Immunization of Rabbits with Spirochaeta aurantia *Does Not Induce Resistance to* Treponema pallidum

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Rabbits were immunized with viable *Spirochaeta aurantia*, a free-living, facultative anaerobic spirochete that is similar in some biochemical characteristics to *Treponema pallidum*, a parasitic, microaerophilic spirochete. Single and multiple immunizations with living *S. aurantia*, with or without Freund's incomplete adjuvant, Freund's complete adjuvant, or heat-killed *T. pallidum*, were carried out over a four-month period. Living *S. aurantia* was neither toxic nor virulent for rabbits. Immunized rabbits produced a high level of agglutinating antibody to *S. aurantia* but no antibody to *T. pallidum*, as determined by the *T. pallidum* hemagglutination test. Immunized rabbits were challenged with multiple intradermal inoculations of 100 viable *T. pallidum* (Nichols strain) and compared to unimmunized rabbits similarly infected. Immunization with *S. aurantia* did not protect against *T. pallidum* infection. Thus *S. aurantia* appears not to be suitable as a potential vaccine against infection with *T. pallidum*.

TREPONEMA PALLIDUM, the causative agent of human syphilis, is highly virulent for both humans and rabbits. No naturally attenuated, avirulent strains are known to exist, but were they available, their use as a potential syphilis vaccine could be readily investigated.

Closely related, pathogenic species within the genus *Treponema* such as *Treponema pertenue*, the causative agent of human yaws, and *Treponema paraluis-cuniculi*, the causative agent of rabbit venereal spirochetosis, do give some cross-protection against syphilis.[1-3] *T. pallidum* and *T. pertenue* are very closely related, based on antigenicity and DNA sequence homology, but neither bacterium is closely related to the cultivable, nonpathogenic members of the genus *Treponema*, such as *Treponema phagadensis* and *Treponema refringens*,[4,5] nor to the porcine pathogen *Treponema hyodysenteriae*.[6] Furthermore, unlike the nonpathogenic treponemes, *T. pallidum* does not contain monoglycosyldiglyceride[7] and is a left-handed helix, whereas the nonpathogenic treponemes are right-handed helices.[8] It is likely that the pathogenic and nonpathogenic treponemes will be separated into distinct genera in the future.

Members of the genus *Spirochaeta* are free-living spirochetes, all species of which are obligate anaerobes, with the exception of *Spirochaeta aurantia*, a facultative anaerobe.[9] *T. pallidum* has recently been shown to be a microaerophile,[10] whereas the nonpathogenic treponemes are strict anaerobes.[11]

The guanine-plus-cytosine content of *T. pallidum* (52.4–53.7%),[5] is closer to that of the spirochetes *Spirochaeta zuelzerae* (56.1%), *Spirochaeta litoralis* (50.6%),[5] and *S. aurantia* (56.8–60.0% or 61.2–65.3%, depending on method used)[11] than to that of other treponemes (38–41.5%).[5] This finding suggests that *T. pallidum* is phylogenetically closer to the *Spirochaeta* than to the anaerobic members of the genus *Treponema*, although DNA homology studies would provide more conclusive data.

Morphologic similarity between *T. pallidum* and *S. aurantia* and their common requirement for low concentrations of oxygen also suggests that they may be related. They may have evolved from a common ancestral spirochete, with one line occupying a low-oxygen niche within certain animal species and becoming the pathogenic treponemes, while another line remained free-living and became *S. aurantia*. Oxygen levels in vivo are very low, especially in the human skin where *T. pallidum* grows so well, and these oxygen levels are similar to those thought to be present in the biosphere about the time of the evolution of the metazoa.[12]

Unfortunately, killed *T. pallidum* does not immunize rabbits against challenge with virulent *T. pallidum*,[13] unless a large number of immunizations are given over a long period of time,[14,15] both of which are impracticable for purposes of vaccinating humans. Likewise, non-*pallidum* (avirulent) treponemes do not protect against challenge with virulent *T. pallidum*.[16]

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On the assumption that *S. aurantia* may be a type of avirulent *T. pallidum*, we became interested in the possible use of *S. aurantia* as an immunizing agent against infection with *T. pallidum*. By means of rabbit immunizations with *S. aurantia* and subsequent challenge with virulent *T. pallidum*, we tested this hypothesis directly.

Materials and Methods

Bacteria

Spirochaeta aurantia (ATCC 25082) was grown in MTY medium (maltose, 3 g/liter; trypticase, 5g/liter; yeast extract, 2 g/liter) at 30 C.[17,18] For anaerobic growth bottles were completely filled with medium, while for aerobic growth 250 ml of medium was used in a 1-liter conical flask. Growth was overnight, and the bacteria harvested by centrifugation (10,000 *g*, 30 min).

Treponema pallidum (Nichols strain) was grown in vivo in rabbit testes.[19] Testes were inoculated with viable *T. pallidum* and examined regularly until orchitis was apparent (usually ten to 14 days). At this time the rabbit was sacrificed, and the testes were removed aseptically. Testicular tissue was minced in sterile eluting fluid for 30 min at 4 C. Eagle's minimal essential medium containing 10% fetal calf serum and 1 mM dithiothreitol was used for elution if *T. pallidum* was to be kept viable. Phosphate-buffered saline was used for elution if killed *T. pallidum* was required.

Concentrations of *S. aurantia* and *T. pallidum* were determined by examination of a sample in a bacterial counting chamber under dark-field microscopy. Adjustments in concentrations were made by centrifugation at 10,000 *g* for 30 min, followed by resuspension in the appropriate volume of fluid.

Only male rabbits were used, both for growth of *T. pallidum* in vivo and for immunization experiments. They were housed at 16–19 C and fed antibiotic-free food.

Preparation of S. aurantia *for Immunization*

When *S. aurantia* alone was used for immunization, cultures were adjusted to 10^{11} cells/ml, and 0.1 ml volumes (10^{10} cells) were used for each immunization. When *S. aurantia* was used in conjunction with incomplete Freund's adjuvant or complete Freund's adjuvant, cultures were adjusted to 1.2×10^{11} cells/ml and mixed with an equal volume of adjuvant. Again, 0.1-ml volumes (6×10^9 cells) were used for immunization.

Preparation of T. pallidum *for Immunization*

Treponema pallidum was harvested in sterile phosphate-buffered saline and killed by heating at 56 C for 30 min. These preparations were tested for residual viability by inoculation into a rabbit and were invariably found to be sterile. Cultures of *T. pallidum* were adjusted to 10^{11} cells/ml and mixed with an equal volume of *S. aurantia* (1.2×10^{11} cells/ml). Rabbits were immunized with 0.1-ml volumes (5×10^9 killed *T. pallidum* plus 6×10^9 viable *S. aurantia*).

Immunization

Seven different immunization schedules were used. (*1*) *S. aurantia* (anaerobically grown; viable; 10^{10} cells given intradermally); one dose was given one month prior to challenge. (*2*) *S. aurantia*, as in (*1*), but aerobically grown; both anaerobically (*1*) and aerobically (*2*) grown bacteria were used in case they synthesized different antigens under these two conditions. (*3*) *S. aurantia* (aerobically grown; viable; 5×10^9 cells given intradermally); one dose given two and one-half months before challenge. (*4*) *S. aurantia* (aerobically grown; viable; 6×10^9 cells given intradermally); four doses were given at four, three, two, and one-half month(s) prior to challenge. (*5*) *S. aurantia*, as in (*4*) but given with incomplete Freund's adjuvant. (*6*) *S. aurantia*, as in (*4*), but given with complete Freund's adjuvant at the first immunization, incomplete Freund's adjuvant at the third immunization, and no adjuvant at the second and fourth immunizations. (*7*) *S. aurantia* (aerobically grown; viable, 6×10^9 cells given intradermally); four doses were given at four, three, one and one-half, and one-half month(s) prior to challenge, each with 5×10^9 heat-killed *T. pallidum*.

Preparation of T. pallidum *for Challenge*

Cultures of *T. pallidum* were harvested in Eagle's minimal essential medium with 10% fetal calf serum and 1 mM dithiothreitol. Cells in the suspension were counted, and the suspension was diluted to 10^3 treponemes/ml. Volumes of 0.1 ml (100 *T. pallidum*) were used for intradermal inoculations of the immunized and control rabbits.

Before challenge the rabbits were shaved and their backs marked into a 3- × 3-grid, approximately 18 cm × 6 cm. Thus nine inoculation sites were used on each rabbit.

Determination of Immune Status of Challenged Rabbits

Inoculation sites were kept shaved and examined daily for appearance of syphilitic lesions, as indicated by the development of induration. The time between challenge inoculation and the first appearance of induration was defined as the latent period. Any lengthening of the latent period, or failure of a lesion to appear (compared to the control rabbits) was taken as immunity (partial or complete) in the immunized rabbits.

TABLE 1. Response to Challenge Infection with *Treponema pallidum* (Nichols strain) in Rabbits Immunized with *Spirochaeta aurantia* Under the Different Conditions Shown

Schedule of Immunization of Rabbits				Challenge of Immunizated Rabbits		Challenge of Control Rabbits		
Rabbit Group, Condition of *S. aurantia* Culture	Months from Immunization to Challenge	Immunization Dose (Cells)	Adjuvant	No. of Lesions/No. of Inoculation Sites	Days of Latency (±SD)	No. of Lesions/No. of Inoculation Sites	Days of Latency (±SD)	Difference Between Immunized and Control Rabbits
1, aerobic	1	10^{10}	None	27/27 (3)*	16.7 ± 0.7	27/27 (3)	17.9 ± 1.3	Shorter latent periods in immunized rabbits ($P < 0.05$)
2, anaerobic	1	10^{10}	None	27/27 (3)	16.6 ± 0.6			
3, aerobic	2½	5×10^9	None	18/18 (2)	16.7 ± 0.7			None
4, aerobic	4, 3, 2, ½	4 doses of 6×10^9	None	9/9 (1)	16.8 ± 0.8			
5, aerobic	4, 3, 2, ½	4 doses of 6×10^9	Incomplete Freund's Adjuvant	18/18 (2)	17.4 ± 1.0	27/27 (3)	16.9 ± 0.8	None
6, aerobic	4, 3, 2, ½	4 doses of 6×10^9	Complete Freund's Adjuvant	18/18 (2)	16.5 ± 0.9			
7, aerobic	4, 3, 1½, ½	4 doses of 6×10^9	Killed *T. pallidum*, 4 doses of 5×10^9 cells	18/18 (2)	15.1 ± 2.0	16/16 (2)	17.7 ± 1.4	None

Note. Each rabbit (control or immunized) was challenged with separate doses of 100 *T. pallidum* (Nichols strain) at eight or nine distinct sites on its shaved back. The time between challenge, infection, and first appearance of the syphilitic lesion was the latent period (days).
* The number of rabbits per group is given in parentheses.

Determination of Antibody Levels in Immunized Rabbits

Antibody to *T. pallidum* was determined by the standard *T. pallidum* hemagglutination (TPHA) test, with details as supplied by the manufacturer (Fujizoki Pharmaceutical Co., Ltd., Tokyo).

Antibody to *S. aurantia* was determined by a microscopic agglutination titration similar to the test used in the serologic diagnosis of leptospirosis. Doubling dilutions 1:2 to 1:2048 of 0.025-ml volumes of rabbit serum were made in phosphate-buffered saline. Equal volumes of an overnight, actively motile culture of *S. aurantia* were added to each dilution of antiserum, yielding a final antiserum dilution in the range of 1:4 to 1:4096. The reaction mixtures were incubated at 30 C for 6 hr, after which time no further agglutination occurred. Mixtures were examined by dark-field microscopy for the presence of agglutination clumps of *S. aurantia.* Any visible agglutination was considered positive, since the negative control mixtures (antiserum-free) were invariably free of agglutination. The titer of the antiserum was defined as the highest final dilution of antiserum that caused any agglutination of *S. aurantia.*

Results

The data in table 1 show that immunization with *S. aurantia* at intervals of from one-half month to four months before challenge with *T. pallidum* did not protect against challenge infection with *T. pallidum.* Addition of incomplete or complete Freund's adjuvant to *S. aurantia* did not confer protection. The rabbits immunized with anaerobically or aerobically grown *S. aurantia* developed syphilitic lesions at every intradermal challenge site. There was no delay in development of lesions in the immunized rabbits; in fact, the converse occurred, with shorter latent periods in the immunized rabbits (16.6 and 16.7 days) than in the control rabbits (17.9 days). This difference was statistically significant by Student's *t* test.

Synthesis of Antibody to S. aurantia *in Immunized Rabbits*

By means of a microscopic agglutination titration test (see Materials and Methods), it was demonstrated that every rabbit immunized with *S. aurantia* developed a high titer of agglutinating antibody to *S. aurantia.* Titers ranged up to 1:4096 (final dilution of serum required for agglutination of *S. aurantia*). Before immunization some of the rabbits had titers of agglutinating antibody to *S. aurantia* of ≤1:64. This activity was presumably due to cross-reacting antibody. Other rabbits had no agglutinating antibodies before immunization with *S. aurantia.* There was no correlation between preimmunization and postimmunization levels of antibody to *S. aurantia.*

It was not possible to relate levels of antibody to *S.*

aurantia to levels of resistance to *T. pallidum*, since no resistance to *T. pallidum* was detected in any of the immunized rabbits.

Synthesis of Antibody to T. pallidum *in Immunized Rabbits*

None of the rabbits immunized with *S. aurantia* (alone or with incomplete or complete Freund's adjuvant) synthesized antibodies to *T. pallidum* as detectable by the TPHA test. However, one might not expect many cross-reacting antibodies to be detected by the TPHA test because an adsorbent of nonpathogenic treponemes is used in this test.

Of the two rabbits immunized with *S. aurantia* plus heat-killed *T. pallidum*, one did produce TPHA antibodies transiently (titer, 1:320). After challenge with viable *T. pallidum*, the immunized rabbits were not tested for TPHA antibodies since it had previously been observed that *T. pallidum* infection in rabbits caused TPHA antibodies to be produced.[3]

Toxicity of Live S. aurantia *for Rabbits*

Preliminary studies showed that living *S. aurantia* was neither lethal nor apparently toxic for rabbits when $\leq 10^{10}$ bacteria (in 0.1 ml) were injected intradermally into one site on the shaved back. Larger doses were not tested. Following immunization in this way, a lesion would appear at the inoculation site, with erythema and induration of ~0.5 cm (maximum) in diameter that lasted for about one week. Apart from the lesion at the inoculation site, the rabbit appeared normal.

Rabbits immunized with *S. aurantia* plus incomplete or complete Freund's adjuvant showed more intense (~1 cm in diameter) and longer-lasting (several weeks) induration at the inoculation site than rabbits immunized with *S. aurantia* alone.

On the other hand, rabbits immunized with *S. aurantia* plus heat-killed *T. pallidum* showed less intense (<0.5 in diameter) and less long-lived induration at the inoculation site than rabbits immunized with *S. aurantia* alone. These inoculation sites returned to normal before those in any of the other rabbits. This observation suggested that an antiinflammatory substance was present in the killed *T. pallidum* culture.

Discussion

The search for a vaccine against syphilis could, in theory, involve either an artificially attenuated strain of *T. pallidum*, derived by growth of *T. pallidum* in vitro under conditions that cause a permanent loss of virulence, or a naturally attenuated strain of *T. pallidum* already present in nature. The problem with the first approach is that *T. pallidum* is not readily grown in vitro, although recent studies have been very promising;[20,21] the second approach is hindered by the fact that no naturally avirulent strains of *T. pallidum* are known. The treponemes pathogenic for humans appear to represent a range of virulence from venereally transmitted *T. pallidum* (syphilis), the most virulent; through *T. pertenue* (yaws); non-venereally-transmitted *T. pallidum* (endemic syphilis); to *Treponema carateum* (pinta), the least virulent. It has been suggested[22] that *T. carateum* could be useful as an immunizing agent against syphilis. Little is known about this organism, but it is a human pathogen and, as such, would not be acceptable as a human vaccine unless it was further attenuated. Unfortunately it cannot be grown in vitro; thus artificial attenuation is unlikely in the near future.

All of the pathogenic treponemes show some degree of cross-protection,[1,2] and *T. paraluis-cuniculi*, which causes a mild and superficial disease (venereal spirochetosis of rabbits) has been shown to give partial protection against *T. pallidum*.[3] However, protection was not complete even against small challenge doses of *T. pallidum*, and the recipient rabbit suffered transient immunosuppression. Thus, for this and other reasons,[23] *T. paraluis-cuniculi* was not considered suitable as a vaccine against syphilis.

A review of the physiology and possible evolution of the spirochetes by Canale-Parola[9] stimulated us to test the hypothesis that *S. aurantia* was phylogenetically related to *T. pallidum* and, as such, might be suitable as a vaccine against syphilis. Because it is a free-living organism, it was considered unlikely to be pathogenic for man. Preliminary experiments using increasing tenfold doses of viable *S. aurantia* injected into rabbits showed it to be nontoxic for rabbits up to the maximum dose tested (10^{10} cells administered intradermally).

On the assumption that *S. aurantia* and *T. pallidum* were related and that they arose from a common ancestral spirochete, the immunization and challenge experiments were undertaken to see whether protection against *T. pallidum* could be induced in rabbits by immunization with *S. aurantia*. Unfortunately, the animals did not become immune; therefore, the possibility that *S. aurantia* could be used as a vaccine against syphilis was ruled out.

The failure of the immunization and challenge experiments to detect any immunity in the rabbits does not, by itself, rule out the possibility of a phylogenetic relationship between these two species. During the process of becoming a parasite, *T. pallidum* may have evolved surface antigens very different from those of *S. aurantia* in order to cope with the animal tissue environment of the vertebrate host and its nonspecific and specific defense mechanisms.

One interesting observation from our study was the antiinflammatory effect of heat-killed *T. pallidum* on the host response to viable *S. aurantia* when both were inoculated into the rabbit from the same syringe. These lesions were much less inflammed and persisted for a much shorter time than inoculations of an identical number of viable *S. aurantia* without the killed *T. pallidum*. This response may be another manifestation of the postulated immunosuppressive property of *T. pallidum*.[24]

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