

Immunization with *Treponema pallidum* Endoflagella Alters the Course of Experimental Rabbit Syphilis

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Rabbits were immunized over a 32-week period with a total of 450 µg of purified *Treponema pallidum* endoflagella. As measured by enzyme-linked immunosorbent assay, sera from immunized rabbits had antiendoflagellar antibody titers that were fivefold greater than titers of sera from infected immune rabbits and patients with secondary disease. Sera from all immunized animals possessed complement-dependent treponemidal activity as measured by in vitro immobilization. Immunized animals challenged with virulent *T. pallidum* were not protected from symptomatic infection but showed an altered course of lesion development.

While complete protection against experimental syphilis has not yet been achieved by immunization with recombinant or native proteins of *Treponema pallidum* subsp. *pallidum* (6, 10), two successful vaccination studies in which whole organisms were used to elicit protective immunity have been reported. Miller (13) demonstrated complete resistance to intradermal challenge in rabbits vaccinated intravenously over a period of 37 weeks with gamma-irradiated living *T. pallidum*. Metzger et al. (12) showed protection in 41% of test rabbits vaccinated intravenously over an 11-week period with organisms "aged" at 4°C. Although these vaccine protocols required a prolonged and impractical course of immunization, the results provide evidence that successful vaccination against syphilis is possible and suggest that protective immunogens exist at or near the surface of *T. pallidum*.

In previous studies, we have demonstrated that antibodies against epitopes shared by the endoflagella of the nonpathogenic *Treponema phagedenis* biotype Reiter and *T. pallidum* can kill *T. pallidum* in vitro and contribute to the in vitro treponemidal activity present in normal human serum (4). However, Hindersson et al. (9) found that immunization of rabbits with purified *T. phagedenis* biotype Reiter endoflagella did not protect animals from challenge infection with *T. pallidum*.

We have recently reported the antigenic and structural characterization of *T. pallidum* endoflagella, including the finding that the endoflagellar filament proteins possess *T. pallidum*-specific epitopes (2). Studies using monoclonal antibodies have also demonstrated the presence of *T. pallidum*-specific epitopes, in particular, those present on the 37-kilodalton protein which comprise the endoflagellar filament sheath (1, 8, 15). Because these specific epitopes may provide a better target for a protective immune response than cross-reactive targets, we initiated this immunization study to determine whether purified *T. pallidum* endoflagella could elicit a greater level of serum complement-dependent treponemidal activity and resistance to challenge in experimental syphilis.

Adult male New Zealand White rabbits with nonreactive Venereal Disease Research Laboratory and *T. pallidum* immobilization tests (18) were used throughout this study. Infected rabbits were housed individually, maintained at 18 to 20°C, and given antibiotic-free food and water ad libitum.

T. pallidum Nichols strain was maintained by rabbit testicular passage twice weekly without the use of cortisone acetate, as described previously (14). Each treponemal suspension was prepared from the orchitis of a rabbit infected for 9 to 11 days by the method described by Miller et al. (14).

T. pallidum endoflagella were isolated and purified as described previously (2). For immunizations with endoflagella, each of four rabbits was given a total of 100 µg of endoflagella in 1 ml of phosphate-buffered saline (pH 7.2) mixed with 1 ml of Freund complete adjuvant; the mixture was divided equally and injected between two intramuscular and two subcutaneous sites. Each rabbit was then boosted seven times by the same routes at 4-week intervals with approximately 50 µg of endoflagella in 1 ml of phosphate-buffered saline mixed with 1 ml of Freund incomplete adjuvant. Rabbits were bled before immunization and before each boost. Sera collected from each bleeding were stored at -70°C.

Immunized and serologically nonreactive control rabbits were challenged intradermally on their clipped backs at each of four sites with 10³ virulent *T. pallidum* organisms 1 week after the final immunization boost. All animals were examined daily to monitor the development, morphological appearance, and progression of lesions. Lesions were classified as typical if they were erythematous, indurated, well circumscribed, and progressed to ulceration or as atypical if they were pale, soft, flat, and nonprogressive. At various times before lesion ulceration, representative lesions from the immunized and control rabbits were aspirated and examined by dark-field microscopy for the detection of motile treponemes.

Anti-*T. pallidum* endoflagellar serum collected from the immunized animals was tested for treponemidal activity by using the in vitro immobilization assay (4). *T. pallidum* extracted in heat-inactivated (56°C for 30 min) normal rabbit serum (NRS) was adjusted in the same medium to a concentration of 5 × 10⁷ organisms per ml. Mixtures (1:9) of treponemal suspension and either unheated or heat-inactivated (56°C for 30 min) test serum were placed in an

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TABLE 1. ELISA titers of anti-*T. pallidum* endoflagellar antibodies in serum from endoflagellum-immunized rabbits and in normal and syphilitic human sera

Serum	ELISA titers of specimen ^a :					
	1	2	3	4	5	6
Rabbit antiendo- flagellar serum	≥32,000	≥32,000	32,000	≥32,000		
NRS ^b	0	0	0	0	0	0
IRS (pool) ^c	6,000					
NHS ^d	50					
SHS ^e	500	1,000	6,000			

^a ELISA titers were calculated from the optical density readings at 690 and 490 nm. An absorbance of ≥0.026, which was >2 standard deviations above the mean determined for six normal sera, was considered positive. The endpoint titer was the reciprocal of the last dilution giving an absorbance of ≥0.026.

^b Obtained from six rabbits, including sera taken from rabbits before immunization with endoflagella.

^c Immune rabbit serum, obtained from three rabbits infected with *T. pallidum* for approximately 6 months and shown to be resistant to intradermal challenge.

^d Obtained from a donor with no history of syphilis and nonreactive in the Venereal Disease Research Laboratory test.

^e Syphilitic human serum, obtained from patients with secondary syphilitic infection.

atmosphere of 95% N₂-5% CO₂ and incubated at 34°C for 16 h. All test mixtures containing unheated serum were shown to have residual hemolytic complement activity after 16 h by using sensitized sheep erythrocytes in a hemolytic assay (20). After incubation, treponemal suspensions were scored for percent motility by counting 25 organisms, using dark-field microscopy.

Sera collected from each immunized rabbit, along with NRS, serum from noninfected humans (NHS), serum from 6-month-infected and immune rabbits, and serum from patients with documented secondary-stage disease, were tested quantitatively by enzyme-linked immunosorbent assay (ELISA) to determine immunoglobulin G antibody specific for *T. pallidum* endoflagella. Each microtiter plate well was absorbed with 250 ng of purified *T. pallidum* endoflagella, an amount which, as determined from preliminary experiments, represents an antigen excess for the serum dilutions used in these tests. Bound immunoglobulin G was detected by the addition of 50 μl of a 1:3,000 dilution of staphylococcal protein A-horse radish peroxidase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) followed by the addition of 50 μl of *o*-phenylenediamine dihydrochloride (80 mg/100 ml of 0.1 M citrate-phosphate buffer, pH 5.0) containing 0.01% hydrogen peroxide (Sigma Chemical Co., St. Louis, Mo.). The reaction was allowed to proceed for 5 min, at which time the reaction was terminated by the addition of 5 N H₂SO₄. ELISA titers were calculated from optical density readings at 690 and 490 nm on a Titertek MCC automated ELISA reader. Optical density measurements were considered to be significant if they were ≥0.026, a value 2 standard deviations above the mean determined for six NRS specimens. The reciprocal of the last dilution with an absorbance of ≥0.026 was considered to be the endpoint.

As determined by ELISA, rabbits immunized with purified *T. pallidum* endoflagella generated serum titers of antiendoflagellar antibodies equal to or greater than 1:32,000 (Table 1). These titers were at least fivefold greater than those present in sera from immune syphilitic rabbits and patients with secondary-stage disease. An NHS specimen

TABLE 2. Induction of in vitro complement-dependent treponemicidal activity in sera from rabbits immunized with *T. pallidum* endoflagella^a

Time after immunization (mo) ^b	Serum treponemicidal activity ^c of rabbit:			
	1	2	3	4
0	NR	NR	NR	NR
3	NR	NR	NR	NR
6	WR	WR	WR	NR
8 ^d	WR	WR	R	WR

^a Unheated and heat-inactivated (56°C for 30 min) anti-*T. pallidum* endoflagellar antisera were combined with 5 × 10⁷ *T. pallidum* organisms per ml in a 9:1 ratio. Treponemal suspensions were incubated under 95% N₂-5% CO₂ for 16 h at 34°C before dark-field observation for motility.

^b Each animal was immunized over 32 weeks with a total of 450 μg of purified *T. pallidum* endoflagella by a combined intramuscular-subcutaneous route.

^c NR, Nonreactive (≤20% difference in motility as compared with controls); WR, weakly reactive (21 to 49% difference in motility as compared with controls); R, reactive (≥50% difference in motility as compared with controls).

^d One week before challenge.

also showed low but detectable levels of antiendoflagellar immunoglobulin G antibody as compared with NRS.

Sera from animals immunized with *T. pallidum* endoflagella were tested for complement-dependent treponemicidal activity by in vitro immobilization. Serum from three of four rabbits immunized and boosted for 24 weeks with *T. pallidum* endoflagella showed treponemicidal activity (Table 2). After 32 weeks of immunization, sera from all four rabbits developed treponemicidal activity; the serum from one animal was judged to be strongly reactive as determined by the criteria for *T. pallidum* immobilization test reactivity (18).

In order to determine the ability of immunization with endoflagella to confer protection against syphilitic infection, immunized and serologically nonreactive control rabbits were challenged as described above. As compared with control rabbits, accelerated lesion development after an incubation period of only 5 days postchallenge was observed at 8 of 16 sites from two of four immunized rabbits (Table 3). Continued lesion development in all of the immunized rabbits was found to be atypical as compared with control rabbits. In addition, dark-field examination of lesion aspirates from immunized animals showed that none of the four representative lesions at day 12 and only one of four representative lesions at day 19 had motile treponemes; in con-

TABLE 3. Response of rabbits to intradermal challenge after immunization with *T. pallidum* endoflagella^a

Group	No. of rabbits	No. of lesions/no. of sites	Lesion development			
			Incubation period (days)	Dark-field examination ^b (no. with motile treponemes/no. tested)		
				Range	Mean	Day 12
Immunized	4	16/16 ^c	5-12 ^c	8.0 ^c	0/4	1/4
Control	3	12/12	11-16	13.6	3/3	3/3

^a Rabbits were challenged intradermally 1 week postimmunization with 10³ virulent *T. pallidum* organisms at each of four sites.

^b Dark-field microscopic examinations were performed on aspirates taken from representative lesions in both groups.

^c Accelerated lesion appearance was seen at 8 of 16 sites representing two of four immunized rabbits. Subsequent lesion development and morphology were observed to be atypical for all 16 lesions as compared with control sites.

trast, each of the three sampled control lesions showed motile treponemes at these time points.

The results of this study corroborate and extend our previous findings regarding the complement-dependent *in vitro* treponemicidal activity of antiendoflagellar antibody. Although the antiserum generated in this study has been shown to possess antibodies specific to *T. pallidum* endoflagellar epitopes (2), no increase in the level of treponemicidal activity by this antiserum was observed as compared with the antisera raised against *T. phagedenis* biotype Reiter endoflagella (4). These results suggest that the majority of endoflagellar targets for treponemicidal activity may be the epitopes common to the genus *Treponema*. This concept is supported by the antiendoflagellar antibody present in NHS, which has been shown to contribute to NHS treponemicidal activity (3, 4). The recent cloning and sequence analysis of genes which encode all of the *T. pallidum* endoflagellar filament proteins (7, 11, 16) can now allow determination of the epitopes which are the targets for this treponemicidal activity.

While immunization of rabbits with *T. pallidum* endoflagella did not prevent lesion appearance, the normal course of lesion development was observed to be altered. It was found that half of all lesions on immunized animals appeared 6 to 8 days before the appearance of lesions on the nonimmunized control animals. Accelerated lesion appearance was not observed by Hindersson et al. (9) after *T. pallidum* challenge of rabbits immunized with *T. phagedenis* biotype Reiter endoflagella. However, this has been observed after challenge of animals immunized with other *T. pallidum* molecules, including the 190-kilodalton recombinant protein 4D (6) and hydrophobic proteins extracted from *T. pallidum* by using the detergent Triton X-114 (Lovett et al., unpublished observation). It is therefore likely that these accelerated lesions represent a specific cell-mediated immune response to these various *T. pallidum* immunogens.

It was also found that subsequent lesion development on the endoflagellum-immunized animals was atypical as compared with the corresponding controls. We believe that these results suggest that accelerated lesion appearance and subsequent altered lesion development represent the combined effects of specific antiendoflagellar antibody and cell-mediated immunity rapidly responding to *T. pallidum* endoflagellar antigens introduced upon challenge. Our inability to consistently identify viable treponemes from lesion aspirates taken from immunized animals further suggests that this early immune response may have effectively reduced the number of viable treponemes in these lesions. It should be noted, however, that postchallenge sera from these animals acquired after the resolution of lesions did not show any quantitative or qualitative differences on *T. pallidum* immunoblots as compared with serum acquired from control infected animals (data not shown). Taken together, these results suggest that *T. pallidum* endoflagellar immunization lessened the initial focal disease but did not significantly inhibit disseminated infection.

While immunization with endoflagella did not confer complete protection of animals against challenge infection, recent studies in our laboratory have provided a possible explanation for this result. It has been shown that prebound antibody to *T. pallidum*, present on treponemes extracted from the infected rabbit, has been an essential cofactor in the demonstration of *in vitro* treponemicidal activity by various antisera, including endoflagellar antiserum (5). We have hypothesized that these prebound antibodies are directed against *T. pallidum* rare outer membrane protein (TROMP),

which was recently identified by freeze-fracture electron microscopy (17, 19) and shown to be antigenic and surface exposed (5). We have also found that antiendoflagellar antibody does not possess *in vitro* treponemicidal activity when treponemes extracted from immunocompromised rabbits are used (5), further suggesting that anti-TROMP antibody is required for this activity. Therefore, the inability to elicit complete protective immunity after endoflagellar immunization may be due to the inability of antiendoflagellar antibodies, in the absence of anti-TROMP, to provide *in vitro* treponemicidal activity. Nevertheless, while the primary target molecules on *T. pallidum* may reside with TROMP, the results of this immunization study suggest that *T. pallidum* endoflagella may be an important secondary target antigen for humoral and cellular immune mechanisms.

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