence of infection, or cure, in individuals who have undergone antisyphilitic treatment (6). Although in human syphilis the procedure, as a test for either the presence or absence of infection, has been shown to be unreliable (7), it is still widely used in the experimental disease.

Does a negative tissue transfer experiment indicate the complete absence of typical Treponema pallidum in the material being tested? In an attempt to throw some light upon this question varying dilutions were made of a testicular saline suspension which contained Treponema pallidum. Rabbits were then inoculated with the several dilutions. It was found that not only did dilutions in which there were no visible organisms give positive results but also that dilutions in which it seemed probable that Treponema pallidum was present, gave negative results. However, it was obvious that no conclusive data could be obtained by this approach. We therefore proceeded to determine whether syphilitic infection could be induced in the rabbit by the inoculation of from one to several morphologically typical organisms. It was thought that if infection resulted, the experiment would afford convincing evidence of the reliability of the tissue transfer method as a means of determining the presence of infection. If infection did not result
from such an inoculation, the broad conclusions based on results of the use of the tissue transfer test to determine the presence of infection are not warranted and we must conclude that either the capacity for initiating infection does not reside in the visible organisms when present in minimal numbers, or that the rabbit's ability to resist infection is adequate for organisms when present in minimal numbers. Moreover, it seemed possible that these inoculations might throw some additional light on theories pertaining to the existence of an ultramicroscopic or granular form of the virus.

With these considerations in mind we have inoculated rabbits with material containing *Treponema pallidum* in numbers varying from one to six organisms.

**EXPERIMENTAL**

The equipment consisted of a Chambers micro manipulator (8) and a special dark-field condenser with moist chamber. Detailed descriptions of the micro manipulator and its adaptation for isolation of single bacteria in the light field have been published by Kahn (10) and Gee and Hunt (11). The dark-field condenser and moist chamber have been described by Hauser (12).

The dark-field condenser is of the biconvex type with a focal length of 10.7 mm. and a working distance of 14 mm. The range of aperture of the illumination rays is from 0.85 mm. to 0.99 mm., which is necessary for use with oil immersion objectives. The diameter of the condenser is 49 mm. and its height is 42.5 mm. It is provided with lateral slots 15.5 mm. wide and 10 mm. high to allow ample room for the vertical and horizontal movements of the micro needles. There is a depression in the base of the condenser which is filled with water to provide moisture in the chamber. A microscope with an opening in the stage of at least 50 mm. in diameter is required.

The moist chamber has a base formed by an 80 mm. square of glass containing a circular aperture in the center. This aperture is partially surrounded by the two curved glass walls of the chamber. The two openings in the walls are opposite each other. The chamber is roofed with glass in such a manner as to leave an open space across which a cover-slip with hanging drop may be placed.

In order to maintain proper moisture in the chamber its walls are lined with moist blotting paper except at the point where the pipettes are to be introduced. The top of the chamber is covered with a mica apron containing an opening slightly smaller than the cover-glass to be used. The apron is sealed to the chamber by vaseline and the cover-slip is likewise sealed to the mica apron by vaseline.

A carbon arc lamp and water filter is used. By properly focussing the condenser so that the light is concentrated at different levels in the hanging drop, and by

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1 The dark-field condenser and moist chamber is manufactured by E. Leitz Co.
properly adjusting the diaphragm in the oil immersion objective, an excellent
dark-field is obtained and the drop and its contents may thus be inspected.

The pipettes used in the experiments are prepared in the manner described by
Kahn (13).

Cover-slips not exceeding 0.17 mm. in thickness are used and a fine grease film
is applied to them in order to prevent spreading of the hanging drops.

Two strains of *Treponema pallidum* were used. One of these, the Nichols
strain, was furnished us by Chesney in 1927 and has regularly produced syphilis
in rabbits during the past 6 years. The other strain which is designated S in
Table I, was isolated in 1932 (14) from a case of gastric syphilis. The lesions pro-
duced by it in the rabbit are, in every way, typical of the experimental disease.
Animals infected with these strains were sacrificed at intervals of from 17 to 36
days. Saline emulsions of early testicular lesions were prepared in the usual way,
and control animals were inoculated with the undiluted emulsions. The emul-
sions were then subjected to the dilutions found appropriate for single cell iso-
lations.

A small amount of the suspension is aspirated into a 1 cc. syringe through a 22
gauge needle. A drop of this material is then examined in the dark-field and
enough warm salt solution is added to obtain the optimum preliminary concentra-
tion of organisms. This can only be determined by experience. By means of a
micro pipette drops of the diluted suspension are placed upon the under surface of
the cover-slip in the moist chamber. This is rapidly inspected in order to deter-
mine the actual number of organisms present in each drop. When a drop is found
which contains the desired number of organisms their morphology and motility is
noted. If this is found to be normal the entire drop is rapidly aspirated into a
sterile micro pipette containing salt solution. The site of the drop on the cover-
slip is then carefully examined to be sure that the organism or organisms have
actually been aspirated into the pipette. The latter is immediately removed
from the manipulator. Its tip is introduced into an 18 gauge needle which is
attached to a 1 cc. syringe filled with salt solution. The tip of the pipette is then
broken off at the bend of the shaft. The needle is then introduced directly into the
testicle of a rabbit and the glass tip with its contents is washed into the testicle
by the injection of the salt solution. The testicle is massaged to distribute the
material.

Eighteen rabbits were thus inoculated, sixteen with single organisms, one with
two, and one with six organisms (Table I). These animals were carefully observed
for evidence of the development of syphilitic infection over periods varying from 6
to 13 weeks. Each animal was then sacrificed. The testicle which had been
inoculated, together with the inguinal lymph nodes when they were readily found,
were ground in a mortar containing salt solution. The resulting saline suspension
was subjected to dark-field examination and injected into the testicles of either one
or two normal rabbits. These rabbits were in turn observed for from 4 to 8½ weeks
for manifestations of syphilitic infection. At the termination of this period each
<table>
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<tr>
<th>Rabbit No.</th>
<th>Date of inoculation</th>
<th>Strain of Trep.</th>
<th>Date of Trep.</th>
<th>Time interval before 2nd transfer experiment</th>
<th>Dark-field examination of inoculum</th>
<th>Rabbit Series No. 2</th>
<th>Time interval before 2nd transfer experiment</th>
<th>Dark-field examination of inoculum</th>
<th>Rabbit Series No. 3</th>
<th>Time interval before inoculation of Experimental Trep.</th>
<th>Dark-field examination of inoculum</th>
<th>Retr. of inoculation with homologous antigen of Trep.</th>
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</table>

* N., negative. P., positive.
† Animals dead or in poor condition.
pair of the second series of rabbits was sacrificed, the injected testicles ground in salt solution, pooled, and injected into the testicles of one or more normal animals. Dark-field examinations were also made of these suspensions. This third series of rabbits was carefully observed for evidence of syphilis. At the end of varying periods of observation all had remained normal. Twenty-one animals of this group were then reinoculated with the homologous strain of Treponema pallidum.

Evidence of syphilis failed to appear in any of the rabbits and no organisms were demonstrated in any of the testicular suspensions by dark-field examination. In those animals of the third series which were tested, no convincing evidence of immunity to the homologous strain was found; only two of the twenty-one rabbits reinoculated failed to develop typical syphilis (see Table I). The animals of the control series, which were inoculated with the usual quantity of undiluted virus, invariably developed syphilitic infection.

DISCUSSION

The consistently negative results of the sixteen experiments indicate conclusively that an inoculation with one morphologically typical, motile Treponema pallidum does not induce syphilis in the rabbit. In two other experiments infection did not occur following the inoculation of either two or six organisms.

This failure to infect can hardly be due to the method employed. The possibility that the organisms were not viable when injected seems remote. They were actually delivered into the testicles within a few moments after they had been seen to be normal morphologically and actively motile in the hanging drop. It seems highly improbable that the short bit of fine glass capillary pipette in which they were injected into the testicles and which was probably distributed by the subsequent testicular massage could have injured the organism or otherwise acted as a factor to prevent infection.

A more reasonable explanation of the failure to infect is to be found in a consideration of other possibilities. The resistance of the rabbit to the syphilitic virus is admittedly weak. However, as has been pointed out, we have seen injections with inocula which we felt certain contained Treponema pallidum, although not demonstrable by dark-field examination, fail to induce infection. That these inocula did actually contain organisms is highly probable in the light of the work of Jahnel and his associates (15) who found that blood suspensions containing 300 Spirochaeta hispanica per c. mm. gave negative dark-field findings but would infect mice when injected in 0.2 cc. quantities. Moreover,
these observers point out that suspensions containing 80 organisms per c. mm., or less, fail to induce infection when injected in 0.2 cc. quantities. These observations indicate that an irreducible number of *Spirochaeta hispanica* must be present before infection can develop and it is probable in the light of our work that this also holds for *Treponema pallidum*.

Our work offers no support to the theory of the existence of an ultramicroscopic or granular form of *Treponema pallidum*. Such negative evidence as our experiments afford cannot be interpreted as indicating that the morphologically typical, motile *Treponema pallidum* is avirulent. It seems necessary to emphasize this since the proponents of this theory have cited positive tissue transfer experiments, with inocula in which organisms were not demonstrable by dark-field examination, as evidence favoring their hypothesis. The negative results obtained by us with typical organisms in a menstrum in which the hypothetical virulent form may be said to have been lost by dilution, might lead to similar erroneous interpretations.

The assumption by workers in experimental syphilis that a negative tissue transfer experiment indicates the absence of *Treponema pallidum* in the inoculum and the absence of syphilitic infection in the source animal is not warranted. This is indicated by the experiments reported herein as well as by a previously reported study of the tissue transfer method in human syphilis (6).

**SUMMARY**

Sixteen rabbits were inoculated intratesticularly with single *Treponema pallidum*. Two other animals were inoculated, one with two, and one with six organisms. All of these animals remained normal. Control animals inoculated with the usual quantity of the same but undiluted virus developed typical lesions of experimental syphilis. The test animals were subjected to a procedure designed to demonstrate the presence of *Treponema pallidum* even in the absence of recognizable syphilitic lesions. At appropriate intervals transfers were made of testicular material from these to a second series and, in many instances, from the second to a third series of rabbits. All of the rabbits remained normal. Moreover, immunity to the homologous strain was not present in those animals of the third series which were tested.
The relation of these observations to (a) the theory of the existence of an ultramicroscopic form of *Treponema pallidum*, and (b) the assumption upon which is based the tissue transfer method of determining the presence or absence of syphilitic infection, is discussed.

CONCLUSIONS

The injection of one or several *Treponema pallidum* into the testicles of rabbits does not induce syphilitic infection.

A negative tissue transfer experiment does not preclude the presence of *Treponema pallidum* in the inoculum nor does it indicate the absence of syphilis in the source animal.

BIBLIOGRAPHY